A mouse model for Li-Fraumeni-Like Syndrome with cardiac angiosarcomas associated to POT1 mutations

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Abstract

The shelterin protein POT1 has been found mutated in many different familial and sporadic cancers, however, no mouse models to understand the pathobiology of these mutations have been developed so far. To address the molecular mechanisms underlying the tumorigenic effects of POT1 mutant proteins in humans, we have generated a mouse model for the human POT1R117C mutation found in Li-Fraumeni-Like families with cases of cardiac angiosarcoma by introducing this mutation in the Pot1a endogenous locus, knock-in for Pot1aR117C. We find here that both mouse embryonic fibroblasts (MEFs) and tissues from Pot1a+/ki mice show longer telomeres than wild-type controls. Longer telomeres in mutant mice are dependent on telomerase activity as they are not found in a telomerase deficient background. As in human Li-Fraumeni patients, heterozygous Pot1a+/ki mice spontaneously develop a high incidence of angiosarcomas, including cardiac angiosarcomas, and this is associated to the presence of abnormally long telomeres in endothelial cells as well as in the tumors. The Pot1a+/R117C mouse model constitutes a useful tool to understand human cancers initiated by POT1 mutations.

Author summary

We have generated a mouse model for the human POT1R117C mutation found in Li-Fraumeni-Like (LFL) families with cases of cardiac angiosarcoma by introducing this mutation in the Pot1a endogenous locus, knock-in for Pot1aR117C. The Pot1a+/ki mice show longer telomeres than wild-type controls. Longer telomeres in mutant mice are dependent on telomerase activity as they are not found in a telomerase deficient background. As in human Li-Fraumeni patients, heterozygous Pot1a+/ki mice spontaneously develop a high incidence of angiosarcomas, including cardiac angiosarcomas, and this is associated to the presence of abnormally long telomeres in endothelial cells as well as in the tumors. The ki-
Introduction

Telomeres are protective structures at the ends of chromosomes essential to ensure chromosome stability [1,2]. Vertebrate telomeres consist of tandem repeats of the TTAGGG DNA sequence bound by a six-protein complex known as shelterin, which protects telomeres from chromosomal aberrations and the activation of a persistent DNA damage response (DDR), as well as regulates telomerase activity at chromosome ends [1–3]. The shelterin complex is formed by TRF1, TRF2, RAP1, POT1, TIN2 and TPP1 [1,2]. Telomerase elongates telomeres at the pluripotent stage but is silenced in the majority of cell types after birth leading to progressive telomere loss, a hallmark of ageing [4]. Telomerase encompasses a reverse transcriptase catalytic subunit (TERT) and an RNA template (Terc), which recognizes the hydroxyl group (OH) at the 3' end of the G-strand overhang and elongates the telomere [5].

Telomere maintenance is a hallmark of cancer cells as it allows for indefinite cell division capability [6]. To avoid telomere loss associated to cell division, the vast majority of cancers reactivate the telomere-elongating enzyme telomerase [5,7]. Indeed, telomerase is a highly mutated gene in many different cancer types [8–10]. Telomerase is not the only telomere component altered in cancer. More recently, mutations in POT1, the gene encoding for a component of the shelterin telomere protective complex, have been identified in several types of human sporadic or familial cancers. These include mantle cell lymphoma, adult T-cell leukemia/lymphoma, parathyroid adenoma, colorectal cancer, pulmonary sarcomatoid carcinoma, sporadic and familial chronic lymphocytic leukemia (CLL), familial melanoma, glioma, thyroid cancer and Li-Fraumeni like- (LFL) syndrome families [11–26]. We and others have shown that POT1 mutations found in CLL, melanoma, Hodgkin lymphoma, T-cell lymphoma and LFL syndrome result in longer telomeres and are associated with higher chromosomal instability [13,16,18,23,27,28], another hallmark of cancer [6]. In particular, we demonstrated that families with Li-Fraumeni Like Syndrome carrying POT1 mutations showed increased incidence of different tumor types including the rare cardiac angiosarcoma (CAS) [13,29]. Initially, a single mutation was found in these LFL families, p.(R117C), which was also different from the POT1 mutations found in other cancer types. The wide-tumor spectrum shown in these LFL families made this POT1 mutation especially interesting since it could be at the origin of very different tumor types. Of particular interest, CAS is a rare and infrequent tumor with very bad prognosis with unknown pathogenesis and in need of new therapies. Generating mouse models carrying LFL-associated POT1 mutations will be instrumental to understand the role of POT1 in the pathobiology of various cancers and to search for new therapeutic opportunities. Humanized mouse models for these diseases are lacking.

Both POT1 and its interacting protein TPP1 have been shown to regulate telomerase activity at chromosome ends [30–33]. While human cells contain only one POT1 gene, mouse cells have Pot1a and Pot1b [34]. The two mouse POT1 proteins are highly homologous and can associate with telomeric DNA but while the Pot1b knock-out (KO) mouse is viable and does not present phenotypes except when combined with telomerase deficiency [35,36], Pot1a deletion in mice is embryonic lethal hampering the study of POT1a role in cancer development [34].
To address the mechanisms underlying the tumorigenic effects of POT1 mutant proteins in humans, we have generated a mouse model for the human POT1R117C mutation found in LFL families with cases of CAS by introducing this mutation in the Pot1a endogenous locus, knock-in for Pot1aR117C, thus generating Pot1aR117C mice. Homozygous Pot1aR117C mice are embryonic lethal while heterozygous Pot1aR117C mice are viable and show longer telomeres in proliferative tissues, i.e. whole blood cells, bone marrow and endothelia. The POT1a p.R117C-mediated telomere shortening was dependent on the presence of telomerase activity in MEFs, as it did not occur in the context of telomerase-deficient Tert−/− mice. The Pot1aR117C mice spontaneously develop angiosarcomas, including cardiac angiosarcomas, at higher frequency than wild-type mice. The knock-in Pot1aR117C+ mouse model constitutes a useful tool to understand human cancers initiated by POT1 mutation.

Results

Embryonic lethality in mice carrying the Li-Fraumeni-like mutation Pot1aR117C/R117C

To understand the role of POT1 in the pathobiology of Li-Fraumeni-Like (LFL) syndrome, here we set to generate a knock-in mouse harboring the mouse equivalent to the POT1R117C mutation found in LFL families [13]. In the mouse there are two Pot1 genes, Pot1a and Pot1b, however, only deletion of Pot1a results in embryonic lethality [34,37], suggesting that Pot1a is the essential shelterin gene analogous to the single human POT1 gene. Residue R117 in human POT1 is conserved in mouse POT1a and POT1b at the same position (Fig 1A). We have previously described an essential shelterin gene analogous to the single human POT1 gene. Residue R117 in human POT1 is conserved in mouse POT1a and POT1b at the same position (Fig 1A). Thus, we generated a Pot1a knock-in allele carrying the same missense Fmutation, Pot1aR117C found in human LFL families (Figs 1B and S1A–S1F; Materials and Methods). Intercrosses between heterozygous mice carrying the Pot1a+/flex allele, referred here as Pot1a+/flex mice, failed to render viable homozygous Pot1a+/flex offspring indicating embryonic lethality of the Pot1a+/flex allele when in homozygosis (S2A Fig). In agreement with early embryonic lethality of the conditional allele, we could not derive mouse embryonic fibroblasts (MEFs) of the Pot1a+/flex/+ genotype (E.11.5) from intercrosses between Pot1a+/flex/+ parental mice (S2B Fig).

