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Spartan deficiency causes genomic instability and progeroid phenotypes

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Spartan (also known as DVC1 and C1orf124) is a PCNA-interacting protein implicated in translesion synthesis, a DNA damage tolerance process that allows the DNA replication machinery to replicate past nucleotide lesions. However, the physiological relevance of Spartan has not been established. Here we report that Spartan insufficiency in mice causes chromosomal instability, cellular senescence and early onset of age-related phenotypes. Whereas complete loss of Spartan causes early embryonic lethality, hypomorphic mice with low amounts of Spartan are viable. These mice are growth retarded and develop cataracts, lordokyphosis and cachexia at a young age. Cre-mediated depletion of Spartan from conditional knockout mouse embryonic fibroblasts results in impaired lesion bypass, incomplete DNA replication, formation of micronuclei and chromatin bridges and eventually cell death. These data demonstrate that Spartan plays a key role in maintaining structural and numerical chromosome integrity and suggest a link between Spartan insufficiency and progeria.
 uninterrupted DNA replication is important for timely duplication of the genome before mitotic entry. However, the replication machinery may stall or slow down on replication stress caused by DNA lesions, limited nucleotide pools and repetitive DNA sequences. Failure to deal with such replication stress could lead to genomic instability, which is associated with cancer and ageing. In fact, genetic defects in replicative stress response have been implicated in genome instability syndromes that are characterized by increased cancer incidence and/or premature ageing phenotypes. Although replication stress has been commonly linked to cancer susceptibility, accumulating evidence from mouse studies also supports the role of replication stress in premature ageing phenotypes and ageing of stem cells.

Genomic DNA lesions are one of the common sources of replication stress. Because high-fidelity replicative polymerases cannot accommodate damaged bases in their active sites, unrepaired DNA lesions pose a risk of replication fork stalling, double-strand DNA breaks and cell death. To avoid such threat to genome stability, cells are equipped with a special replication system called transcription synthesis (TLS), which allows continuous replication across DNA lesions. During TLS, monoubiquitination of proliferating cell nuclear antigen (PCNA), a sliding clamp for DNA polymerases, induces switching from replicative DNA polymerases to specialized TLS polymerases, thereby allowing bypass of DNA lesions. Although TLS protects replication forks from stalling at DNA lesions, it is potentially mutagenic because it utilizes error-prone DNA polymerases.

Spartan was recently identified as a novel regulator of TLS that guards against TLS-associated mutagenesis. Spartan is recruited to DNA damage sites through the interaction with PCNA via its PIP (PCNA-interacting peptide) motif and the interaction of the zinc-finger domain UBZ4 with ubiquitin on PCNA or other proteins. Several groups reported that Spartan promotes monoubiquitination of PCNA, thereby facilitating recruitment of TLS polymerases, whereas other groups showed that Spartan recruits an ATP-dependent segregase p97 (also known as valosin-containing protein (VCP)) to the sites of TLS to facilitate dissociation of TLS polymerases. In addition, our previous study linked Spartan to regulation of the error-prone TLS mechanism involving the DNA polymerase Pol θ. While these studies clearly implicated Spartan in TLS regulation, whether Spartan is required for TLS is unknown.

In this study, we create Sprtn-targeted mouse models and investigate the physiological importance of Spartan. We demonstrate essential roles of Spartan in lesion bypass, completion of DNA replication, genome stability and cell viability. Interestingly, Sprtn insufficiency causes senescence and progeria in mice. Our findings establish the critical role of Spartan in the cell cycle and the maintenance of genome integrity and provide the link between Spartan insufficiency and progeria.

**Results**

**Sprtn knockout causes embryonic lethality in mice.** To explore the physiological relevance of Spartan, we generated a series of mice with graded reduction in Spartan expression using hypomorphic (H) and knockout (KO) Sprtn alleles that we generated by gene targeting (Fig. 1a,b). Intercrossing of Sprtn+/− mice failed to produce Sprtn−/− live offspring and no Sprtn−/− embryos were found from embryonic day (E) 7.5 to E13.5 (Table 1), suggesting that Sprtn KO in mice causes early embryonic lethality. To examine the effect of Sprtn KO on early embryogenesis, we isolated blastocysts at E3.5 and cultured them in vitro for 6 days. Genotyping was successful for all the blastocysts collected, and Sprtn−/− blastocysts were obtained at normal Mendelian frequency (Fig. 1c; Table 2). However, Sprtn−/− blastocysts failed to hatch and enlarge the inner cell mass when cultured in vitro (Fig. 1d; Table 2), indicating that death occurred prior to the implantation stage.

