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The SMC5/6 complex maintains telomere length in ALT cancer cells through SUMOylation of telomere-binding proteins

Patrick Ryan Potts & Hongtao Yu

Most cancer cells activate telomerase to elongate telomeres and achieve unlimited replicative potential. Some cancer cells cannot activate telomerase and use telomere homologous recombination (HR) to elongate telomeres, a mechanism termed alternative lengthening of telomeres (ALT). A hallmark of ALT cells is the recruitment of telomeres to PML bodies (termed APBs). Here, we show that the SMC5/6 complex localizes to APBs in ALT cells and is required for targeting telomeres to APBs. The MMS21 SUMO ligase of the SMC5/6 complex SUMOylates multiple telomere-binding proteins, including TRF1 and TRF2. Inhibition of TRF1 or TRF2 SUMOylation prevents APB formation. Depletion of SMC5/6 subunits by RNA interference inhibits telomere HR, causing telomere shortening and senescence in ALT cells. Thus, the SMC5/6 complex facilitates telomere HR and elongation in ALT cells by promoting APB formation through SUMOylation of telomere-binding proteins.

Telomeres are proteinaceous, repetitive DNA elements (5′-TTAGGG-3′ in humans) that comprise 5–15 kilobases (kb) of the ends of each chromosome[1]. These sequences are shortened after every cell division owing to the end-replication problem of the lagging strand[2]. Critically short telomeres result in cellular senescence[3]. Therefore, telomeres have been proposed to act as a counting mechanism for cellular proliferation[3]. Telomerase is the enzyme responsible for synthesis of new telomeric repeats[4]. Normal human somatic cells repress telomerase to limit their own proliferative capacity[5]. Cancer cells overcome this limited proliferative potential, generally by transcriptional upregulation of telomerase[6]. However, telomerase is not activated in a subset of tumors, including many sarcomas and astrocytomas, and tumors of Li-Fraumeni syndrome[7,8]. Cells in a portion of these tumors rely on an alternative mechanism to lengthen telomeres, termed alternative lengthening of telomeres (ALT)[9–11]. Whereas telomerase synthesizes telomeric repeats using an RNA template, ALT relies on HR between telomeric sequences to elongate telomeres[12–14]. A hallmark of ALT cells is their extreme heterogeneity in telomere length (<1 to >20 kb) as a result of HR[15]. Another defining feature of ALT cells is the localization of telomeres in promyelocytic leukemia (PML) bodies, termed ALT-associated PML bodies (APBs)[16]. However, some ALT cells do not show these hallmarks, suggesting the potential existence of multiple ALT pathways[10].

APBs appear upon the activation of ALT and disappear when it is repressed[16,17]. The recruitment of telomeres to PML bodies is enriched in cells with sister chromatids—that is, cells in late S/G2 phase[18]. A number of proteins involved in DNA repair are present in APBs, including RAD51, RAD52, RPA, MRE11, NBS1, BRCA1, RAD9, BLM and WRN[16,19–22]. The recruitment of factors involved in the repair of DNA double-strand breaks (DSBs) to APBs is distinct from their recruitment to irradiation-induced nuclear foci[23]. Recent studies have suggested that NBS1, a subunit of the MRE11–RAD50–NBS1 (MRN) complex, is required for APB formation[23,24]. It has been proposed that APBs are the sites for telomere recombination that elongates telomeres to allow unlimited proliferative potential, although the molecular mechanisms for this process are largely unknown.

Proteins of the 'structural maintenance of chromosomes' (SMC) family (SMC1 through SMC6) form three multisubunit protein complexes that regulate chromosomal dynamics[25]. The cohesin complex consists of the SMC1–SMC3 heterodimer, which promotes the proper segregation of sister chromatids to daughter cells during mitosis[26]. The condensin complex consists of the SMC2–SMC4 heterodimer, which promotes condensation of chromosomes during mitosis[27]. The SMC5/6 complex consists of the SMC5–SMC6 heterodimer and at least six 'non-SMC element' proteins (NSE1 through NSE6)[28]. It is required for efficient repair of DNA damage[28–30] and for proper segregation of the repetitive DNA elements of ribosomal DNA (rDNA) in yeast[31].

MMS21/NSE2 (herein referred as MMS21 for simplicity) is a small ubiquitin-like modifier (SUMO) ligase that promotes the covalent attachment of SUMO to proteins[32]. SUMOylation often changes protein-protein interactions, regulates subcellular localization or mediates transcriptional repression[33]. The SUMO ligase activity of MMS21 is required for proper DNA damage repair[32,34,35]. The SMC5/6

Department of Pharmacology, The University of Texas Southwestern Medical Center, 6001 Forest Park Road, Dallas, Texas 75390-9041, USA. Correspondence should be addressed to H.Y. (hongtao.yu@utsouthwestern.edu).

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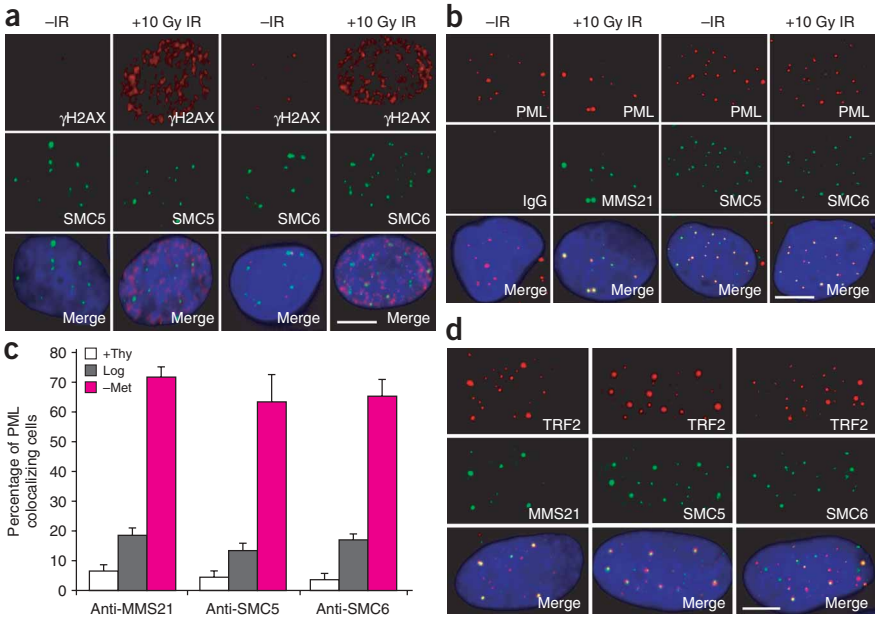

Figure 1 The SMC5/6 complex localizes to PML nuclear bodies. (**a**) SMC5/6 localizes to nuclear foci in U2OS cells that do not depend on or colocalize with DNA damage. Cells were untreated or treated with ionizing radiation (IR; 10 Gy) and stained 1 h later with the indicated antibodies. (**b**) The SMC5/6 complex localizes to PML bodies in U2OS cells. U2OS cells were stained with the indicated antibodies. (**c**) Percentages of cells with colocalization of the SMC5/6 complex and PML bodies in log-phase, G1/S (+Thy) or G2/M (–Met) U2OS cells. Results from three separate experiments were averaged; error bars show s.d. (**d**) The SMC5/6 complex localizes to a subset of telomeres (TRF2) in U2OS cells. Scale bars represent 5 μm.

complex is recruited to DSBs and promotes sister-chromatid HR by facilitating the recruitment of cohesin to DSBs[36–39]. In addition to its localization to DSBs, the SMC5/6 complex also localizes to repetitive DNA elements, such as rDNA and telomeres in yeast[31,35,38]. Mutation of MMS21 in yeast impairs clustering of telomeres during meiosis[35]. Here we investigate the function of the human SMC5/6 complex in telomere recombination and maintenance.