Pot1a+/flex heterozygous mice were crossed with transgenic mice expressing the Cre recombinase under the control of the adenovirus EIIa promoter, which targets expression of the Cre recombinase to the early stages of embryonic development, oocytes, and preimplantation embryos [38] to generate heterozygous Pot1a+/ki-R117C mice, referred here as Pot1a+/ki mice. Expression of the Cre recombinase leads to replacement of the wild-type exon7 for the mutated exon harbouring the R117C missense mutation (Fig 1B). Intercrosses of Pot1a+/ki heterozygous mice, however, did not render any Pot1a+/ki offspring indicating embryonic lethality of the mutant Pot1a+/ki allele when in homozygosis (Fig 1C). We have previously described that some phenotypes associated to deletion of shelterin components, can be rescued by simultaneous deletion of p53 [33,39], thus we set to generate Pot1a+/ki p53−/− double mutant mice to rescue lethality. However, p53 deficiency did not rescue the lethality associated to the Pot1a+/ki allele when in homozygosis (Fig 1D). In agreement with embryonic lethality, we also failed to obtain Pot1a+/ki p53−/− double mutant MEFs (E.11.5 and E13.5) (S2C Fig). Thus, we set to study the effects of the mutant allele when in heterozygosis.

To understand the lethality associated to the Pot1a mutant allele, we analyzed the transcriptional expression of different Pot1a exons in Pot1a+/+ and Pot1a+/ki MEFs (Fig 1E, 1F and S1 Table). We found that the Pot1a+/ki allele is expressed to similar levels than the Pot1a+/ allele in exons 3 to 5, exon 8 to 9 and exon 9 to 10 in Pot1a+/+ and Pot1a+/+ MEFs (Fig 1E and 1F). As expected, the mutated exon 7 (E7+) is only detected in Pot1+/ki MEFs and the wild-type exon 7 (E7) is reduced by 50% in Pot1a+/+ MEFs compared to Pot1a+/+ controls (Fig 1F). We...
Fig 1. Embryonic lethality of Pot1a<sup>R117C/R117C</sup> (Pot1a<sup>ki/ki</sup>) mice is not rescued by p53 deficiency. A. Alignment among human POT1, mouse POT1b and mouse POT1a from residue 111 to 123 in the three proteins. The conserved R117 is indicated. B. Schematic representation of Pot1a wild-type and targeted locus. The loxP, loxP511 and FRT sites are indicated. The genomic location of the 5’- and 3’- probes for southern analysis are drawn. Genomic DNA was digested with EcoR1 restriction enzymes for southern analysis and the size of the fragments are indicated. C-D. Expected and observed number of mice of the offspring from Pot1a<sup>+</sup>/ki (C) and Pot1a<sup>+</sup>/ki p53<sup>+/−</sup> (D) intercrosses. The Fisher’s exact test was used to determine statistical significance. p-values are indicated. E. Schematic representation of Pot1a and Pot1a<sup>ki</sup> mRNA. The R117C substitution is within exon 7 (E7). Five different primers pairs were used to quantify transcripts levels corresponding to E3-E5 (green arrows), E7 (black and red arrows for E7 wildtype and E7<sup>ki</sup>, respectively), E8-E9 (purple arrows) and E9-E10 (brown arrows). The scheme is not drawn to scale. F. Quantitative qRT-PCR analysis of Pot1a and Pot1a<sup>ki</sup> mRNA levels in Pot1a<sup>+/+</sup> and Pot1a<sup>+/ki</sup> MEFs. mRNA expression levels were normalized to wild-type. G. Representative Western blot images and quantification of POT1a and TRF1 protein levels.
confirmed these results in vivo in the tail of Pot1a+/+, Pot1a+/flex and Pot1a+/ki mice (S2D and S2E Fig). Of note, mutant exon 7 was expressed to similar levels in Pot1a+/flex and Pot1a+/ki mice, indicating that intron 6 is not properly processed in Pot1a+/flex mice, thus providing an explanation for the embryonic lethality observed in Pot1a+/flex mice which behaves as a Pot1a knock-out allele [34] (S2A, S2D, and S2E Fig). In addition, we sequenced the exon 3 to exon 9 PCR amplification products from Pot1a+/+ and Pot1a+/ki MEFs cDNA (S1 Table). While the sequencing reaction from Pot1a+/+ and Pot1a+/ki MEFs cDNA rendered a single spectrum in the region corresponding to exon7, a double spectrum was observed in the reaction from Pot1a+/ki cDNA, confirming that both Pot1a+/+ and Pot1a+/ki alleles are transcribed (S3A and S3B Fig).

To confirm correct expression of the mutant POT1a protein, we analyzed POT1a protein levels by western blot in nuclear extracts from of Pot1a+/+ and Pot1a+/ki MEFs using a homemade monoclonal rat antibody specific for POT1a (Clone name POP148C, CNIO Monoclonal Core Unit catalogue, www.cnio.es) No differences in total POT1a protein levels were detected between Pot1a+/+ and Pot1a+/ki MEFs, indicating that the mutant POT1a p.R117C protein is properly transduced and stable (Fig 1G). As negative control for antibody specificity, we used MEFs deleted for Pot1a and either wild-type (Pot1a+/flex Pot1b+/+) or knock-out for Pot1b (Pot1a+/flex Pot1b+/ki) (Materials and Methods) (Fig 1G). As a positive control, total TRF1 levels were similar in Pot1a+/ki and wild-type MEFs (Fig 1G).

To assay whether the Pot1a+/ki and wild-type MEFs present different levels of POT1a bound to telomeres and whether expression of POT1a p.R117C was altering the binding of other shelterin components to the telomeres, we next performed a Chromatin Immunoprecipitation (ChIP) with anti-POT1a, anti-TRF1 and anti-TRF2 antibodies (Materials and Methods). We did not detect significant differences in immunoprecipitated telomeric DNA with any of the antibodies used between Pot1a+/ki p53+/+ and Pot1b+/+ p53+/+ MEFs, suggesting that the mutant POT1a-p.R117C protein is able to bind telomeres and that this binding does not disturb the binding of TRF1 and TRF2 shelterin proteins (S4A–S4D Fig). However, it should be pointed out that in these heterozygous Pot1a+/ki cells we cannot distinguish between POT1a wild-type and mutant variants. Potential compensatory effects could therefore not be ruled out. In agreement with ChIP data, we found similar TRF1 protein levels in the intestine and liver of Pot1a+/+ and Pot1a+/ki mice as determined by immunofluorescence with anti-TRF1 antibody (S4E and S4F Fig).

**POT1a p.R117C expression leads to slightly higher numbers of TIFs but does not elicit a strong DDR at telomeres**

POT1a represses the DNA damage checkpoint at telomeres by preventing ATR activation as well as aberrant homologous recombination at telomeres [34,37]. In order to understand the pathobiology of the increased telomere length and increased cancer susceptibility found by us in Li-Fraumeni-Like patients carrying the POT1R117C mutation [13], we set to study the in vivo phenotypes of Pot1a knock-in mutant mice. In particular, we previously described that lymphocytes from human POT1R117C carriers present longer telomeres and higher incidence of multitelomeric signals, a chromosomal aberration associated to increased telomere fragility [13]. Thus, we first analyzed the telomeric phenotypes of MEFs expressing the POT1a-p.R117C protein. Global genomic DNA damage was determined by quantifying the percentage
of cells positive for the DNA damage marker γH2AX and telomeric DNA damage was determined by quantification of the so-called telomere induced foci (TIFs), which were detected by immunocolocalization of γH2AX and the TRF1 telomere-binding protein (Fig 2A–2C). We did not observe significant differences in global DNA damage between Pot1a+/ki and Pot1a+/+ MEFs (Fig 2A). We found a mild increase in the number of cells presenting 1–2 TIFs in Pot1a+/ki compared to Pot1a+/+ MEFs (Fig 2B). For comparison purposes, we performed similar analysis in immortalized Pot1b, Pot1a and in Pot1a Pot1b deleted MEFs (Fig 2A–2C) (kindly provided by S. Chang). We find that Pot1a deletion induces a significant higher increase in the percentage of damaged cells as well as in the number of TIFs compared to Pot1a+/ki cells (Fig 2A–2D), thus suggesting that the POT1R117C mutation does not induce a strong DNA damage response (DDR) at telomeres.