**Effects of Sprtn KO on cell proliferation and survival.** To further investigate the role of Sprtn at the cellular level, we generated a conditional Sprtn KO system in mouse embryonic fibroblasts (MEFs). Sprtn+/+, SprtnF/F, and SprtnF−/− MEFs were immortalized and transduced with retroviruses expressing Cre-ER. Treatment with 4-hydroxytamoxifen (4-OHT) resulted in excision of exon 2, efficiently converting the floxed allele to a KO allele (Fig. 2a). SprtnF−/− MEFs, but not Sprtn−/− and SprtnF+/− MEFs, showed markedly reduced proliferation and increased apoptosis after exposure to 4-OHT (Fig. 2b,c), suggesting that Sprtn is essential for cell proliferation and survival.

**Cell cycle effects from the loss of Sprtn.** To determine whether Sprtn KO has any effects on the cell cycle, we examined the cell cycle profile of MEFs with various genotypes by flow cytometry. A large portion of 4-OHT-treated SprtnF−/− MEFs accumulated with 4C DNA content after treatment, whereas Sprtn+/+ and SprtnF+/+ cells were unaffected (Fig. 2d). To examine S-phase progression of Sprtn−/− MEFs more closely, we performed 5-bromodeoxyuridine (BrdU) pulse-chase experiments, in which S-phase cells were pulse labelled with BrdU and chased in BrdU-free media every 2h (Supplementary Fig. 1a). Sprtn−/− MEFs initially progressed through S phase at a similar rate to wild-type cells, but subsequently arrested with 4C DNA content at high rates (Supplementary Fig. 1b). These results suggest that Sprtn−/− cells exhibit normal S-phase progression, but accumulate in late S or G2/M phases. A relatively small proportion of Sprtn−/− MEFs were positive for p-H3ser10, a well-established mitotic marker, indicating that Sprtn−/− cells accumulate before entry into mitosis (Supplementary Fig. 1c). Collectively, these results suggest that Spartan is essential for cell proliferation and faithful progression through late S and G2 phases.

The Spartan protein possesses multiple domains including SprT (a zinc metalloprotease-like domain), a SHP box (a P97-interacting motif), a PIP box (PCNA-interacting motif) and UBZ4 (a ubiquitin-binding domain) (Fig. 2e, top). We asked whether these domains are necessary for the function of Spartan in the cell cycle. Wild-type human Spartan, which was expressed at the similar level to endogenous Spartan in a human fibroblast cell line (Supplementary Fig. 2a), suppressed accumulation of cells in late S or G2 phase following loss of Sprtn in MEFs (Fig. 2e). On the other hand, SpartanE112A, a mutant with a substitution in the putative active site of the metalloprotease-like domain SprT, failed to rescue the cell cycle defect in Sprtn−/− MEFs despite the similar expression level to wild-type Spartan (Fig. 2e; Supplementary Fig. 2a). In contrast, the ectopic expression of human SpartanP10 and SpartanUBZ, which exhibit diminished interactions with PCNA and ubiquitin, respectively, efficiently suppressed the cell cycle defect in Sprtn−/− MEFs (Supplementary Fig. 2b,d). Similarly, mouse SpartanSHP, which harbours the mutations that diminish interaction with p97 (ref. 13), also suppressed the cell cycle defect in Sprtn−/− MEFs as efficiently as wild-type Spartan (Supplementary Fig. 2c,e). Altogether, these results suggest that the putative active site of the SprT domain is essential for the cell cycle function of Spartan, while the PIP, UBZ and SHP domains are dispensable.
Loss of Spartan causes DNA damage and checkpoint activation. To determine the cause of the cell cycle defect in Sprtn−/− MEFs, we asked whether Spartan deficiency engages the DNA damage response pathway. γH2AX foci, a marker of DNA damage, markedly increased in Sprtn+/− and Sprtn+/+ MEFs (Fig. 3a). Consistent with increased DNA damage, Sprtn−/− MEFs showed high rates of double-strand DNA breaks as measured by immunostaining of the DNA recombination protein Rad51 (Fig. 3b) and profound activation of the checkpoint kinase Chk2 (Fig. 3c). Thus, loss of Sprtn in MEFs seemingly induces DNA damage, most likely DNA breaks, leading to activation of the DNA damage checkpoint.

Depletion of Spartan causes genomic instability. The elevated levels of DNA damage accompanied by checkpoint activation in Sprtn−/− MEFs prompted us to assess whether loss of Spartan induces genomic instability. Microscopic analysis of DAPI-stained MEFs revealed that a substantial fraction of mitotic Sprtn−/− MEFs exhibited chromatin bridges and micronuclei, structures that have been associated with unresolved replication intermediates and unrepaired DNA breaks (Fig. 4a–c). In addition, these MEFs had increased numbers of 53BP1 nuclear bodies that signify incomplete DNA replication (Fig. 4d). These data raise the possibility that the genome of Sprtn-deficient cells is subject to under-replication. Consistent with this interpretation, the vast majority of chromosomal abnormalities observed in the mitotic spreads of Sprtn−/− MEFs were chromosome gaps, which might...
represents under-replicated regions (Fig. 4e,f). In summary, these results suggest that Spartan is crucial for maintaining genome integrity.