RESULTS

The SMC5/6 complex localizes to PML bodies in ALT cells

To determine whether the SMC5/6 complex forms nuclear foci coinciding with DSBs, we treated U2OS cells with 10 Gy of irradiation and stained the cells for SMC5, SMC6 and γH2AX (a DSB foci marker). To our surprise, a subset of cells formed SMC5/6 nuclear foci in the absence of DNA damage, and these foci did not overlap with γH2AX in the presence or absence of irradiation (**Fig. 1a**). To determine the nature of these SMC5/6 nuclear bodies, we stained U2OS cells with antibodies against the components of the SMC5/6 complex (SMC5, SMC6 and MMS21) and markers of other known nuclear structures, such as centromeres and PML nuclear bodies. We observed specific colocalization of the SMC5/6 complex with PML nuclear bodies in U2OS cells (**Fig. 1b** and data not shown). Approximately 75% of PML foci contained the SMC5/6 complex (**Table 1**). Because only a subset of U2OS cells showed colocalization of the SMC5/6 complex with PML bodies, we speculated that the recruitment of the SMC5/6 complex might be regulated during the cell cycle. We arrested U2OS cells in G1/S with thymidine or enriched cells in G2/M by methionine deprivation and tested for the colocalization of the SMC5/6 complex with PML bodies. Flow-cytometry analysis confirmed that thymidine treatment resulted in the enrichment of G1/S cells and methionine deprivation resulted in the enrichment of G2/M cells (**Supplementary Fig. 1d** online). In cells arrested in G1, the SMC5/6 complex did not localize to PML bodies and showed diffuse nuclear staining (**Fig. 1c** and data not shown). The percentage of cells with colocalization of the SMC5/6 complex and PML bodies greatly increased in cells enriched in G2/M (**Fig. 1c**), suggesting a cell cycle–regulated recruitment of the SMC5/6 complex to PML.

Next, we examined whether the SMC5/6 complex localized to PML bodies in other cell types. To our surprise, we discovered that the SMC5/6 complex localized to PML bodies in only a subset of cell lines (**Table 2**). The localization of the SMC5/6 complex to PML bodies did not depend on the cell type, tissue of origin or immortalization technique (**Table 2**, **Supplementary Fig. 1**, and data not shown). The only characteristic that was correlated with SMC5/6 complex localization to PML bodies was the mechanism of telomere length maintenance (**Table 2**). The SMC5/6 complex localized to PML bodies in cells in which the telomeres are maintained by recombination (ALT cells), but not in telomerase-positive cells. To confirm that the localization of the SMC5/6 complex to PML bodies is indeed due to ALT, we examined the localization of SMC6 in SW26 and SW39 cells. These cells are derived from the same parental cell line (IMR90 primary lung fibroblast) but rely on either ALT (SW26) or telomerase (SW39) to maintain telomere length[40]. As expected, SMC6 formed nuclear foci corresponding to PML bodies only in SW26 cells and not in SW39 cells (**Supplementary Fig. 2** online). Therefore, the SMC5/6 complex specifically localizes to PML bodies in cells that use telomere recombination to elongate telomeres (ALT) and not in telomerase-positive cells.

It has previously been demonstrated that telomeres are recruited to PML bodies in ALT, but not telomerase-positive, cells. To determine whether the PML bodies that contain the SMC5/6 complex also contain telomeres, we stained U2OS ALT cells for the SMC5/6 complex and the telomere-binding protein TRF2. Approximately 50% of TRF2 foci indeed colocalized with the SMC5/6 complex (**Fig. 1d** and **Table 1**). We also examined whether SMC5 colocalized with telomeres in generation 5 (G5) *Terc*$^{-/-}$ *Wrn*$^{-/-}$ Ras^{v12}-expressing mouse embryo fibroblast (MEF) ALT cells[41]. These cells have previously been shown to display ALT characteristics, such as elevated telomere recombination, telomere length heterogeneity and APB

Table 1 The SMC5/6 complex localizes to PML bodies and telomeres in ALT cells

SMC5/6 subunit	PML foci with SMC5/6 (%)	SMC5/6 foci with PML (%)	TRF2 foci with SMC5/6 (%)	SMC5/6 foci with TRF2 (%)
MMS21	71.5	87.3	51.5	75.1
SMC5	73.9	90.3	58.4	72.3
SMC6	78.9	91.4	55.1	76.0

Table 2 The SMC5/6 complex localizes to PML nuclear bodies in ALT cells

Cell line	Cell type	Immortalization	SMC5/6 foci	Telomere maintenance
U2OS	Epithelial; bone osteosarcoma	Tumor	+	ALT
SUSM1	Fibroblast; liver	Chemical	+	ALT
Saos-2	Epithelial; bone osteosarcoma	Tumor	+	ALT
SW-26	Fibrobast; lung	SV40	+	ALT
SW-39	Fibroblast; lung	SV40	–	Telomerase
293	Epithelial; kidney	Adenovirus	–	Telomerase
HeLa	Epithelial; cervical carcinoma	Tumor	–	Telomerase
H1299	Epithelial; non–small cell lung carcinoma	Tumor	–	Telomerase
MCF7	Epithelial; breast adenocarcinoma	Tumor	–	Telomerase

formation[41]. Smc5 indeed colocalized with a subset of telomeres in these MEF ALT cells (**Supplementary Fig. 1c**). These results suggest that the SMC5/6 complex localizes to PML bodies in which telomeres are present (referred to as APBs hereafter).

The SMC5/6 complex is required for telomere recombination

ALT cells rely on recombination of telomeric sequences to elongate and maintain telomere length[10]. Previously, we have demonstrated that the SMC5/6 complex is specifically required for sister-chromatid HR[36]. To determine whether the SMC5/6 complex is required for telomere HR, we examined the frequency of telomere HR using the chromosome orientation fluorescence *in situ* hybridization (CO-FISH) assay in G5 *Terc*$^{-/-}$ *Wrn*$^{-/-}$ Ras^{v12} MEF ALT cells[41]. These cells have previously been shown to display substantial levels of telomere recombination[41]. CO-FISH using differentially labeled fluorescent probes that recognize the telomere leading-strand sequence (G-rich, Cy3 probe) or the lagging-strand sequence (C-rich, FITC probe) yields opposing telomere signals on each sister chromatid (**Fig. 2a**, top). However, if telomere sister-chromatid exchange (T-SCE) occurs, leading and lagging strand sequences will be exchanged, typically in unequal portions, resulting in an overlap of G-rich and C-rich telomeric sequences (**Fig. 2a**, bottom). Treatment of G5 *Terc*$^{-/-}$ *Wrn*$^{-/-}$ Ras^{v12} MEF ALT cells with short interfering RNAs (siRNAs) targeting the mouse *Mms21* gene resulted in substantial knockdown of Mms21 protein (**Fig. 2b**). We observed an approximately 80% reduction in the number of unequal T-SCE events in cells treated with *Mms21* siRNA (**Fig. 2c,d**). In addition to examining whether the SMC5/6 complex is required for telomere recombination in mouse ALT cells, we determined this in the classical human SUSM1 ALT cell line, which has robust T-SCE activity. Treatment of SUSM1 cells with siRNAs targeting *SMC5* resulted in substantial knockdown of both SMC5 and MMS21, suggesting that MMS21 requires binding to SMC5 for its stability (**Fig. 2e**), a common observation for multi-subunit complexes. Knockdown of MMS21, SMC5 or SMC6 resulted in approximately 75% reduction of telomere recombination (**Fig. 2f**). These results suggest that the SMC5/6 complex is required for T-SCE in ALT cells.