We next generated induced pluripotent stem cells (iPS cells) from Pot1a+/ki p53−/− and Pot1a+/+ p53−/− MEFs by transduction of the Yamanaka factors, OCT4, SOX2 and KLF4 [40]. To confirm induction of pluripotency, we analyzed the expression of the pluripotent marker nanog, of the telomerase catalytic subunit TERT, as well as of the shelterin protein TRF1, previously shown to be greatly induced during induction of pluripotency (S5A and S5B Fig) [41]. In agreement with induction of pluripotency, iPS showed a 8-fold increase in TRF1 protein level, nanog expression, and increased Tert mRNA expression compared to the parental MEFs (S5A and S5B Fig). Of note, induction of pluripotency and expression of pluripotency markers was not altered in Pot1a+/ki p53−/− cells compared to the Pot1a+/+ p53−/− controls (S5A and S5B Fig). Next, we set to address chromosomal aberrations in metaphase spreads from MEFs and iPS cells of both Pot1a+/ki p53−/− and Pot1a+/+ p53−/− genotypes. Occurrence of the so-called “multitelomeric signals” or MTS has been previously associated to increased telomere damage and telomere fragility as the consequence of defective telomere capping [39,42,43]. We found a mild but significant increased abundance of MTS in both MEFs and iPS cells of the Pot1a+/ki p53−/− genotype compared to wild-type control cells (Fig 2D and 2E).

To address induction of DNA damage associated to the POT1R117C mutation, we checked the levels of phospho-CHK1 and phospho-RPA in MEFs and iPS cells expressing mutant POT1a p.R1171C protein as a read out of ATR activation (S6A and S6B Fig). As positive control for replicative stress, we treated the cells with 2mM hydroxyurea for three hours. In agreement with the low levels of telomeric DNA damage found in Pot1a+/ki p53−/− cells (Fig 2B–2E), we did not detect either pCHK1 or pRPA in MEF or iPS cells carrying Pot1a+ki allele (S6A and S6B Fig).

Altogether, these results indicate that POT1a-p.R117C protein results in alterations in telomere capping structure that are not sufficient to elicit a strong DNA damage response.

**POT1a.R117C mutant protein expression leads to telomere lengthening in a telomerase-dependent manner**

POT1 has been previously shown to have an important role regulation of telomerase activity at chromosome ends [30–33]. Thus, we set to address the telomere length in MEFs and induced pluripotent stem cells (iPS) expressing the POT1a-p.R117C protein. To this end, we performed Quantitative Fluorescence In Situ Hybridization (Q-FISH) on metaphase spreads, which allows to measure individual telomere fluorescence signals at each chromosome end as well as the percentage of undetectable telomere signals or “signal-free ends”. We used two independent MEFs from each genotype in a p53-null background, Pot1a+/+ p53−/− and Pot1a+/ki p53−/−. Interestingly, mean telomere length per metaphase was significantly higher in Pot1a+ki carriers compared to the Pot1a wild-type controls (Fig 3A). Importantly, the percentage of “signal-free ends” was also significantly lower in the Pot1a+/ki p53−/− compared to Pot1a+/+ p53−/− MEFs (Fig
As telomerase has been shown to preferentially elongate the shortest telomeres both in mice and yeast [44,45], these results suggest increased elongation of short telomeres by telomerase as the consequence of the Pot1a<sup>−/−</sup> mutation in MEFs.

Induction of Pluripotent Stem cells (iPS) from differentiated parental cells leads to a net telomere elongation that is dependent on telomerase activity [46]. To study the effect of the Pot1a<sup>−/−</sup> mutation in net telomere elongation, we measured telomere length by Q-FISH in iPS cells at passage 10. We observed a similar telomere lengthening in iPS cells of both genotypes.
**Fig 3. POT1a p.R117C expression results in longer telomeres in a telomerase dependent-manner.**

**A,B.** Mean telomere intensity (A) and number of signal-free ends (B) per metaphase in MEFs and iPES of the indicated genotype. 
\( n \) = number of metaphases.

**C.** Representative Q-FISH images of metaphase spreads in MEFs and iPES cells of the indicated genotypes. Green arrowheads indicate signal free ends.

**D,E.** Mean telomere intensity per metaphase (D) and mean spot intensity (E) in MEFs of the indicated genotype. The percentage of short and long telomeres are indicated. The percentage of short and long telomeres was defined as fluorescence intensity below the 20\(^{th}\) and above 80\(^{th}\) percentile, respectively of the fluorescence intensity values of the wild type.

**F.** Telomere restriction fragment (TRF) analysis in MEFs of the indicated genotype. The red line represents median intensity.

**G.** Telomeric sister chromatid exchange (T-SCE) events in MEFs of the indicated genotype and a representative CO-FISH images of metaphases hybridized with probes against the leading (green fluorescence) and lagging (red fluorescence) telomere. T-SCE events are indicated with arrows. A T-SCE was considered positive when it was observed with both the leading and lagging strand probes and involved a reciprocal exchange of telomere signal. 
\( n \) = number of metaphases. A t-test two tailed was used for statistical analysis. Error bars represent standard error. The p-values are indicated.

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compared to the parental MEFs (Fig 3A). Accordingly, the percentage of “signal-free ends” was also similar in Pot1a<sup>+/-</sup>p53<sup>+-</sup> and the Pot1a<sup>+/+</sup>p53<sup>+-</sup> controls (Fig 3B and 3C), in agreement with elongation of short telomeres as the consequence of telomerase over-expression and increased access of telomerase to the telomeres during pluripotency in the wild-type iPSCs [46]. The fact that telomeres were not further elongated in Pot1a<sup>+/+</sup>p53<sup>+-</sup> iPSCs compared to the Pot1a<sup>+/+</sup>p53<sup>+-</sup> controls, and that both had the same percentage of “signal-free ends”, further supports that longer telomeres in Pot1a<sup>+/+</sup>p53<sup>+-</sup> MEFs are due to increased telomerase access to the telomeres prior to induction of pluripotency (Fig 3A).

To demonstrate the role of telomerase in POT1a p.R117C-induced telomere lengthening, we generated Pot1<sup>+/-</sup>Tert<sup>+-/-</sup> MEFs. Analysis of telomere length by telomere Q-FISH and by Telomeric Restriction Fragment Southern analysis (TRF) in these cells, clearly shows that the telomere lengthening of Pot1<sup>+/+</sup> MEFs is dependent on telomerase activity, as it does not occur in the double mutant Pot1<sup>+/+</sup>Tert<sup>+-/-</sup> cells (Fig 3D–3F). We observed a decrease in the percentage of short telomeres (<20<sup>th</sup> percentile) as well as an increase in the percentage of long telomeres (>80<sup>th</sup> percentile) in Pot1a<sup>+/+</sup> compared to Pot1a<sup>+/+</sup> MEFs that disappeared in a telomerase deficient background (Fig 3E).