**Lesion bypass defects in Sprtn \(^{-/-}\) cells.** To examine the effect of Sprtn inactivation on DNA replication more directly, we measured movement of replication forks in Sprtn \(^{-/-}\) MEFs using DNA fiber assays, in which replication tracts were sequentially labelled with the thymidine analogues IdU and CldU (Fig. 5a). Lengths of CldU-labelled DNA in Sprtn \(^{-/-}\) MEFs were not significantly different from those of Sprtn \(^{+/+}\) MEFs (Fig. 5b), suggesting that the majority of replication forks are not affected by Spartan loss, an observation consistent with the normal S-phase progression in Sprtn \(^{-/-}\) cells (Supplementary Fig. 1b).

Given that Sprtn \(^{-/-}\) cells have mostly intact replication forks but still accumulate in late S/G2 phases accompanied by under-replication phenotypes, we hypothesized that Spartan might be important for DNA replication under stress, for example, imposed by endogenously created DNA lesions. Previous reports have indeed linked Spartan’s function to TLS\(^{9–15}\), although whether Spartan is necessary for the bypass of DNA lesions during DNA replication has not been established. We therefore tested the requirement of Spartan for lesion bypass during DNA replication. DNA fiber assays were performed with ultraviolet light exposure, which introduces lesions acted on by TLS, between IdU and CldU labelling (Fig. 5a). Lesion bypass efficiency is expressed as elongation ratios (CldU/IdU), which would be reduced with lesion bypass defects. Strikingly, Sprtn KO in MEFs caused reduced fork progression at ultraviolet-induced lesions (Fig. 5c), suggesting that Spartan is important for ultraviolet-induced lesion bypass. We also examined whether Spartan plays a role in the stability of stalled forks by measuring replication fork restart after hydroxyurea (HU) treatments (Supplementary Fig. 3a). Sprtn KO did not increase the amount of collapsed forks on HU treatments (Supplementary Fig. 3b), suggesting that Spartan is not necessary for replication restart after fork stalling.
ubiquitination in response to ultraviolet irradiation and dissociation of Pol η from ultraviolet-induced DNA damage sites, the two processes that have been reported to be impaired in Spartan-depleted human cells.\(^9\)\(^{10}\)\(^{12}\)\(^{13}\) PCNA ubiquitination after ultraviolet irradiation was largely intact in Sprtn\(^{-/}\) cells (Supplementary Fig. 3c), suggesting that Spartan is not absolutely necessary for ultraviolet-induced PCNA ubiquitination. On the other hand, in agreement with previous reports,\(^{12}\)\(^{13}\) we observed prolonged retention of enhanced green fluorescent protein (EGFP)-Pol η foci after ultraviolet irradiation in Sprtn\(^{-/}\) cells (Supplementary Fig. 3d), although it remains to be determined whether this is due to a failure to extract Pol η from DNA damage sites as proposed previously\(^{12}\)\(^{13}\) or if it reflects the lesion bypass defects we report in this study (Fig. 5c).

**Figure 3** | Sprtn KO causes DNA damage and checkpoint activation. 
(a) \(γ\)H2AX focus formation. The indicated MEFs treated with MeOH or 4-OHT for 48 h were stained with anti-\(γ\)H2AX. At least 300 cells were scored for \(γ\)H2AX foci and percentages of cells with 5 or more foci are shown. Values are mean ± s.d. of three independent experiments. NS, not significant; *\(P<0.05\); ****\(P<0.0001\) (two-tailed unpaired t-test). (b) Rad51 focus formation. The indicated MEFs were stained with anti-Rad51 after 48h treatment with MeOH or 4-OHT. At least 300 cells were scored for Rad51 foci. Experiments were performed in triplicate and mean ± s.d. is shown. ****\(P<0.0001\) (two-tailed unpaired t-test). (c) Western blot analyses of phospho-Chk1 and Chk2. The indicated MEFs were treated with 4-OHT and harvested at various time points. Sprtn\(^{-/}\) (H7) cells treated with ultraviolet (\(40\) J m\(^{-2}\)) or ionizing radiation (10 Gy) are shown as positive controls for checkpoint kinases activation. Chk1 is used as a loading control. P-Chk1, phospho-Chk1 (Ser345); P-Chk2, phospho-Chk2. Uncropped blots are shown in Supplementary Fig. 7.