Inhibition of the SMC5/6 complex disrupts APB formation

A hallmark of ALT cells is the association of some of their telomeres with PML bodies (APBs)[16]. It has been suggested that APBs are the site of telomere recombination in ALT cells. Inhibition of APB formation causes progressive telomere shortening in ALT cells[24]. Given the localization of the SMC5/6 complex to telomeres and

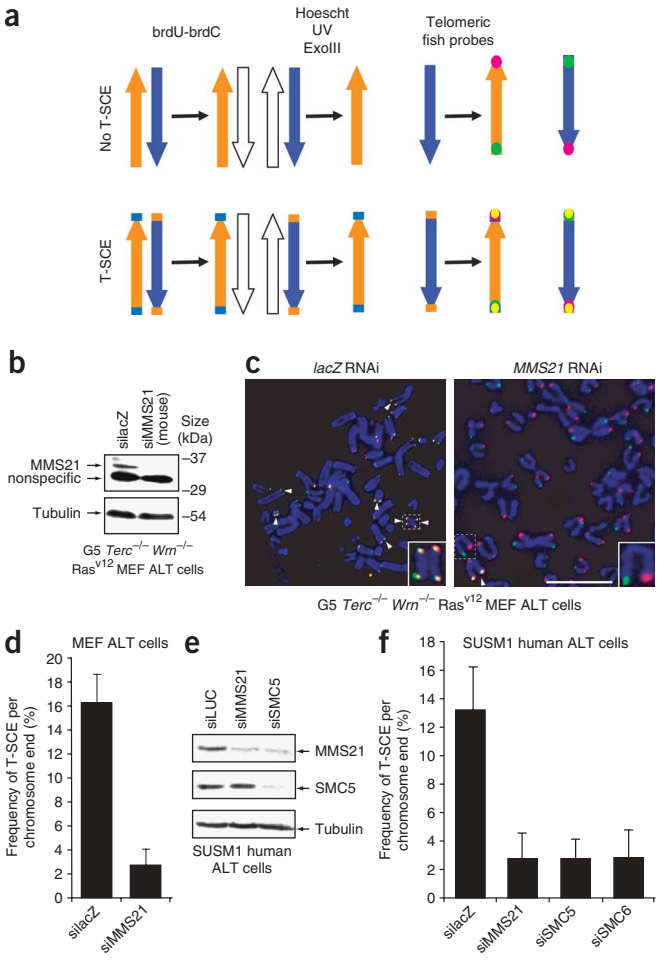

Figure 2 Inhibition of the SMC5/6 complex decreases telomere recombination (T-SCE). (**a**) Schematic illustration of the CO-FISH T-SCE assay. (**b**) Western blot analysis showing the efficiency of RNAi-mediated knockdown in G5 *Terc*$^{-/-}$ *Wrn*$^{-/-}$ Ras^{v12} MEF ALT cells. (**c**) Chromosome spreads from *lacZ*- or *MMS21*-RNAi G5 *Terc*$^{-/-}$ *Wrn*$^{-/-}$ Ras^{v12} MEF ALT cells were processed for CO-FISH as described in **a** with G-rich (red) and C-rich (green) telomeres stained. Arrowheads mark unequal T-SCE events (yellow). Scale bar represents 5 μm. (**d**) Quantification of the frequency of unequal T-SCE events per chromosome end, as depicted in **c**. Results from three separate experiments were averaged; error bars show s.d. (**e**) Western blot analysis showing the efficiency of RNAi-mediated knockdown in human SUSM1 ALT cells. (**f**) Quantification of the frequency of unequal T-SCE events per chromosome end in SUSM1 cells treated with SMC5/6 RNAi. Results from three separate experiments were averaged; error bars show s.d.

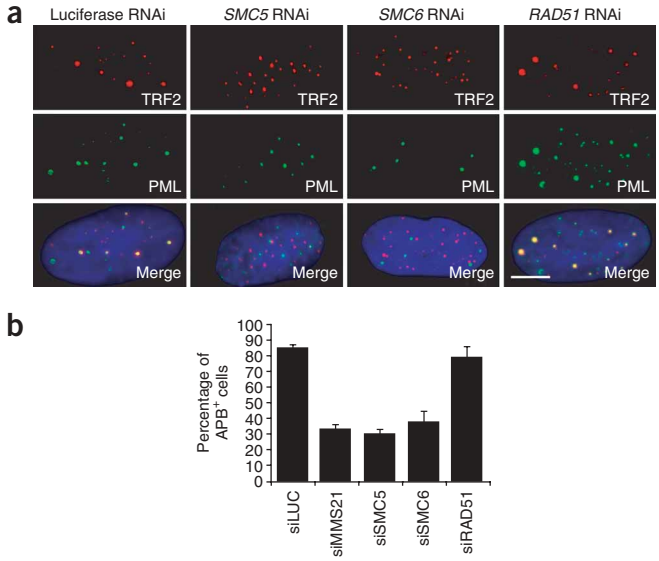

Figure 3 The SMC5/6 complex is required for APB formation. (**a**) U2OS cells were treated with the indicated siRNAs and APBs were analyzed by examining colocalization of TRF2 (red) and PML (green). Scale bars represent 5 μm. (**b**) Quantification of results from cells shown in **a**. Results from three separate experiments were averaged; error bars show s.d.

PML bodies in ALT cells (**Fig. 1**) and the requirement of the SMC5/6 complex for telomere recombination (**Fig. 2**), we examined whether the SMC5/6 complex is required for the recruitment of telomeres to PML bodies in ALT cells. Knockdown of MMS21, SMC5 or SMC6 by RNA interference (RNAi) blocked the recruitment of telomeres to PML bodies in U2OS cells (**Fig. 3a**,**b**).

It has previously been reported that inhibition of NBS1, a component of the MRN complex that facilitates HR, disrupts APBs[23,24]. To determine whether the requirement for the SMC5/6 complex in APB formation, like that for NBS1, is an indirect result of its role in HR, we determined the effect of inhibiting the essential HR factor, RAD51, on APB formation. Treatment of U2OS cells with *RAD51* siRNA resulted in substantial knockdown of the RAD51 protein (**Supplementary Fig. 2c**) but did not alter APB formation (**Fig. 3a**,**b**). Notably, this level of knockdown has previously been shown to inhibit HR[36]. Therefore, APB formation is not disrupted by inhibition of any HR protein.

Previously, we have shown that the SMC5/6 complex is required to recruit the cohesin complex to DSBs[36]. We thus examined whether the SMC5/6 complex also recruited cohesin to PML bodies. We immunostained U2OS cells for endogenous or Myc-tagged SMC1 and SCC1, two cohesin subunits. In both cases, we did not observe localization of the cohesin complex to PML bodies (**Supplementary Fig. 3** online), suggesting that the SMC5/6 complex might facilitate APB formation using a cohesin-independent mechanism.

MMS21 SUMOylates the shelterin complex

To gain insights into the mechanism by which the SMC5/6 complex promotes APB formation, we tested whether the SUMO ligase activity of MMS21 is required for APB formation. As expected, RNAi knockdown of MMS21 produced cells defective in APB formation (**Fig. 3b** and **Fig. 4a**,**b**). Cells treated with *MMS21* siRNA were transfected with plasmids expressing wild-type MMS21 or a mutant with no SUMO ligase activity (C215A). The plasmids also contained silent mutations in the siRNA-binding site to prevent knockdown of the transgenes. Overexpression of wild-type MMS21 substantially rescued APB formation in *MMS21* RNAi–treated cells (**Fig. 4a**,**b**). Although the SUMO ligase–dead mutant of MMS21 was still recruited to PML bodies in ALT cells (data not shown), it did not efficiently restore APB formation in *MMS21* RNAi–treated cells (**Fig. 4a**,**b**). These results strongly suggest that the SUMO ligase activity of MMS21 is required for APB formation.