Finally, to study a potential role of telomere recombination in the telomere elongation observed in Pot1a<sup>+/+</sup> cells, we next analyzed whether POT1a p.R117C mutant variant affected the recombination rates at telomeres. To this end, we measured the frequency of telomere sister chromatid exchanges (T-SCEs) in MEFs by using the chromosome-orientation FISH (CO-FISH) technique [47]. We found a mild but significant increased numbers of T-SCEs events in Pot1a<sup>+/+</sup>p53<sup>+-</sup> MEFs compared to Pot1b<sup>+/+</sup>p53<sup>+-</sup> controls (Fig 3G). The presence of C-circles, partially double-stranded telomeric circles consisting of a complete C-rich strand and an incomplete G-rich strand, has also been associated to increased telomere recombination [48]. Thus, we next analyzed the presence of C-circles in Pot1a<sup>+/+</sup>p53<sup>+-</sup> and Pot1b<sup>+/+</sup>p53<sup>+-</sup> MEFs (S6C Fig). As positive and negative controls, we used the osteosarcoma U2OS cell line and HEK293T that use telomere recombination-based and telomerase-dependent mechanisms for telomere lengthening, respectively [49]. We found increased C-circles in Pot1a<sup>+/+</sup> MEFs that was further enhanced in the absence of p53 although much lower than in ALT+ U2OS cells (S6C Fig) [50]. This difference disappeared in Pot1a<sup>+/+</sup>Tert<sup>+-/-</sup> MEFs (S6C Fig). these results indicate that POT1a p.R117C mutant protein is not inducing a <i>bona fide</i> ALT mechanism in MEFs.

We also studied the colocalization of PML nuclear bodies with telomeres (ALT-associated PML nuclear bodies or APBs), a phenomenon previously described in cells with increased telomere recombination [7]. We observed higher numbers of APBs in Pot1a<sup>+/+</sup>p53<sup>+-</sup> MEFs as compared to Pot1a<sup>+/+</sup>p53<sup>+-</sup> controls, a difference that was not detected in IPS cells, in agreement with telomerase-dependent telomere lengthening upon induction of pluripotency (S6D Fig). However, the <i>bona fide</i> ALT+ U2OS presented a much higher numbers of APBs as compared to Pot1a<sup>+/+</sup> p53<sup>+-</sup>, ruling out that expression of POT1a p.R117C mutant protein is favoring the onset of the ALT-mechanism in MEFs.

### Dominant-negative effects of POT1a.R117C mutant protein

In order to address the nature of the POT1a.pR117C mutant variant we retrovirally transduced Pot1a<sup>flox/flox</sup> MEFs with either empty pBabe, pBabe harboring HA-tagged wild-type Pot1a or pBabe harboring HA-tagged mutant Pot1a<sup>R117C</sup> alleles, pBabe-HA-Pot1a or pBabe-HA-Pot1a<sup>ki</sup>, respectively. After puromycin selection, cells were cultured for 5 days with or without 4-hydroxy-tamoxifen (TMX) to induced the Cre-mediated excision of the floxed flanked exon
4 to exon 5 fragment of the *Pot1a*<sup>flox</sup> allele [37]. We performed a double immunofluorescence using antibodies against TRF1 and HA to address whether the overexpressed HA-*Pot1a* alleles localized to telomeres. The results showed proper co-localization of both HA-POT1a and HA-POT1-KI proteins with TRF1 in both conditions with and without TMX (Fig 4A). These results confirm that the R117C mutation does not affect the capacity of POT1-KI mutant protein to localize to telomeres independently of the presence or absence of POT1 wild-type protein.

We next analyzed global genomic DNA damage and telomeric DNA damage (TIFs), which were detected by immunocolocalization of γH2AX and the TRF1 telomere-binding protein (Fig 4B–4D). Cells deleted for *Pot1a* showed a significant increase in global DNA damage and in the number of TIFs. Overexpression of wild-type HA-*Pot1a* and HA-*Pot1a*<sup>ki</sup> alleles rescue both global and telomeric DNA damage, indicating that the R117C substitution does not result in a complete loss of function mutation. Of note, the overexpression of HA-POT1a-KI mutant protein led to a significant 2-fold increase in the number of damaged telomeres as compared to basal levels in pBabe transduced control wild-type cells, a phenomenon that was not detected upon overexpression of wild-type HA-POT1a (Fig 4D). These results indicate a dominant-negative effect of the mutant protein as it was also observed in the presence of the endogenous POT1a.

We next set to address the telomere length in wild-type MEFs overexpressing HA-*Pot1a* and HA-*Pot1a*<sup>ki</sup> alleles by Q-FISH on metaphase spreads (Fig 4E–4G). Interestingly, overexpression of HA-*Pot1a*<sup>ki</sup> allele led to a significant mean telomere length increase and reduction in the percentage of short telomeres (<20<sup>th</sup> percentile) compared to control cells and to cells overexpressing the wild type allele (Fig 4E and 4F). In contrast, overexpression of wild type HA-*Pot1a* did not lead to significant changes in the percentage of short telomeres (Fig 4F). No significant differences in the percentage of long telomeres (>80<sup>th</sup> percentile) were detected among the three experimental conditions (Fig 4G). These results reinforce the previous results showing elongation of short telomeres by telomerase as the consequence of the *Pot1a*<sup>ki</sup> expression and indicate that expression of POT1a-R117C mutant variant leads to telomere lengthening.

**Pot1-ki mice show longer telomeres**

We generated different mouse cohorts carrying the *Pot1a*<sup>ki</sup> mutant allele in heterozygosis both in a *p53*-proficient and in a *p53*-deficient background, a situation that is analogous to the human patients carrying this mutation. In particular, we generated mouse cohorts for the *Pot1a*<sup>+/</sup>*p53<sup>−/−</sup>, *Pot1a*<sup>+/+</sup>*p53<sup>−/−</sup>, *Pot1a*<sup>+/−</sup>*p53<sup>/−</sup>, *Pot1a*<sup>+/−</sup>*p53<sup>/+</sup>, *Pot1a*<sup>+/+</sup>*p53<sup>/−</sup> and *Pot1a*<sup>+/−</sup>*p53<sup>/−</sup> genotypes. Importantly, we found no significant differences in either median or maximal survival between the *Pot1a*<sup>+/−</sup> cohorts compared to their *Pot1a*<sup>++/−</sup> counterpart controls either in the presence or in the absence of p53 (S7A Fig).

Next, we set to address whether mice carrying the *Pot1a*<sup>ki</sup> allele showed similar telomeric phenotypes to those shown by Li-Fraumeni-Like Syndrome patients carrying the homologous mutation in humans. In particular, we have previously shown that Li-Fraumeni-Like patients show increased telomere length and telomere fragility in peripheral blood mononuclear cells (PBMCs) [13]. Thus, we isolated PBMCs from healthy *Pot1a*<sup>+/+</sup> and *Pot1a*<sup>+/−</sup> mice at 2 years of age and measured telomere length by using High throughput Telomere Q-FISH (HT-QFISH) which measures telomere fluorescence on interphase nuclei [51]. We found that mean telomere fluorescence was significantly increased in *Pot1a*<sup>+/−</sup> PBMCs compared to those of control *Pot1a*<sup>++/−</sup> mice, corresponding to a mean length of 51.8 kb compared to 44.7 kb, respectively (Fig 5A). In agreement with longer telomeres in PBMCs from *Pot1a*<sup>++/−</sup> mice, we
Fig 4. POT1a p.R117C mutant protein exerts dominant-negative effects. A. Pot1a<sup>flox/flox</sup> MEFs were retrovirally transduced with either empty pBabe, pBabe-HA-Pot1a or pBabe-HA-Pot1a<sup>ki</sup>. After puromycin selection, cells were cultured for 5 days with or without 4-hydroxy-tamoxifen (TMX) to induce the Cre-mediated excision of the floxed Pot1a allele. Representative images of double immunofluorescence with antibodies against TRF1 and HA.

B. Representative images of γH2AX and TRF1 immunofluorescence staining. TIFs were detected by γH2AX and TRF1 co-localizing foci (white arrowheads).