Next we assessed the requirement of the Spartan domains for the bypass of ultraviolet-induced lesions by re-expressing Spartan mutants in conditional Sprtn KO MEFs. The lesion bypass defect in Sprtn\(^{-/}\) cells was rescued by the introduction of wild-type human Spartan, but not the mutant that harbour the E112A mutation in the putative active site (Fig. 5d), suggesting that the role of Spartan in lesion bypass requires an intact SprT domain. On the other hand, the PIP\(^*\), UBZ4\(^*\) or SHP\(^*\) mutants of Spartan exhibited partially diminished abilities to rescue the lesion bypass defects in Sprtn\(^{-/}\) MEFs (Fig. 5e,f). Taken together, these results suggest that Spartan is dispensable for the replication of the bulk of the genome, but is crucial for replication past DNA damage. This function of Spartan in lesion bypass depends on the putative active site of the SprT domain, but only partially on the PIP, UBZ4 and SHP domains.

To obtain more insight into the role of Spartan in lesion bypass, we examined the effect of Sprtn KO on PCNA growth defects and genome instability in Sprtn\(^{H/-}\) cells. Finally, we examined the effect of Sprtn insufficiency by producing Sprtn hypomorphic mice. The targeted Sprtn\(^{neo}\) allele is hypomorphic (hereafter referred to as Sprtn\(^{H/-}\)), producing wild-type mRNAs at reduced levels due to the cryptic exon in the Neo cassette (Supplementary Fig. 4a). In contrast to Sprtn\(^{-/}\) MEFs, Sprtn\(^{H/-}\) MEFs, which contain one KO and one hypomorphic allele that produce reduced amounts of wild-type Spartan, were viable, but proliferated at reduced rates (Supplementary Fig. 4b–d). Furthermore, Sprtn\(^{H/-}\) MEFs had increased rates of aneuploidy and micronuclei (Supplementary Fig. 4e,f; Supplementary Table 1), indicating that, in addition to structural chromosome damage, Spartan insufficiency leads to numerical chromosome instability.

**Growth defects and genomic instability in Sprtn\(^{H/H}\) mice.** Although no viable Sprtn\(^{H/-}\) offspring were found in crossing of Sprtn\(^{+/}\) and Sprtn\(^{H/-}\) mice, Sprtn\(^{H/-}\) mice were born at near Mendelian frequency from Sprtn\(^{+/}\) breeding pairs (Sprtn\(^{+/}\), Sprtn\(^{++/}\) and Sprtn\(^{H/-}\) at 26.6, 53.3 and 20.1%, respectively), despite substantially reduced Sprtn expression (Fig. 6a,b). At weaning, Sprtn\(^{H/-}\) mice appeared healthy but were overly smaller than control littermates. Dwarfism was observed in both sexes and persisted as Sprtn\(^{H/-}\) mice developed to adulthood (Fig. 6c). To investigate in more detail the effects of Sprtn insufficiency in Sprtn\(^{H/H}\) mice, we isolated primary lung fibroblasts and prostate epithelial cells from Sprtn\(^{+/}\) and Sprtn\(^{H/-}\) mice. Sprtn expression was reduced to less than 10% in the Sprtn\(^{H/H}\) cells (Supplementary Fig. 5a,b). Sprtn\(^{H/-}\) lung fibroblasts showed elevated structural and numerical chromosomal instabilities, as evidenced by increased rates of \(γ\)H2AX foci, aneuploidy and micronuclei (Fig. 6d,e; Table 3). In addition, Sprtn\(^{H/H}\) lung fibroblasts displayed defects in fork progression at ultraviolet-induced lesion, which was also confirmed in Sprtn\(^{H/H}\) prostate epithelial cells (Fig. 6f; Supplementary Fig. 5c). Taken together, these results indicate that cells from Sprtn hypomorphic mice exhibit genome instability and lesion bypass defects.