We next tested whether MMS21 stimulated the SUMOylation of the telomere-binding proteins TRF1, TRF2, TIN2, TPP1, POT1 and RAP1. These proteins are components of the shelterin complex, or telosome (**Fig. 4c**), which protects telomeres from being recognized as DSBs and prevents telomere HR[42]. Overexpression of MMS21 in HeLa cells stimulated the SUMOylation of four of the six ectopically expressed shelterin components, including TRF1, TRF2, TIN2 and RAP1 (**Supplementary Fig. 4a** online). This is noteworthy, as we have shown previously that MMS21 stimulates the SUMOylation of only two out of 30 SUMO substrates, many of which were identified in an *in vitro* expression cloning (IVEC) screen[34,43]. The MMS21-induced SUMOylation of TRF1, TRF2, TIN2 and RAP1 was not further enhanced by irradiation (**Supplementary Fig. 4a**), suggesting that this activity is not regulated by DNA damage. To test the specificity of SUMOylation of the shelterin complex by MMS21, we transfected cells with Myc-TRF1 in the presence of various SUMO ligases, such as MMS21, PIAS1, PIASxβ or PIASy. We observed a dose-dependent increase of TRF1 SUMOylation in the presence of wild-type MMS21, but not its SUMO ligase–dead C215A mutant or other SUMO ligases (**Fig. 4d**). Overexpression of the SUMO isopeptidase SENP2, or the use of a SUMO mutant (SUMO-ΔGG) incapable of conjugation, greatly reduced the amounts of slow-migrating TRF1 species, confirming their identities as SUMO conjugates (**Fig. 4d**). These results suggest that MMS21 is sufficient to SUMOylate several shelterin components in the telomerase-positive HeLa cell line. We next examined whether these shelterin components were SUMOylated in U2OS ALT cells without the overexpression of MMS21. Indeed TRF1, TRF2 and RAP1 were SUMOylated in ALT cells without overexpression of MMS21 (**Fig. 4e** and data not shown). We next determined whether SUMOylation of TRF2 or RAP1 in ALT cells was dependent on MMS21 by knocking down MMS21 by RNAi. Knockdown of MMS21 substantially decreased the SUMOylation of both TRF2 and RAP1 in U2OS ALT cells. This decreased SUMOylation of TRF2 and RAP1 was rescued by expression of an siRNA-resistant wild-type MMS21, but not SUMO ligase–dead MMS21 C215A (**Fig. 4e**). Therefore, the SUMO ligase activity of the SMC5/6 complex component MMS21 is required for APB formation and is necessary and sufficient for the SUMOylation of several subunits of the shelterin complex.

TRF1 and TRF2 SUMOylation is required for APB formation

Previous studies have shown that SUMOylation controls the recruitment of several proteins (such as DAXX) to PML bodies[44]. This SUMOylation-dependent recruitment of proteins to PML bodies is dependent on the conserved SUMO-binding motif of PML[44]. We examined whether SUMOylation of shelterin subunits is required for their recruitment to APBs. SUMOylation often occurs on lysines within a loosely defined consensus motif, ΨKXE (Ψ denotes a hydrophobic residue)[45]. TRF1 contained two such potential SUMOylation sites, with multiple lysines in each (**Fig. 5a**). We mutated both motifs by replacing the lysines with arginines. Although mutation of either SUMOylation motif (TRF1 M1 or M2) alone was not sufficient to decrease SUMOylation, mutation of both (TRF1

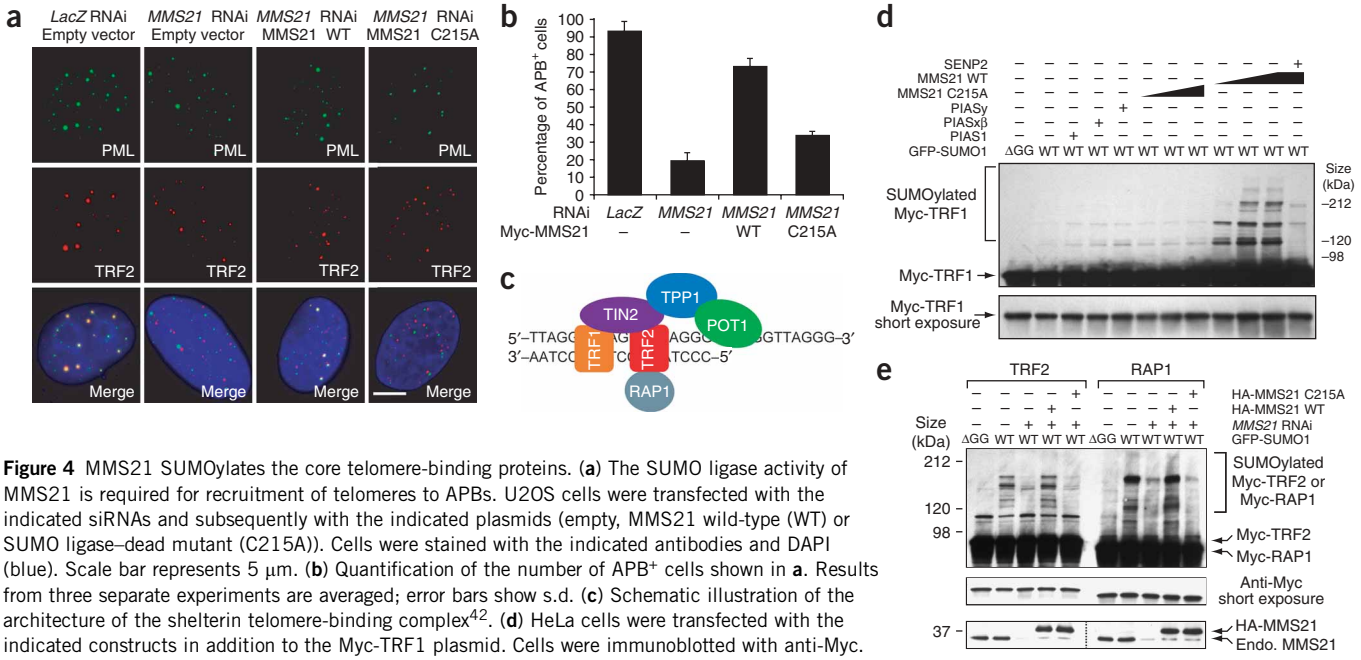

Figure 4 MMS21 SUMOylates the core telomere-binding proteins. (**a**) The SUMO ligase activity of MMS21 is required for recruitment of telomeres to APBs. U2OS cells were transfected with the indicated siRNAs and subsequently with the indicated plasmids (empty, MMS21 wild-type (WT) or SUMO ligase–dead mutant (C215A)). Cells were stained with the indicated antibodies and DAPI (blue). Scale bar represents 5 μm. (**b**) Quantification of the number of APB$^+$ cells shown in **a**. Results from three separate experiments are averaged; error bars show s.d. (**c**) Schematic illustration of the architecture of the shelterin telomere-binding complex[42]. (**d**) HeLa cells were transfected with the indicated constructs in addition to the Myc-TRF1 plasmid. Cells were immunoblotted with anti-Myc. GFP–SUMO1-ΔGG lacks the diglycine C-terminal motif required for its conjugation to substrates. (**e**) U2OS cells were transfected with the indicated constructs in addition to either Myc-TRF2 or Myc-RAP1. HA-MMS21 WT or C215A contained silent point mutations in the siRNA-binding region to prevent knockdown of the transgenes. Cell lysates were immunoblotted with anti-Myc or anti-MMS21.