C. Percentage of γH2AX positive cells.

D. Number of Telomere-Induced Foci (TIFs) per γH2AX positive cells.

E-G. Mean spot intensity (E) percentage of short (F) and long (G) telomeres in wild type MEFs overexpressing either HA-Pot1a or HA-Pot1a<sup>ki</sup> alleles. The percentage of short and long telomeres was defined as fluorescence intensity below the 20<sup>th</sup> and above 80<sup>th</sup> percentile, respectively of the fluorescence intensity values of cells transduced with the pBabe empty vector. n = number of metaphases. A t-test two tailed was used for statistical analysis. Error bars represent standard error. The p-values are indicated.

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Fig 5. Pot1a+/+ peripheral blood mononuclear cells show longer telomeres. A-C. Whisker plot representation of telomere length (A), percentage of short telomeres (B) and percentage of long telomeres (C) in PMBCs from two-year-old mice of the indicated genotype. The percentage of short and long telomeres was defined as fluorescence intensity below the 20th and above 80th percentile of the fluorescence intensity values of Pot1a+/+ cells, respectively. The ends of the box are the upper and lower quartiles so that the box spans the interquartile range. The middle line represents the median and bars the standard deviation. n = number of mice. D. Mean telomere length in PMBCs from mice at different ages from the indicated genotype. Mouse ages range from 15- to 76-week-old. Linear regression analysis was used to determine the rate of telomere shortening. n = number of mice. R² coefficient and the p value from the comparison of both linear regression analysis are indicated. E-F. Percentage of γH2AX (E) and 53BP1 (F) positive cells.
observed a significant decrease in the percentage of short telomeres (< 20th percentile) as well as a significant increase in the percentage of long telomeres (> 80th percentile) in Pot1a+/ki PBMCs compared to those from Pot1a+/+ mice (Fig 5B and 5C). Again, the significant decrease in short telomeres in the PBMCs from Pot1a+/ki mice, suggest a telomerase-dependent telomere elongation in this genotype.

To further confirm longer telomeres in Pot1a+/ki mice compared to those of control Pot1a+/+ mice, we determined telomere length in PMBCs from Pot1a+/+ p53+/- and Pot1a+/ki p53+/- mice at different ages by using HT-QFISH. Again, we observed longer telomeres in Pot1a+/ki mice compared with Pot1a+/+ mice at all ages analyzed. As expected, the rate of telomere shortening was not significantly different between both mouse cohorts (Fig 5D).

As previously shown in the case of MEFs, PBMCs derived from Pot1a+/ki mice showed a 2-fold increase in telomeric damage (TIFs) as determined by immunocolocalization of γH2AX and the telomeric protein TRF1 compared to the wild-type controls, while the total levels of DNA damage did not vary between genotypes (Fig 5E–5G).

As human Li-Fraumeni-Like patients show a higher incidence of cardiac angiosarcomas [13], we next analyzed telomere length by Q-FISH in the vascular endothelium. To identify endothelial cells (EC) we used the anti-CD31 marker in heart sections from healthy 30-week-old Pot1a+/+ and Pot1a+/ki mice. We observed a significantly higher mean nuclear telomere fluorescence and mean telomere spot fluorescence in the vascular endothelium of Pot1a+/ki compared to wild-type mice indicative of longer telomeres, while no differences in telomere length were detected in CD31 negative cells composed mainly of cardiomyocytes and cardiac fibroblasts [52] (Fig 6A–6C).

Finally, we also analyzed telomere length in bone marrow, liver and intestine from the same mouse cohorts. We found longer telomeres in the bone marrow of Pot1a+/ki mice compared to that of Pot1a+/+ mice while no significant differences in telomere length between both genotypes were observed in liver and intestine (Fig 6D–6F).

**Higher incidence of angiosarcomas in Pot1a+/ki mice**

As the human POT1R117C heterozygous mutation is associated with higher incidence of cancers including familial cardiac angiosarcomas [13], here we set to address whether the equivalent mouse Pot1aR117C mutation also led to increased tumors in the context of mice carrying the Pot1a+/+ allele in heterozygosis. To this end, we performed a full histopathological analysis of mice of the different genotypes at death point and analyzed the frequency of spontaneous tumors. Mice carrying the Pot1a+/+ allele showed an increased angiosarcoma incidence within the thorax cavity, including heart angiosarcomas (Fig 7A and 7B). Of note, while none of the Pot1a+/+ mice in a p53 wild-type background developed angiosarcoma, a total of 14.3% of the Pot1a+/ki mice developed these tumors (Fig 7A). This higher incidence of angiosarcomas in Pot1a+/ki mice compared to wild-type mice was also observed in the p53 heterozygous and homozygous genetic backgrounds (Fig 7A). We used a positive CD31 staining, a marker for vascular endothelial cells, for diagnosis of angiosarcoma incidence (Fig 7B). The thoracic angiosarcomas developed by Pot1a+/ki mice were characterized by invasion of malignant endothelial cells to surrounding tissues and the presence of anaplastic endothelial cells with large

G. Number of Telomere-Induced Foci (TIF) per damaged cells of the indicated genotype. n = number of analyzed cells. Representative images of γH2AX and TRF1 immunofluorescence staining in cells from the indicated genotype are shown to the right. TIFs were detected by γH2AX and TRF1 co-localizing foci (white arrowheads). A t-test two tailed was used for statistical analysis. Error bars represent standard error. The p-values are indicated.

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and hyperchromatic nuclei forming capillary and sinusoidal channels (see “black arrow” in Fig 7B). These tumors also showed a number of mitotic figures (see “green arrow” in Fig 7B).

Of note, the incidence of thoracic angiosarcomas is low and the size of these tumors are very

Fig 6. Longer telomeres in the vascular endothelium and in bone marrow cells of Pot1α+/ki mice. A-B. Mean nuclear and mean spot intensity in endothelial cells (A) and in cardiac myocytes and fibroblast (B) from healthy 30-week-old mice of the indicated genotype. C. Representative images of immune-FISH from heart sections. The heart sections were stained with anti-CD31 for identification of vascular endothelial cells. D-F. Mean nuclear and mean spot intensity in bone marrow (D), liver (E) and intestine (F) from healthy 30-week-old mice of the indicated genotype. Representative images of Q-FISH of bone marrow sections are shown in D. Insets correspond to higher magnification images. n = number of mice. A t-test two tailed was used for statistical analysis. The p-values are indicated.

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Fig 7. Higher incidence of angiosarcomas in Pot1a<sup>+/−</sup> mice. A. Percentage of mice presenting angiosarcomas at time of death. Chi squared test was performed for statistical analysis. The number of mice and the p values are indicated. B. Macroscopic and representative images of hematoxylin & eosin (H&E) and CD31 staining of angiosarcomas developed in vascular endothelium within the thorax cavity. Dark-red angiosarcoma protrudes from right ventricular wall (upper panels), on the pleural surface of the sternum (middle panels) and in the pulmonary artery (lower panels). Malignant endothelial cells have invaded surrounding tissues (asterisk). High magnification images show anaplastic endothelial cells with large and hyperchromatic nuclei (black arrows) forming capillary and sinusoidal channels. Mitotic figures were observed (green arrows). CD31 was used as a vascular marker for diagnosis. Scale bars are shown. C-D. Mean telomere fluorescence intensity (C), percentage of short telomeres (D) and percentage of long telomeres (E) in healthy vascular endothelium (VE) and in angiosarcomas (AS) from mice of the indicated genotype at death point. The percentage of short and long telomeres was defined as fluorescence intensity below the 20<sup>th</sup> and above 80<sup>th</sup> percentile.
small, making it impossible to check for angiosarcoma at earlier time points. It is of interest to note the fact that the increased angiosarcoma incidence is in accordance with increased telomere length in endothelial cells expressing the $Pot1a^{R117C}$ mutation, and indicate that mice carrying this mutation are a bona fide model to study the pathobiology of these tumors, as well as potential effective treatments.