**Reduced expression of Sprtn causes accelerated ageing in mice.** As young adults, Sprtn\(^{H/H}\) mice exhibited no overt phenotypes other than dwarfism. However, by 12 months of age, all Sprtn\(^{H/H}\) mice (seven out of seven mice) had developed lordokyphosis (concavity in the curvature of the lumbar and cervical spine), cataracts and cachexia (Fig. 7a,b), three phenotypes often seen in mouse models of progeria.\(^{3}\)–\(^{5}\) The total fat mass and percentage of body fat of these mice were dramatically reduced (Fig. 7c). In-depth analysis of individual fat depots revealed that the relative weights of the paraovarian, inguinal adipose tissue (IAT), perirenal and subscapular adipose tissue were all significantly reduced in size (Fig. 7c). Consistent with fat tissue atrophy, fat
cells in Sprtn<sup>H/H</sup> IAT were significantly smaller in size (Fig. 7d). Furthermore, IAT stained highly positive for senescence-associated-β-galactosidase (SA-β-GAL) and showed increased levels of key senescence markers, including p16<sup>Ink4a</sup>, p19<sup>Arf</sup> and PAI-1 (Fig. 7e,f). These data suggest that increased accumulation of cellular senescence in fat causes dysfunction and atrophy of this tissue. Lordokyphosis in progeroid mouse models is often associated with osteoporosis and/or muscle wasting<sup>24,26</sup>. However, neither disorder seemed to apply to Sprtn<sup>H/H</sup> mice as their bone mineral content and density (Supplementary Fig. 6a,b) and gastrocnemius, abdominal and paraspinal muscle fibre diameters (Supplementary Fig. 6c–e) were normal. Consistent with segmental progeria, Sprtn<sup>H/H</sup> mice showed impaired exercise ability as measured by the use of a treadmill: the duration of exercise, the distance travelled and the overall amount of work performed were all reduced at the age of 12 months (Fig. 7g). Taken together, these data suggest that the reduced expression of Sprtn leads to development of various progeroid phenotypes, including dwarfism, cataracts, lordokyphosis, fat tissue dysfunction and accumulation of senescent cells.

**Discussion**

In this study, we investigate the physiological roles of Spartan using a series of mice with graded reduction in Sprtn expression. Our data uncover a striking requirement of Spartan in cell survival and mouse development. Specifically, Sprtn KO causes early embryonic lethality in mice, while conditional KO in MEFs leads to incomplete DNA replication and cell death. In contrast, Sprtn hypomorphic mice are viable but develop premature ageing phenotypes accompanied by elevated levels of DNA damage and genome instability. Extending previous studies that implicated Spartan in TLS, we demonstrate that lesion bypass is impaired in Sprtn<sup>−/−</sup> cells when assayed with external DNA damage. Taken together, our data best fit a model whereby Spartan is essential for DNA replication under stress, which can...
To our knowledge, the current study is the first to demonstrate the requirement of Spartan in lesion bypass. This suggests that Spartan plays a more fundamental function than just suppressing damage-induced mutations. Our experiments using a variety of Spartan mutants provide mechanistic insight into the role of Spartan in lesion bypass. The results indicate that bypass of ultraviolet-induced lesions is dependent on the SprT domain of Spartan but only partially on the SHP, PIP and UBZ4 domains (Fig. 5d–f). The dependence on the SprT domain is consistent with our previous observation that suppression of ultraviolet-induced mutagenesis by Spartan required an intact SprT domain13. On the other hand, the partial requirement of the PIP and UBZ4 domains indicates that recruitment of Spartan to ubiquitinated PCNA is important, but not absolutely necessary for Spartan’s function in lesion bypass. This is consistent with the report that PCNA ubiquitination is important but not absolutely required for TLS27. Although not absolutely required, the SHP box of Spartan clearly has an important role in lesion bypass. Previous reports suggested a model in which the Spartan-p97 interaction facilitates p97-mediated removal or degradation of Pol η from DNA damage sites. Consistent with this model, Sprtn−/− MEFs showed prolonged retention of ultraviolet-induced Pol η foci (Supplementary Fig. 3d). Our findings that both the SprT domain and the SHP box are involved in lesion bypass raise an intriguing possibility that p97 and the SprT domain of Spartan cooperate in polymerase switching at the sites of TLS.

Although it is not clear why Sprtn KO cells die, it is tempting to speculate that failure to execute TLS is a major contributing factor given that the catalytic subunit of the TLS polymerase Pol ζ has been shown to be essential in mice28–33. Consistently, our previous study implicated Spartan in regulation of Pol ζ15. Therefore, Spartan might be required for efficient TLS in response to stalled replication at endogenous DNA damage sites, thereby allowing cells to complete S phase. In addition, given that TLS polymerases are important for efficient replication and stability of difficult-to-replicate sequences34–37, it is possible that Spartan-deficient cells exhibit cell cycle defects and genome instability due to replication failure at the sites of inherent replication stress such as difficult-to-replicate regions. Alternatively, given the previous observation that Spartan interacts with the DNA polymerase δ subunit10,15, it is possible that Spartan has a more general role in DNA replication unrelated to the TLS pathway. In this case, Spartan might be dispensable for replication of the majority of the genome, with only certain regions requiring Spartan function. Our experiments found that distinguishing these two possibilities might be challenging, because PIP and UBZ4, two TLS-linked domains of Spartan, were not only dispensable for cell viability (Supplementary Fig. 2d), but also not completely required for the function of Spartan in lesion bypass (Fig. 5e). In contrast, we found that the SprT is necessary for both lesion bypass and cell viability (Fig. 5d). Therefore, functional studies of the putative zinc metalloprotease domain in TLS or other processes will clarify the cause of incomplete DNA replication and cell death following Sprtn KO.