ΔSUMO) appreciably inhibited MMS21-induced SUMOylation of TRF1 (**Fig. 5b**). Mutation of all six lysines in M1 and M2 to arginines (TRF1 ΔSUMO) did not disrupt TRF1 localization to telomeres or binding to TIN2 (**Supplementary Fig. 4b,c**). Notably, TRF1 ΔSUMO did not localize to PML bodies, whereas wild-type or single SUMO–site mutant TRF1 did (**Fig. 5c,d**). Therefore, SUMOylation of TRF1 is required for its recruitment to PML bodies.

We next asked whether SUMOylation of other shelterin components is required for their recruitment to APBs. We identified three potential SUMOylation sites (**Supplementary Fig. 5a** online) in another shelterin component, TRF2 (**Fig. 4c**). Mutation of all three potential SUMOylation sites (TRF2 ΔSUMO) substantially decreased MMS21-induced SUMOylation of TRF2 (**Supplementary Fig. 5b** and data not shown). As observed for TRF1 ΔSUMO, TRF2 ΔSUMO also did not localize to PML bodies (**Supplementary Fig. 5c,d**). These results suggest that the SUMOylation of multiple subunits of the shelterin complex is required for APB formation in ALT cells.

The SMC5/6 complex is required for telomere maintenance

To determine whether the reduction in telomere recombination and APB formation in SMC5/6-knockdown ALT cells affects telomere length, we tested chromosomes for telomere signal free ends,

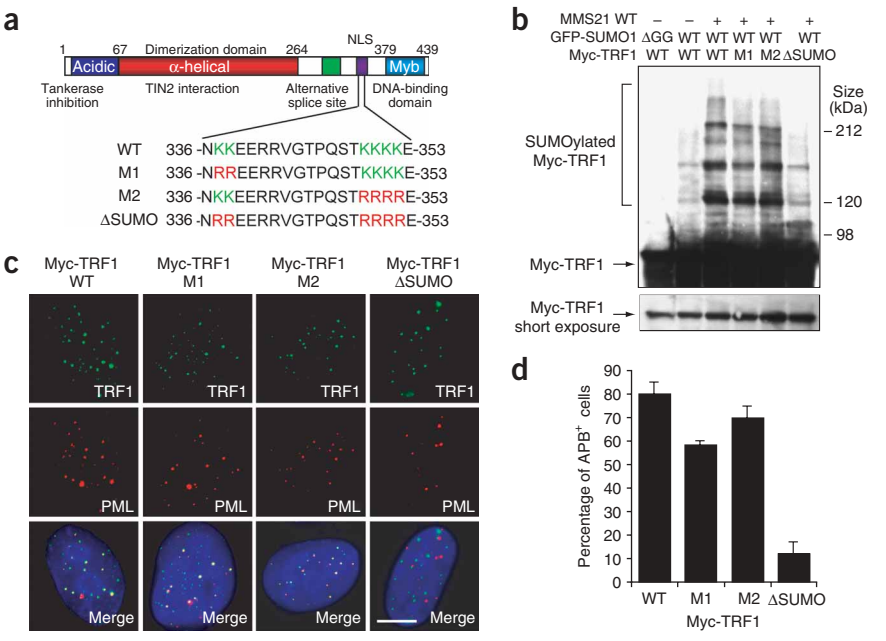

Figure 5 MMS21-induced SUMOylation of telomere-binding proteins is required for APB formation. (**a**) Schematic illustration of putative TRF1 SUMOylation sites and the mutations created. (**b**) Mutation of TRF1 SUMOylation sites (ΔSUMO) decreases MMS21-induced SUMOylation of TRF1. Myc-TRF1 WT, M1, M2 or ΔSUMO mutants were expressed in HeLa cells with the indicated plasmids. Cell lysates were immunoblotted with anti-Myc. (**c**) Myc-TRF1 ΔSUMO mutant does not localize to PML bodies in U2OS cells. U2OS cells were stained with anti-Myc (green), anti-PML (red) and DAPI (blue). Scale bar represents 5 μm. (**d**) Quantification of the percentage of APB$^+$ cells shown in **c**. Results from three separate experiments were averaged; error bars show s.d.

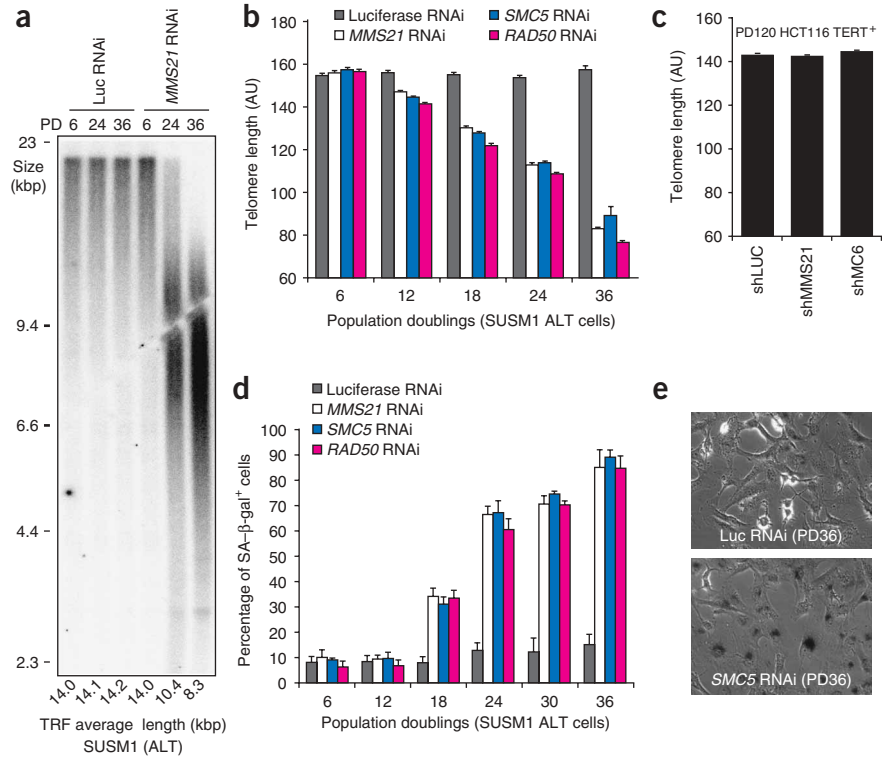

Figure 6 Inhibition of the SMC5/6 complex results in telomere shortening and cellular senescence. (**a**) Telomere restriction fragment analysis of SUSM1 cells treated with either siLuciferase or siMMS21 RNAi oligonucleotides for the indicated number of population doublings. Genomic DNA was probed with ^{32}P-$(TTAGGG)_4$ oligonucleotide. Average telomere lengths are indicated below the gel. kbp, kilo–base pairs. (**b**) SUSM1 cells were treated with the indicated siRNAs for the indicated number of population doublings. Relative telomere lengths (AU, arbitrary units) were determined by Q-FISH and the average intensities from two separate experiments are shown for the indicated siRNAs and population doublings. Error bars show s.e.m. (**c**) Stable shRNA-knockdown cell lines were made in telomerase-positive HCT116 cells with the indicated shRNAs and were maintained in culture for 120 population doublings. Relative telomere lengths were determined by Q-FISH and the average intensities from two separate experiments are shown for the indicated shRNAs. Error bars show s.e.m. (**d**) SUSM1 cells were treated with the indicated siRNAs for the indicated number of population doublings. Senescent cells were observed by SA–β-gal staining and the average number of SA–β-gal–positive cells from two separate experiments is shown. Error bars show s.d. (**e**) Micrograph of cells tested in **d**, showing SA–β-gal–positive cells (dark cells).