Mice carrying the $Pot1a^{R117C}$ mutation also presented rare carcinomas in mice such as lung bronchoalveolar carcinoma and hypophysis carcinomas, although the frequency was not significantly different between genotypes (S7B Fig). Also, the incidence of benign, sarcoma and hematopoietic tumors was not significantly different when comparing $Pot1a^{+/+}$ with $Pot1a^{+/ki}$ mouse cohorts in none of the p53 background (S7C–S7E Fig).

### Longer telomeres in $Pot1a^{ki}$ angiosarcomas

As endothelial cells from the heart of $Pot1a^{+/ki}$ mice showed longer telomeres, we next set to determine telomere length in angiosarcomas from both $Pot1a^{+/+}$ and $Pot1a^{+/ki}$ mice at death point. Angiosarcoma tumors expressing POT1a p.R117C showed significantly longer telomeres compared to similar tumors found in wild-type mice (Fig 7C). Non-tumoral vascular endothelium surrounding the tumors were also analyzed and we found that $Pot1a^{+/ki}$ endothelial cells have longer telomeres compared to wild-type endothelial cells (Fig 7C; see also Fig 6A). Accordingly, the percentage of short telomeres (< 20th percentile) was lower in $Pot1a^{ki}$ angiosarcomas (AS) and healthy vascular epithelium (VE), while the percentage of long telomeres (> 80th percentile) was higher in $Pot1a^{ki}$ AS and in VE as compared to those in wild-type mice (Fig 7D and 7E). The longer telomeres in $Pot1a^{ki}$ AS are consistent with the telomerase-mediated telomere elongation found in both MEFs and iPS cells carrying the POT1a p.R117C mutation (Fig 3A–3F).

Interestingly, we did not find longer telomeres in hypophysis and lung adenomas and carcinomas compared to their surrounding healthy tissue from $Pot1a^{+/+}$ mice (S8A–S8C Fig). Of note, telomeres where shorter in tumor samples compared to their corresponding non-pathological tissue (S8A–S8C Fig), which is consistent with previous studies [53]. We did not detect PML positive cells within the carcinomas (S8D Fig).

As found in $Pot1a^{+/+}$ MEFs, we also observed increased PML-positive cells and higher number of APBs in $Pot1a^{+/ki}$ VE and AS compared to $Pot1a^{+/+}$ control samples, respectively (S9A–S9C Fig). Of note, the number of APB foci per cell increased significantly in $Pot1a^{+/ki}$ angiosarcomas compared to non-pathological vascular epithelium (S9A–S9C Fig).

Finally, we also analyzed total DNA damage and telomeric damage by $\gamma$H2AX and TRF1 double immunofluorescence in angiosarcoma tumors. Overall DNA damaged was low, 5% $\gamma$H2AX positive cells of total tumoral cells. Of note, no differences either in the percentage of damaged cells or in Telomere-Induced DNA damage foci (TIFs) between $Pot1a$ wild-type and knock-in angiosarcomas were detected (S9D and S9E Fig), indicating that telomeric DNA damage in $Pot1a^{ki}$ angiosarcomas is not contributing to tumorigenesis.

### Discussion

The availability of mouse models that recapitulate the human disease is instrumental to understand the origin and molecular mechanisms associated to the development of a particular
disease, as well as to develop effective treatments. In this work we have generated a mouse model for humans carrying the hPOT1$^{R117C}$ mutation associated to Li-Fraumeni syndrome by generating a Pot1a$^{R117C}$ knock-in mouse. Among other tumor types, families carrying the hPOT1$^{R117C}$ mutation show an elevated incidence of cardiac angiosarcomas, a rare tumor type [13]. Although additional POT1 variants have been found in cardiac angiosarcomas and cardiac sarcomas, *in silico* studies predict that these variants are affecting the POT1 function in a similar way than the p.R117C mutation studied here [12]. In all cases, these mutations acted as dominant-negative alleles, as indicated by absence of loss of heterozygosity in the corresponding tumors [12,13]. Thus, here we generated a heterozygous Pot1a$^{+/ki}$ mouse model and showed that mouse POT1a pR117C exerts dominant-negative effects at telomeres. Importantly, we show that these mice spontaneously develop angiosarcomas, including cardiac angiosarcoma, at a higher frequency than wild-type mice. These observations indicate that the homologous missense mutation R117C found in hPOT1 introduced in mPot1a has similar consequences in angiosarcoma development in human and mouse.

It is of particular relevance that POT1 mutations are mainly associated to increased angiosarcomas both in mice and humans. In this regard, a recent pan-cancer study aimed at to identify associations between POT1 mutation frequency and tumor type, found that 1834 tumors out of a total of 62,368 tumors harbored a non-benign mutation in POT1 (2.94%) of which angiosarcomas turned out to be 11 times more likely to carry a POT1 mutation than other cancer types [22]. The highest frequency of POT1 mutation was found in pulmonary sarcomatoid carcinomas (28%) and angiosarcomas (23%) [21], which are particularly aggressive malignancies compared with other types of sarcomas in which the frequency of POT1 mutations was not elevated. Thus, pathological POT1 mutant variants seem to be associated to the increased aggressiveness of pulmonary sarcomatoid carcinomas and angiosarcomas [21]. In an independent study, the Angiosarcoma Project (ASCproject) in which altered genes in a cohort of 47 angiosarcomas were studied, POT1 mutations were the fourth most frequent after mutations in TP53, KDR and PIK3CA genes [54].

We and others have shown that at the molecular level, the hPOT1 mutations found in human tumors cause telomere dysfunction, genomic instability and telomere lengthening [13,16,18,20,23,27,28], which might facilitate survival of cells with chromosomal instability and the acquisition of aditional somatic mutations leading to malignant transformation [29]. Here, we demonstrated that Pot1a$^{R117C}$ mice carrying the p.R117C mutation in heterozygosis recapitulate the longer telomere phenotype found in humans. In particular, we observed longer telomeres in lymphocytes, as well as in mouse embryonic fibroblasts (MEFs), cardiac endothelial cells and bone marrow cells. We did not find longer telomeres, however, in liver, intestines and cardiac muscle. It would be of interest to know whether human carriers of these mutation show increased telomere length in other tissues other than lymphocytes.

Longer telomeres as the consequence of the Pot1a$^{R117C}$ mutation could be the consequence of increased recruitment or accessibility of telomerase to the telomeres. Human POT1 functions both as a positive and a negative regulator of telomerase since POT1 and its interacting partner TPP1 have been shown to have a role in recruiting telomerase to telomeres as well as to inhibit telomerase access to telomeres [33,35,55–57]. Alternatively, POT1 mutations may also affect telomere length throughout its role in recruitment of the CST complex that binds to telomeric DNA and regulates the fill-in synthesis of the telomeric C-strand [28,30,58]. In mice, POT1b interacts with TPP1 to promote telomerase recruitment to telomeres to elongate telomeric G-strand as well as C-strand fill-in through CST complex recruitment while POT1a negatively regulates telomerase access to telomeres [59]. The fact that telomerase deficient MEFs carrying the Pot1a$^{R117C}$ mutation do not present longer telomeres compared to Pot1a wild-
type counterparts demonstrate that telomere lengthening induced by POT1a pR117C is telomerase dependent. Given that the \( \text{ki-Pot1a}^{R117C} \) mouse conserves intact POT1b, the longer telomeres observed are likely due to a defect of POT1a p.R117C in preventing telomerase access to telomeres and/or an enhanced function as a telomerase activator/processivity factor bound to TPP1. Also, the reduced abundance of undetectable telomere signals at chromosome ends (“signal-free ends”) in \( \text{Pot1a}^{+/R117C} \) compared with wild-type MEFs suggests a higher telomerase activity at telomeres as it has previously been shown that telomerase preferentially acts on the shortest telomeres both in mice and yeast [44–46]. In agreement with this, the difference in the percentage of “signal-free ends” between wild-type and \( \text{Pot1a}^{R117C} \) genotypes is lost upon induction of pluripotency, which involves an opening of the telomeric chromatin and a net telomerase-mediated telomere elongation [46].