We find that Spartan deficiency leads to both structural and numerical chromosome instabilities, both of which have been associated with cancer predisposition. SprtnH/H mice sacrificed at 1 year of age were thoroughly screen for the presence of tumours, but none were detected. On the other hand, with 100% penetrance, these mice presented multiple age-related and progeroid phenotypes, suggesting that the genomic stress resulting from Spartan insufficiency predominantly engages signalling pathways that have been associated with cell fate decisions that negatively impact tissue homeostasis and repair, such as the p53 and the p16INK4a tumour suppressor pathways38. Consistent with this, we observed a profound accumulation of senescent cells in fat tissue of Spartan-insufficient mice, as well as

Figure 5 | Effects of Sprtn KO on DNA replication forks. (a) Schematic representation of DNA fiber assays. MEFs were treated with MeOH or 4-OHT for 48 h and sequentially labelled with IdU and CldU to mark ongoing replication. A picture of a representative replication track is shown. At least 100 fibres were scored for each sample in all of the DNA fiber experiments. (b) A box plot showing distribution of the lengths of DNA fiber tracts in Sprtn−/− MEFs treated with MeOH or 4-OHT for 48 h. NS, not significant (P = 0.829, two-tailed unpaired t-test). (c) Effect of ultraviolet irradiation on replication forks. DNA fiber assays were performed with Sprtn−/− MEFs with or without ultraviolet irradiation (40 J m−2) between IdU and CldU labelling. Distribution of replication forks at different CldU/IdU ratios is shown. (d–f) Effect of ultraviolet irradiation on replication forks. Experiments were performed as in (c) using SprtnH/H, Cre-ERΔ2 MEFs (K3) expressing wild-type human Spartan or the E112A mutant (d), wild-type human Spartan, the PIP∗ or the UBZ∗ mutant (e), and wild-type mouse Spartan or the SHP∗ mutant (f). Horizontal red lines in c–f indicate median values.

be imposed by DNA lesions or difficult-to-replicate sequences such as repetitive DNA elements. This model is also consistent with previous studies that have linked replication stress with age-related phenotypes in mice4–6. In summary, our findings reveal the fundamental role of Spartan in the cell cycle and genome stability and support the link between deficiency in replication stress response and progeria.
assays were performed with significance is \( P \). Distribution of replication forks at different CldU/IdU ratios is shown. A horizontal red line indicates median value. 

However, it will be important to screen a larger cohort of data suggest that Spartan insufficiency primarily causes progeria; leads to genome instability and progeria. In conclusion, the current study provides the evidence that Spartan is essential for the normal cell cycle and cell viability. Our data strongly suggest that Spartan deficiency causes cell cycle defects through accumulation of incompletely replicated genomic regions. Further studies on the potential enzyme activity of the SprT domain will reveal the full function of Spartan and provide insight into the mechanism by which insufficiency of Spartan leads to genome instability and progeria.

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<th>Table 3</th>
<th>Karyotypes of primary lung fibroblasts.</th>
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<td>Lung fibroblast genotype ( (n) )</td>
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<tr>
<td>( \text{Sprtn}^{+/+} ) (2)</td>
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<td>( \text{Sprtn}^{+/H} ) (2)</td>
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Karyotyping was performed with lung fibroblasts isolated from two mice per genotype (#45 and #46 for \( \text{Sprtn}^{+/+} \), #84 and #87 for \( \text{Sprtn}^{+/H} \)) at passage 5. Twenty-five mitotic figures were inspected for each sample.

In conclusion, the current study provides the evidence that Spartan is essential for the normal cell cycle and cell viability. Our data strongly suggest that Spartan deficiency causes cell cycle defects through accumulation of incompletely replicated genomic regions. Further studies on the potential enzyme activity of the SprT domain will reveal the full function of Spartan and provide insight into the mechanism by which insufficiency of Spartan leads to genome instability and progeria.

**Methods**

**Generation of Sprtn-targeted mice.** The Sprtn-targeting vector was constructed in modified pNTKV1901-frt/loxP22 with a new MfeI site for left homology arm clamping. Mouse Sprtn genomic sequences were amplified by PCR from a BAC clone (RPCI-23 BMG384-H14) using following primers. Left homology arm forward, 5’-GCATCAAGATATCTAAGGCTTCTCCACATGCTAC-3’; left homology arm reverse, 5’-GCATCACAATTGATCTCCGAGTTCCAGGCCA-3’; exon 2 forward, 5’-GCATCAAGCTTGAATTCCCTGC-3’; exon 2 reverse, 5’-GCATCACAATTGATCTCCGAGTTCCAGGCCA-3’.
CTTCCAGC-3'; exon 2 reverse, 5'-GCATCACTGCCATGGGCTGCTAT CAAAGACC-3'; right homology arm forward. 5'-GCATCACCACGGG AAGCTTTGCTTCAACCAAGATCGTCCT-3'; right homology arm reverse, 5'-GCATCAGTCAGCTGAGGAGCTCCAGATAAGCTAC-3'. After electroporation into 129Sv/E mouse embryonic stem cells and selection under G418, correctly injected clones were injected into blastocysts and resulting chimeric mice were used to generate SprtnNeo+/+ mice. SprtnH/H mice were generated by crossing SprtnNeo+/+ mice and Cre-transgenic mice. A conditional KO allele, 278 bp for the KO allele, 527 bp for the floxed allele and 442 bp for the H allele. All of the animal procedures were approved by Mayo Clinic Institutional Animal Care and Use Committee.