end-to-end fusions and telomere length by quantitative FISH (Q-FISH) and telomere restriction fragment (TRF) analysis. SUSM1 cells were transfected with siRNAs targeting luciferase, *MMS21* or *SMC5* every 3 d for a total of 36 population doublings. The knockdown level of MMS21 was followed during the course of the siRNA treatment (**Supplementary Fig. 6a** online). We observed an increase in the percentage of both telomere signal free ends and chromosomal end-to-end fusions in *MMS21* or *SMC5* RNAi–treated SUSM1 ALT cells at late population doublings (**Supplementary Table 1** online). In addition, we observed progressive shortening of telomeres over time in SUSM1 ALT cells treated with *MMS21*- or *SMC5*-specific RNAi, using both TRF and Q-FISH analyses (**Fig. 6a**,**b** and **Supplementary Fig. 6b**). This decrease in telomere length was similar to that observed in cells treated with *RAD50* RNAi (**Fig. 6b**), a known regulator of telomere length in ALT cells[23,24]. Consistent with the Q-FISH and TRF analyses, we observed an increased number of telomeres colocalizing with γH2AX, a marker of DSBs, in *MMS21* RNAi ALT cells (**Supplementary Fig. 6c**). However, this result could also be attributed to the increased number of unrepaired DSBs in *MMS21* RNAi cells[36]. These findings suggest that the SMC5/6 complex is required to maintain telomere length in ALT cells.

To examine whether this progressive shortening of telomeres in SMC5/6-knockdown ALT cells is specific to ALT cells, we generated stable HCT116 telomerase-positive cell lines in which MMS21 or SMC6 was knocked down by short hairpin RNAs (shRNAs). shMMS21 or shSMC6 HCT116 cells contained markedly reduced amounts of MMS21 or SMC6 even after 120 population doublings (**Supplementary Fig. 6d**). Unlike ALT cells, these telomerase-positive SMC5/6-knockdown cells did not show changes in telomere lengths, signal free ends or chromosomal end-to-end fusions (**Fig. 6c**, **Supplementary Fig. 6e** and **Supplementary Table 1**). Previous findings in yeast also support our observation that the SMC5/6 complex has minimal role in telomere length maintenance in telomerase-positive cells[35,46].

Critically short telomeres result in cellular senescence[3]. We next examined whether the shortened telomeres in SMC5/6-knockdown ALT cells led to cellular senescence. To do so, we determined whether these cells showed senescence-associated β-galactosidase (SA–β-gal) activity, a classical marker of cellular senescence. Knockdown of the SMC5/6 complex in SUSM1 ALT cells caused a progressive increase in the number of SA–β-gal–positive cells over time (**Fig. 6d**,**e**). This increase correlated with the substantial decrease in telomere lengths in these cells (**Fig. 6a**,**b**). ALT cells treated with SMC5/6-specific RNAi showed other expected senescence phenotypes, such as morphological changes and an upregulation of the cyclin-dependent kinase inhibitor p21 (**Fig. 6e**, **Supplementary Fig. 6f** and data not shown). Therefore, these results suggest that in the absence of the SMC5/6 complex, telomeres undergo progressive shortening in ALT cells, leading to cellular senescence.

DISCUSSION

The molecular mechanisms underlying the ALT pathway for telomere homologous recombination and telomere lengthening are largely unknown. Our results support a role for the SMC5/6 complex in telomere maintenance in ALT cells. More notably, the MMS21 SUMO ligase within the SMC5/6 complex stimulates the SUMOylation of multiple subunits of the shelterin complex, and this SUMOylation is required for APB formation.

The SMC5/6 complex and APB formation

The recruitment of telomeres to PML bodies (APBs) is a hallmark of ALT cells[16]. PML bodies are nuclear structures composed of PML aggregates and a diverse set of other proteins[47]. These nuclear bodies are very dynamic in nature, transiently recruiting and releasing

proteins[48]. PML bodies have been proposed to be involved in numerous cellular processes[48]. Though the roles of PML nuclear bodies in most of these processes are not clearly established, they are thought to facilitate post-translational modifications and localization of proteins to sites of action[48]. APBs are a specialized type of PML bodies that contain telomeres as well as factors involved in DNA repair[11,16]. Disruption of APBs results in progressive shortening of telomeres, suggesting that APBs promote telomere elongation in ALT cells[24].

We observed the localization of the SMC5/6 complex to APBs in ALT cells. Like other HR proteins in ALT cells[18,49], the SMC5/6 complex localizes with PML bodies in cells with sister chromatids—that is, cells in the late S/G2 phase of the cell cycle. The mechanism for cell cycle–regulated localization of the SMC5/6 complex and other HR proteins to APBs is unclear at present. However, extensive evidence suggests that the recruitment of HR proteins to APBs is functionally linked with telomere HR. Thus, the fact that telomeres and HR proteins localize to PML bodies only after replication of telomeres suggests that the majority of telomere HR occurring in APBs is through uneven sister-chromatid HR, instead of inter- or intra-chromatid telomere HR. The mechanism by which APB formation is restricted to ALT (telomerase-negative) cells is also unclear.

Previously, we have reported that the SMC5/6 complex facilitates sister-chromatid HR by promoting the recruitment of the cohesin complex to DSBs[36]. Here, we did not observe localization of cohesin to APBs in ALT cells. This was perhaps to be expected, as telomeres assemble into specialized t-loop structures resembling Holliday junctions that can potentially undergo replication-induced HR, resulting in elongation[11]. The proposed function of cohesin in HR is to promote strand invasion and exchange (Holliday-junction formation) by holding sister chromatids in close proximity. Therefore, cohesin may not be required for telomere HR, although this remains to be formally tested.

MMS21-dependent SUMOylation of shelterin and APB formation

SUMOylation is a post-translational modification that involves the covalent conjugation of SUMO to lysine residues on protein substrates[33]. Many components of PML nuclear bodies are SUMOylated[50]. PML itself is highly SUMOylated, and its SUMOylation is required for the formation of PML bodies[51]. The presence of a number of SUMOylated and SUMO-binding proteins in PML bodies has led to the proposal that PML bodies are cellular storage sites for SUMOylated proteins[50]. We show here that several subunits of the shelterin telomere-binding complex are SUMOylated, and this is enhanced by MMS21. Furthermore, depletion of MMS21 in ALT cells inhibits shelterin SUMOylation. Mutation of either TRF1 or TRF2 SUMOylation sites substantially blocks their MMS21-mediated SUMOylation and recruitment to PML bodies. This is consistent with the notion that SUMOylation of at least the TRF1 and TRF2 components of shelterin by MMS21 is important for APB formation in ALT cells.

The detailed mechanism by which MMS21-dependent SUMOylation of shelterin promotes APB formation is unknown. We envision two nonexclusive models (**Fig. 7**). In the recruitment model, MMS21-induced SUMOylation of shelterin in the nucleoplasm promotes the subsequent recruitment of shelterin and telomeres to PML bodies by the binding of SUMO to the SUMO-binding motifs of PML itself or other proteins in PML bodies. In the maintenance model, MMS21 localizes to PML bodies independently of telomeres. Upon the recruitment of shelterin to PML bodies, MMS21 SUMOylates shelterin to facilitate the maintenance of telomeres at PML bodies. Both mechanisms may occur in ALT cells and reinforce each other, thereby establishing a positive feedback loop. In either model, SUMOylation of shelterin facilitates localization of telomeres to PML bodies to promote telomere HR. Unequal HR between the two sister telomeres generates one long and one short sister telomere (**Fig. 7**). Two cells with differing replicative potentials are generated after sister-chromatid separation and cell division. The daughter cell with an elongated telomere will have an extended replicative potential.