Interestingly, we also find longer telomeres in \( \text{Pot1a}^{R117C} \) angiosarcomas, which are also likely the consequence of telomerase elongation associated to the \( \text{Pot1a}^{R117C} \) mutation as shown here for \( \text{Pot1a}^{R117C} \) MEFs. Of interest, although telomerase over-expression is the most widely used telomere elongating mechanism in tumors, some cancers of mesenchymal and neuroepithelial cell origin [60], such as angiosarcomas [61,62], have been reported to show an Alternative Lengthening of Telomeres (ALT) phenotype. Interestingly, here we find higher numbers of APBs in angiosarcomas forming in \( \text{Pot1a}^{R117C} \) mice, suggesting that increased recombination could also be partially contributing to telomere elongation in these tumors. We cannot rule out that the cancer phenotype associated to \( \text{Pot1}^{R117C} \) in the mouse model could also be due to telomere dysfunction resulting from suboptimal end protection by the POT1a pR117C variant that could cause telomere recombination and other forms of genome instability that are causative of cancer. Other cancer-associated POT1 mutations when introduced in stem cells do not cause significant telomere damage but led to telomere elongation [63]. Based on the fact that the \( \text{Pot1a}^{+/R117C} \) mice do not present an increase incidence in either carcinomas, sarcomas or hematological tumors, and only a significant increase in angiosarcomas in adulthood, we propose that POT1a p.R117C variant is not the driver but rather a facilitator of neoplastic transformation in endothelial cells. Cancer-associated POT1 mutations by their ability to lengthen telomeres might endow cancer cells with proliferative advantages.

Finally, it is interesting to note that although different hPOT1 variants studied lead to similar telomeric phenotypes, i.e. longer telomeres and telomere fragility [13,16,18,20,23,27,28], the families carrying a particular POT1 mutation do not show a wide spectrum of POT1-associated tumors as it could be expected [11–20,22,24–26,64]. This is also the case of the \( \text{Pot1a}^{+/R117C} \) mice generated here, which spontaneously develop angiosarcomas but the incidence of other type of sarcomas, carcinomas or hematological tumors was not altered compared to wild-type mice.

In summary, the \( \text{ki-Pot1a}^{R117C} \) mouse constitutes a potential pre-clinical mouse model for LFL syndrome presenting with high angiosarcoma incidence that could provide in the future a very useful tool for the study of treatments for these tumors.

**Materials and methods**

**Ethics statement**

All mice were generated and maintained at the Spanish National Cancer Centre under specific pathogen-free conditions in accordance with the recommendation of the Federation of European Laboratory Animal Science Associations. All experiments and animal procedures were approved by our Institutional Animal Care and Use Committee (IACUC) and by the Ethics Committee for Research and Animal Welfare (CEIyBA) (PROEX 065/16).
Generation of Pot1a<sup>R117C</sup> knock-in mouse model

The knock-in for Pot1a<sup>FRT-Neo/flexR117C</sup> was generated following a conditional strategy by the Flex technology [65]. The targeting construct was produced by Gene Bridges (www.genebridges.com). G4-ES cells were electroporated with 10 μg of Ascl linearized targeting vector. ES clones showing homologous recombination at the Pot1a locus was identified by PCR. The results of PCR screening were confirmed by Southern blot analysis of EcoR I restricted genomic DNA using a 5'- and a 3'- external probes, respectively (S1A and S1B Fig). Unique insertion of the targeting vector was confirmed using a probe within the Neomycin marker (S1C Fig). The integrity of the distal loxP511 site was confirmed by PCR amplification of genomic DNA from the selected ES clones using primers Pot1a-1Fand Pot1a-R1 (S1 Table) and sequencing the PCR products (S1D Fig). Chimeric mice were generated by microinjection of two independently targeted ES clones into B6N-Tyr<sup>C-Brd</sup> host blastocyst, which were then implanted into pseudopregnant CD1 foster females. The resulting offspring showed a high level of chimerism as shown by coat color, and were mated to C57BL/6J mice to assess germ line transmission. The resulting heterozygous Pot1a<sup>+</sup>/FRT-Neo<sup>/</sup>flexR117C mice were then bred to transgenic mice expressing the Flpe recombinase to induce excision of the Neo marker to generate Pot1a<sup>+</sup>/flexR117C mice (S1E Fig).

Pot1a<sup>+</sup>/flexR117C heterozygous mice were then intercrossed to generate Pot1a<sup>+</sup>/ki<sup>R117C</sup> resulting in embryonic lethality. Pot1a<sup>+</sup>/flexR117C heterozygous mice were crossed with transgenic mice expressing the Cre recombinase under the control of the adenovirus EIIa promoter [38] to generate Pot1a<sup>+</sup>/ki<sup>R117C</sup> mice (S1E Fig). Pot1a<sup>+</sup>/flexR117C heterozygous mice were then intercrossed to generate Pot1a<sup>+</sup>/ki<sup>R117C</sup> resulting in embryonic lethality. Pot1a<sup>+</sup>/flexR117C heterozygous mice were crossed with transgenic mice expressing the Cre recombinase under the control of the adenovirus EIIa promoter [38] to generate Pot1a<sup>+</sup>/ki<sup>R117C</sup> mice (S1E Fig). Pot1a<sup>+</sup>/flexR117C heterozygous mice were then intercrossed to generate Pot1a<sup>+</sup>/ki<sup>R117C</sup> resulting in embryonic lethality. Pot1a<sup>+</sup>/flexR117C heterozygous mice were crossed with transgenic mice expressing the Cre recombinase under the control of the adenovirus EIIa promoter [38] to generate Pot1a<sup>+</sup>/ki<sup>R117C</sup> mice (S1E Fig).

Cell culture and Induced Pluripotent stem (iPs) cell generation

Primary embryonic fibroblasts (MEFs) were isolated from E11.5 or E13.5 embryos according to standard protocols. Briefly, after removal of the head and organs the whole embryo was minced and rinsed in ice-cold PBS, incubated in trypsin/EDTA (Gibco, Grand Island, NY) before dissociating in complete medium. MEFs were grown in high-glucose DMEM supplemented with 10% FBS. For iPS cell generation, MEFs were transduced with retroviral supernatants produced in 293T cells transfected with the ecotropic packaging plasmid pCL-Eco and the Yamanaka factors, OCT4, SOX2 or KLF4 [40]. Pot1a<sup>flex/flox</sup> MEFs were transduced with retroviral supernatants produced in 293T cells transfected with the ecotropic packaging plasmid pCL-Eco and with either empty pBabe, pBabe-HA-Pot1a<sup>-6xHIS</sup> or pBabe-HA-Pot1aki<sup>-6xHIS</sup>. Cells were cultured for 5 days with or without 4-hydroxy-tamoxifen (1 μM). The HA-Pot1a-6xHIS and HA-Pot1aki-6xHIS alleles were synthetically synthesized by Integrated DNA Technologies, IDT (https://eu.idtdna.com) and cloned in pBabe vector.