In vitro culture of mouse blastocysts. All blastocysts were generated by natural mating of 2- to 3-month old Sprtn+/− male and Sprtn+/+ female mice. The morning of the day on which a vaginal plug was detected was designated as day 0. Blastocysts were collected on E3.5 by flushing the uteri with M2 medium (Sigma, M7167) and cultured on 35-mm glass-bottom dishes (FloUoroDish FD35, World Precision Instruments) in G1™ vs PLUS medium (Vitrolife, #10128) under paraffin oil (Vitrolife, #10029). Each blastocyst was photographed daily using a Zeiss Axio Observer Z1 system with CO2 Module S (5% CO2), Temp-Module S (37°C), Heating Unit XL S (37°C), and camera (AxioCam MRm camera and AxioVision 4.8 software). Images were exported as jpeg files, subsequently cropped and adjusted for brightness and contrast. At day 6, the blastocyst-outgrowing cells or the undeveloped blastocysts were collected using a microneedle and suspended in 50 μl lysis buffer (60 mM Tris–HCl, pH 9.0, 15 mM [NH₄]₂SO₄, 2 mM MgCl₂, 0.5% Tween-20 (v/v) and 250 μg/ml proteinase K). For PCR-based genotyping, 3–10 μl from each sample was used in 25 μl reactions. Genotyping was performed in duplicate and was successful for all of the blastocysts collected.

Analysis of ageing-associated phenotypes. Body composition was determined using an EchoMRI-100TM QNMR instrument (Echo Medical Systems, Houston, Texas) in 12-month-old female mice. Total body fat and lean mass were measured. Total body fat percentage is equal to fat mass divided by body mass. Bone mineral
Three consecutive days of acclimation occurred before testing. Treadmill exercise (N) was determined using a grip strength meter from Columbus Instruments. Cell density and bone mineral content were determined with dual-energy X-ray absorptiometry scanning using a Lunar PIXimus densitometer. Forelimb strength (N) was measured using a grip strength meter from Columbus Instruments. 

Plasmins and viral infection. Complementary DNA fragments encoding wild-type, the E112A mutant, the VFAA mutant (Pip*) and the D473A mutant (UBZ4*) of human Spartan11,12 were subcloned into a retrovirus vector pMSCV-puro. Wild-type mouse Spartan and Spartan219 (harbouring F254A and L261A mutations), were cloned into the pMSCV-puro vector with a XhoI 3Flag tag. Full-length human 3Flag-tagged β-gal was subcloned from pEGFP-C1 (a gift from Chikahide Masutani and Fumi Hanoaka) into a lentiviral expression vector pLVX6-IRES-Neo (N-terminal EGFP tag). Retroviruses and lentiviruses were packaged by cotransfecting viral and packaging plasmids a human embryonic kidney cell line 293T (American Type Culture Collection, CRL-1586). Cells were infected with viral vector in the presence of 2 μg/ml polybrene and selected with 3 μg/ml puromycin or 400 μg/ml G418.

Cell culture. MEFs were cultured in Dulbecco’s modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Primary Sprtn+/+ MEFs were isolated from E13.5 embryos by crossing Sprtn+/+ and Sprtn+/− mice. The H-line MEFs (H2, H3, H5 and H7) were obtained from E13.5 embryos produced by crossing Sprtn+/+ females and Sprtn−/− males and immortalized23. For Cre-ERT2 expression in the H-line MEFs, cells were infected with retroviral vectors and selected with 3 μg/ml G418. To analyze S-phase progression, cells were pulse labelled with 10 μM BrdU for 1 hour and then fixed with fixative 1:40 and concentrations were adjusted to 3–5 μg/ml BrdU and analysed by the flow cytometer.