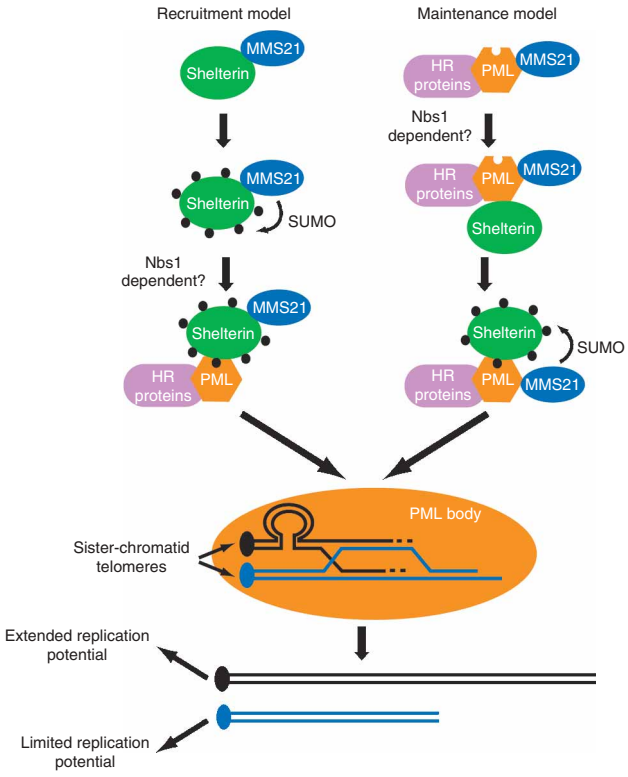

Figure 7 Two proposed models for how MMS21-induced shelterin SUMOylation promotes telomere length maintenance in ALT cells. In the recruitment model, MMS21 SUMOylates the shelterin complex in the nucleoplasm, resulting in the subsequent recruitment of telomeres to PML bodies for telomere recombination and lengthening. In the maintenance model, MMS21 localizes to PML bodies independently of telomeres. Upon recruitment of telomeres to PML bodies, MMS21-induced SUMOylation of shelterin maintains telomeres in PML bodies for telomere recombination.

We cannot rule out the possibility that the SMC5/6 complex has roles in telomere HR in addition to the recruitment of telomeres to PML bodies. Recent evidence suggests a role of the SMC5/6 complex in the late steps of HR to prevent the accumulation of lethal intermediates[52–54]. Furthermore, we observed a slightly higher rate of telomere shortening (~175 base pairs per population doubling) than is normally attributed to replicative attrition in telomerase-negative cells. Although the underlying reasons for this discrepancy are unknown at present, this observation is consistent with the SMC5/6 complex having T-SCE–independent functions at telomeres in ALT cells. For example, the SMC5/6 complex might protect telomeres against excessive levels of inappropriate HR, such as t-loop HR that can result in telomere shortening. In addition to the SMC5/6 complex, the MRN-complex protein NBS1 is also required for APB formation[23,24], although the mechanism by which it promotes APB formation is unknown. Future studies are needed to examine the interplay between the SMC5/6 complex and the MRN complex in promoting APB formation.

SUMOylation of shelterin and telomere HR

Notably, MMS21 stimulates the SUMOylation of four of the six known components of shelterin, including TRF1, TRF2, TIN2 and RAP1. There are numerous other examples in which multiple subunits of a given macromolecular complex are SUMOylated[43]. SUMOylation of multiple components in the same complex may alter its stability, potentially leading to its disassembly. It is conceivable that MMS21-dependent SUMOylation of shelterin may cause transient disassembly of the complex and the dissociation of its components from telomeres in APBs. This is an attractive model, as previous studies have shown that the shelterin complex prevents HR through binding to telomeric t-loops, therefore safeguarding telomerase-positive cells from potentially dangerous telomere HR[55]. ALT cells need to overcome this inhibition to allow HR to lengthen telomeres. MMS21-induced SUMOylation of shelterin may provide such a mechanism, deprotecting telomeres to facilitate telomere recombination in ALT cells. Consistent with this model, one putative SUMOylation site of TRF2 is located in the TRFH domain, which mediates the dimerization of TRF2 and its binding to TIN2. Therefore, SUMOylation of TRF2 could potentially alter TRF2's interactions with itself or TIN2.

Whether SUMOylation is a post-translational regulatory mechanism of shelterin in telomerase-positive cells remains to be determined. Recent reports have demonstrated the involvement of SUMOylation in the control of telomere length maintenance in telomerase-positive yeast[46]. Inactivation of Smt3 (yeast SUMO) or the Pli1 E3 SUMO ligase, but not MMS21, results in increased telomere length in yeast[46]. Therefore, it is likely that MMS21-independent SUMOylation controls telomere length regulation in telomerase-positive cells.

METHODS

Cell culture, transfections, short interfering RNAs and flow cytometry. All cell lines were maintained in DMEM (Invitrogen) supplemented with 10% (v/v) FBS (Gemini) and 100 μg ml^{-1} penicillin and streptomycin (Invitrogen). At 40%–50% confluence, cells were transfected with plasmids or siRNAs with either Effectene (Qiagen) or Oligofectamine (Invitrogen), respectively, according to the manufacturers' instructions. The siRNA oligonucleotides used in this study are specific to *lacZ* (5′-GCGCCGAAAUCCCGAAUCUdTdT-3′), luciferase (5′-CUUACGCUGAGUACUUCGAdTdT-3′), human *MMS21* (5′-CUCUGGUAUGGACACAGCUdTdT-3′), mouse *Mms21* (5′-GGAGUUGACGAAGAUAUGAdTdT-3′), *NBS1* (5′-AAGAAGCAGCCTCCACAAAdTdT-3′), *RAD50* (5′-GGAUCUUCAGACAGGAUUCdTdT-3′), *SMC5* (5′-GAAGCAAGAUGUUAUAGAAdTdT-3′) and *SMC6* (5′-AGAGCGGCTTACTGAACTAdTdT-3′). *RAD51* siRNAs were obtained from Dharmacon's SMARTpool service. Knockdown efficiency of at least 75% with these oligonucleotides was confirmed, as described[34,36]. For cell-cycle arrest in G1/S or G2/M, cells were treated for 24 h with 2 mM thymidine or for 4 d with media lacking methionine, respectively. Flow-cytometry analysis of cell-cycle profiles was done as described[34].

Immunofluorescence, immunoblotting, immunoprecipitation and antibodies. For immunofluorescence, cells were plated in four-well chamber slides (LabTek) and treated as indicated. Cells were washed in PBS and fixed in 1% (w/v) paraformaldehyde for 15 min at room temperature. The fixed cells were washed in PBS and then permeabilized and blocked for 20 min at 4 °C in incubation buffer (PBS containing 0.2% (v/v) Triton X-100 and 3% (w/v) BSA). After blocking, cells were incubated in primary antibody (2 μg ml^{-1}) for 1 h at room temperature in incubation buffer. Cells were then washed with PBS and incubated with fluorescent secondary antibodies (Alexa Fluor 488 or 647, Molecular Probes) in incubation buffer for 30 min at room temperature. After incubation, cells were washed with PBS and their nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; 1 μg ml^{-1}). Slides were mounted and viewed with a ×63 objective on a Zeiss Axiovert 200M fluorescence microscope. Images were acquired with a CCD camera using the Slidebook imaging software (Intelligent Imaging Innovations). All images were taken at 0.5-μm intervals, deconvolved using the nearest-neighbor algorithm and stacked to better resolve foci. For quantification, multiple random fields were captured and 50–100 cells were counted in each of three independent experiments.

For immunoblotting, cells were lysed in SDS sample buffer, sonicated, boiled, separated by SDS-PAGE and blotted with the indicated antibodies. Horseradish peroxidase–conjugated goat anti-rabbit or goat anti-mouse IgG (Amersham Biosciences) was used as a secondary antibody, and immunoblots were developed using the ECL reagent (Amersham Biosciences) according to the manufacturer's protocols.

For immunoprecipitation, whole-cell lysate was made by lysing cells in NP-40 lysis buffer (50 mM Tris-HCl (pH 7.7), 150 mM NaCl, 0.5% (v/v) Nonidet P-40, 1 mM DTT and 1× protease inhibitor cocktail) on ice for 15 min, sonicating three times, then centrifuging the lysed cells at 16,000*g* for 15 min at 4 °C. Anti-Flag affinity gel (Sigma) was incubated with the supernatants for 2 h at 4 °C. The beads were then washed five times with the NP-40 lysis buffer. The Flag-tagged proteins bound to the beads were eluted with Flag peptide (Sigma), dissolved in SDS sample buffer, boiled, separated by SDS-PAGE and blotted with the indicated antibodies as described above.

The commercial antibodies used in this study were as follows: anti-Flag M2 (Sigma, F1804), anti-γH2AX (Upstate, 05-636), anti-Myc (Roche, 11667203001), anti-p21 (Santa Cruz, SC-6246), anti-PML (Santa Cruz, SC-5621 or SC-966), anti-RAD50 (GeneTex, GTX70228), anti-SCC1 (Bethyl Laboratory, A300-080A), anti-SMC1 (Bethyl Laboratory, A300-055A), anti-SMC5 (Bethyl Laboratory, A300-236A), anti-SMC6 (Bethyl Laboratory, A300-A237A) and anti-TRF2 (Calbiochem, OP129 or Upstate, 05-521). The production of polyclonal anti-MMS21 has been described[34].

Telomeric sister-chromatid exchange fluorescence *in situ* hybridization and quantitative telomeric fluorescence *in situ* hybridization. For CO-FISH analysis, human SUSM1 or G5 *Terc*$^{-/-}$ *Wrn*$^{-/-}$ Ras^{v12} MEF ALT cells[41] (see Acknowledgments) were transfected with the indicated siRNAs for 48 h before addition of a 3:1 ratio of bromodeoxyuridine to bromodeoxycytidine (brdU/brdC, 7.5 μM/2.5 μM) for 16 h. Chromosome spreads were prepared as described[36] and treated with 0.5 mg ml^{-1} RNase A at 37 °C for 10 min. Slides were incubated in 2× SSC containing 10 μg ml^{-1} Hoescht 33258 for 15 min, then exposed to 365-nm UV light (Stratalinker 1800) for 30 min. Nicked DNA was then degraded by incubation of slides in 1.6% (v/v) ExoIII (Promega) for 10 min. Slides were then treated with 5 μg ml^{-1} pepsin for 7.5 min at 37 °C and washed in PBS. Chromosome spreads were then dehydrated through an ethanol series (70% (v/v), 85% (v/v) and 100% ethanol). Slides were allowed to air-dry in the dark before incubation with a fluorescein isothiocyanate (FITC)-labeled G-rich telomeric peptide–nucleic acid (PNA) probe (amino-FITC-TTAGGGTTAGGGTTAGGG-carboxyl; Panagene) for 1 h at room temperature. Slides were washed in PBS containing 0.02% (v/v) Tween-20, then incubated in cyanine 3 (Cy3)-labeled C-rich telomeric PNA probe (amino-Cy3-CCCTAACCCTAACCCTAA-carboxyl; Applied Biosystems) for 1 h at room temperature. Finally, slides were washed in PBS containing 0.02% (v/v) Tween-20 for 20 min at 57 °C, stained with 1 μg ml^{-1} DAPI in 2× SSC containing 0.02% (v/v) Tween-20 for 5 min and mounted. Chromosomes were imaged as described above, and the number of T-SCEs was quantified in 50 cells in multiple experiments by counting the frequency of unequal exchange events that result in unequal signal intensity of both FITC and Cy3 on the two sister telomeres. Chromosomes with closely associated or indistinguishable sister telomeres were not counted. Control experiments confirmed that signals generated were not due to unintended denaturation of DNA and were dependent on brdU and brdC incorporation.

For Q-FISH analysis, SUSM1 cells were treated with the indicated siRNAs for the indicated number of population doublings. Cells were treated with siRNAs every third day and passaged as needed. To stably knock down SMC5/6 complex components in HCT116 cells, cells were transfected with pSuperior-neo plasmid (Oligoengine) containing shRNA sequences for either luciferase, *MMS21* or *SMC6* (as described above), then selected with G418. Colonies were expanded and efficiency of knockdown was analyzed by western blotting. Cells were maintained in culture under G418 selection for up to 120 population doublings. Chromosome spreads from either SUSM1 siRNA-transfected or HCT116 shRNA-stable cell lines were made as described[36]. Slides were treated with pepsin, washed and dehydrated as described above. Telomeres

were stained with a Cy3–telomere PNA probe by incubation at 70 °C for 7 min, then incubated for up to 60 min at room temperature. Slides were washed and processed after probe incubation as described above. Slides were imaged as described above, and the intensity of telomeres was quantified in 50 cells in multiple experiments. Additionally, the number of chromosomes containing telomere signal free ends and end-to-end fusions was quantified.

For telomere restriction-fragment analysis, SUSM1 cells were transfected with siRNAs for the indicated number of population doublings as described above. Genomic DNA was collected and digested with HinfIII and RsaI for 8 h. The digested DNA, along with a radiolabeled DNA ladder, was run on a 25-cm, 0.7% (w/v) agarose gel for 18 h. The gel was then denatured and dried for 1 h at 50 °C. The dried gel was neutralized, then prehybridized for 1 h at 42 °C in hybridization solution (5× SSC, 5× Denhardt Solution, 10 mM Na_2HPO_4, 1 mM $Na_2H_2P_2O_7$). After prehybridization, the gel was incubated overnight at 42 °C in hybridization solution containing ^{32}P end-labeled $(TTAGGG)_4$ oligonucleotide. The gel was then washed for 15 min in 2× SSC, then three times for 10 min each in 0.1× SSC containing 0.1% (w/v) SDS. The gel was exposed overnight to a phosphorimaging screen and scanned on Fuji Imager. Approximate telomere lengths were quantified using ImageQuant software (Molecular Dynamics) and TELORUN.

Senescence-associated β-galactosidase assay. Cells were transfected with the indicated siRNAs for the indicated number of population doublings as described above and then plated in chamber slides for 24 h. Slides were washed in PBS and fixed in PBS containing 2% (v/v) formaldehyde and 0.2% (v/v) glutaraldehyde for 5 min, then extensively washed in PBS and incubated in X-gal staining solutions (40 mM citric acid/sodium phosphate (pH 6.0), 1 mg ml^{-1} X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, 2 mM $MgCl_2$) for 16 h at 37 °C. Slides were washed extensively, mounted and imaged as described above. The number of SA–β-gal–positive cells was quantified as the percentage of blue cells in random fields from multiple experiments.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

P.R.P. and H.Y. designed the research. P.R.P. performed the experiments and analyzed the results. P.R.P. and H.Y. wrote the paper.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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