DNA fiber assays. Cells were isolated as described above in 2N HC1 with 0.5% Triton X-100 for 30 min at room temperature. After neutralization with 0.1 M sodium borate (pH 8.5), cells were labelled with 1 μg of fluorescein isothiocyanate-conjugated anti-BrdU antibody (Roche, #1120693001) for 30 min at room temperature. Finally, cells were stained with PI solution (50 μg/ml −1 PI, 10 μg/ml −1 RNase A, 0.05% NP-40) for at least 30 min and analysed by flow cytometry. For phospho-histone H3 (Ser10), cells were incubated with 40 ng/ml −1 nocodazole (Sigma) for 4h, harvested and fixed with 70% ethanol at −20 °C overnight. Cells were washed once with PBS containing 1% BSA (1% BSA/PBS) and incubated with 1% BSA/PBS solution for 0.25% Triton X-100 for 5 min at 4°C. After washing with 1% BSA/PBS, cells were resuspended in 1 μl of anti-phospho-H3 (Millipore, #06-570) in 1% BSA/PBS containing 10% normal goat serum and incubated overnight at 4°C. Cells were washed once with 1% BSA/PBS and incubated with 7 μg of Alexa Fluor 488-labelled goat-anti-rabbit IgG (Invitrogen, #A11034) in PBS containing 10% normal goat serum for 1 h at 4°C. After washing with 1% BSA/PBS, cells were incubated in PI solution for at least 30 min and analysed by flow cytometry.

Cytogenetics. Mitotic cells were enriched by colcemid treatment for 2h and harvested by tapping plates. Cells were then swollen in hypotonic buffer for 25 min, fixed and dropped on glass slides. Slides were baked at 90°C for 30 min and stained with methanol’s stain solution for 1–3 min. After a brief rinse with running water, slides were dried at room temperature and analysed using a bright-field microscope. A metaphase spread was considered positive for breakage if any minor anomalies, major anomalies or radial configurations were observed.
antibodies (Abcam, ab6326, 1:1,000 in PBS containing 5% goat serum) overnight at 4°C. Slides were washed three times with PBS and incubated with secondary antibodies (Invitrogen, Alexa fluor 594 anti-mouse IgG, A11032, Alexa fluor 488 anti-rat IgG, A11006, 1:2,000 in PBS containing 5% goat serum) for 30 min at 37°C. After washing with PBS, slides were mounted with ProLong Gold (Invitrogen). Slides were viewed and photographed on a Zeiss fluorescence microscope with a ×100 objective. The lengths of IDU and CILU tracts were measured using the ImageJ software.

**Quantitative reverse transcription-PCR.** Total RNA was isolated from the kidneys and lungs of Sprn−/− and SprnH1/H1 mice using TRIzol (Invitrogen) and from MEFs and IAT using RNeasy Kit (Qiagen). cDNAs were generated using oligo(dT) primers and SuperScript III (Invitrogen). Quantitative PCR was performed in triplicate for each sample on CFX96 (Bio-Rad) using iTaq Universal SYBR Green Supermix (Bio-Rad) or on ABI PRISM 7900 Sequence Detection System (Applied Biosystems) using SYBR green PCR Master Mix (Applied Biosystems). To quantify the wild-type Sprn transcripts, primers were designed at the junction of exon 2 and exon 3 (forward primer: 5′-GGAACCTTGTAAGACCTGTTGTC-3′) and in exon 4 (reverse primer: 5′-CATCCTATCGGAAATGTTGGTC-3′) and used for normalization between samples. For senescence-associated markers, following primers were used: pteBP1 forward, 5′-CCCAACGCGCGCCGA-3′; pteBP1 reverse, 5′-GACGAGAGGCGTCAATCGGTA-3′; pteBP1 forward, 5′-AATCCTTTGGCTGATTACC-3′; pteBP1 reverse, 5′-GCCGAGAAGCTGCT-3′; p16 forward, 5′-TTAATAATGCTGCT-3′; p16 reverse, 5′-CACAGCAACAACTGCTACAGT-3′; p16 forward, 5′-CCACCACCTTGGTTGCT-3′; p16 reverse, 5′-TCCACCACCTTGGTTGCT-3′. Reverse primer (5′-CAGATGAGCTGCTA-3′) and forward primer (5′-AACTCCTATCGGAAATGTTGGTC-3′) were used for normalization for cDNAs construction.

**References**

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Author contributions
Y.J.M. directed and designed the study. M.K. generated and characterized Sprtn knockout and hypomorphic mice and isolated MEFs. R.S.M. and Y.M. analysed the phenotypes of conditional Sprtn knockout in MEFs. L.A.M. isolated and characterized blastocysts and performed chromosome counts. D.J.B. and B.C. analysed Sprtn hypomorphic mice for ageing-associated phenotypes. K.B.J. performed isolation, culture and karyotyping of lung fibroblasts and prostate epithelial cells. R.S.M., M.K., J.M.v.D. and Y.J.M. wrote the manuscript with contributions from L.A.M. and D.J.B.

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