Immunohistochemistry and Immunofluorescence analysis

Tissue samples were fixed in 10% buffered formalin, dehydrated, embedded in paraffin wax and sectioned at 2.5 mm. Tissue sections were deparaffinized in xylene and re-hydrated.
through a series of graded ethanol until water and then stained with hematoxylin and eosin for pathological examination. Immunohistochemistry (IHC) and immunofluorescence (IF) were performed on de-paraffined tissue sections processed with 10 mM sodium citrate (pH 6.5) cooked under pressure for 2 min. IHC staining of angiosarcoma sections was performed with rabbit polyclonal anti-CD31 (1:100; Abcam ab28354), counterstained with hematoxylin and analyzed by light microscopy. For IF, tissue sections were permeabilized with 0.5% Triton in PBS and blocked with 5% BSA in PBS. Lymphocytes, MEFs and iP cells were plated in Poly-L-lysine-coated coverslips, treated for 5 min with Triton-100 buffer for nuclear extraction, fixed 10 min in 4% buffered formaldehyde, permeabilized with 0.2% PBS-Triton for 10 min and blocked with 5% BSA in PBS for 1h. Samples were incubated O/N at 4°C with rabbit polyclonal anti-TRF1 (1:500; CNIO homemade), with rabbit polyclonal anti-CD31 (1:50; Abcam ab28354), mouse monoclonal anti-phospho-histone γH2A.X-Ser139 (1:300) (Millipore, 05–636), rabbit-polyclonal anti-53BP1 (1:300; Novus Biologicals, NB100-304) or with rabbit polyclonal anti-PML (1:100; Santa Cruz Biotechnology, H-238). When indicated, a Q-Fish was performed on IF stained slides fixed with 4% formaldehyde for 20 minutes.

MEFs and IPS cells were treated for 5 min with Triton-100 buffer for nuclear extraction, fixed 10 min in 4% buffered formaldehyde, permeabilized with 0.2% PBS-Triton for 10 min and blocked with 5% fetal bovine serum in PBS for 1h. Samples were incubated O/N at 4°C with rabbit anti-TRF1 (1:500) (CNIO homemade antibody), mouse anti-γH2A.X-Ser139 (1:500) (05–636, Millipore), with rabbit anti-PML (1:100) (Santa Cruz Biotechnology, H-238) and mouse anti-HA-Tag (1:100) (Cell Signalling, 2367). Cells were then washed and incubated with 488-Alexa or 555-Alexa labeled secondary antibodies (Thermo Fisher Scientific) for 1 h at RT in a humid chamber. Samples were mounted in Prolong Gold with DAPI (Invitrogen).

Immunofluorescence images were obtained using a confocal laser-scanning microscope (Leica TSC SP5) using a Plan Apo 63Å-1.40 NA oil immersion objective (HCX). Maximal projection of z-stack images generated using advanced fluorescence software (LAS) were analyzed with Definiens XD software package.

Quantitative Fluorescence In Situ Hybridization (Q-FISH) analysis

For quantitative telomere fluorescence in situ hybridization (Q-FISH), paraffin-embedded tissue sections were deparaffinized and fixed with 4% formaldehyde, followed by digestion with pepsin/HCl and a second fixation with 4% formaldehyde. Slides were dehydrated with increasing concentrations of EtOH (70%, 90%, 100%) and incubated with the telomeric (TTAGGG) probe labelled with Cy3 at 85°C for 3 min followed by 2h at room temperature in a wet chamber. The slides were extensively washed with 50% formamide and 0.08% TBS-Tween 20. Confocal microscopy was performed at room temperature with a laser-scanning microscope (Leica TSC SP5) using a Plan Apo 63Å-1.40 NA oil immersion objective (HCX). Maximal projection of z-stack images generated using advanced fluorescence software (LAS) were analyzed with Definiens XD software package. The DAPI images were used to detect telomeric signals inside each nucleus.

High-throughput (HT)-QFISH on peripheral blood leukocytes was done using 150 μl of blood as described [51]. Confocal images were captured using the Opera High-Content Screening system (Perkin Elmer).

MEFs and iP cells were incubated with 0.1 μg/ml colcemide during 4 h and 2h, respectively. After hypotonic swelling in 0.03 M sodium citrate for 30 min at 37°C, cells were fixed in methanol:acetic acid (3:1). Quantitative telomere fluorescence in situ hybridization (Q-FISH) was performed as described. Images were captured using microscope Leica DM6B using a 100x oil objective. Telomere length was analyzed using Leica Application Suite X Software.
The incidence of chromosomal aberrations per metaphase was determined by eye. The images were analyzed blindly.

**Telomere recombination measurements using chromosome orientation FISH (CO-FISH)**

Exponentially growing primary MEFs were sub-cultured in the presence of 5'-bromo-2'-deoxyuridine (BrdU; Sigma) at a final concentration of $1 \times 10^{-5}$ M, and then allowed to replicate their DNA once at 37°C for 12 hours. Colcemide was added at a concentration of 0.1 μg/ml during the last 4 hours. Cells were then recovered and metaphases prepared as described [44].

**Western blot analysis**

Total cell and nuclear protein extracts were obtained by RIPA extraction buffer MERK, R0278) or using a Nuclear/Cytosolic Fractionation Kit (Biovision, K266-100) and protein concentration was determined using a Bradford Reagent (B6916, Sigma Aldrich). Forty micrograms of nuclear extracts were separated in 4–12% SDS-PAGE gels (NuPAGE Invitrogen) and transferred to nitrocellulose membranes (Amersham Protan). Blots were incubated with the indicated antibodies. Antibody binding was detected after incubation with a secondary antibody coupled to horseradish peroxidase using a chemiluminescence with ECL detection kit (GE Healthcare). The primary antibodies used were rat monoclonal anti-POT1a (1:200, Clone name POP148C, CNIO homemade), rat monoclonal anti-TRF1 (1:500, Clone name 572C, CNIO homemade), rabbit polyclonal anti-nanog (1:1000, Cell Signaling, 8822), rabbit polyclonal antiphospho-CHK1 (Ser345) (1:1000, Cell Signaling, 2348), mouse monoclonal anti-CHK1 (1:1000, Cell Signaling, 2360), rabbit polyclonal anti-phosphoRPA32 (1:1000, Bethyl), A300-245A), rat monoclonal anti-RPA32 (1:1000, Cell Signalling, 2208) and rabbit polyclonal anti-SMC1 (1:8000; Bethyl, A300-055A).

**RNA and qPCR**

Total RNA from cells was extracted with the RNeasy kit (74106, QIAGEN) and reverse transcribed was using the iSCRIPT cDNA synthesis kit (1708891, BIO-RAD) according to manufacturer’s protocol. Quantitative real-time PCR was performed with the QuantStudio 6 Flex (Applied Biosystems, Life Technologies) using Go-Taq Green Master Mix (M7123, Promega) according to the manufacturer’s protocol. All values were obtained in triplicates. Primer sequences can be found in S1 Table. We determined the relative expression in each sample by calculating the $2^{\Delta \text{CT}}$ value. For each sample, $2^{\Delta \text{CT}}$ was normalized to control $2^{\Delta \text{CT}}$ mean.

**Chromatin Immune Precipitation assay (ChIP)**

Formaldehyde was added directly to MEFs culture medium to a final concentration of 1% and incubated for 15 min at room temperature (RT) on a shaking platform. Cross-linking was then stopped by addition of glycerine to a final concentration of 0.125 M for 5 min at RT. Cross-linking cells were washed twice with cold PBS containing 1 μM PMSF and protease inhibitors and then pelleted. Cells were lysed in lysis buffer (1% SDS, 10 mM EDTA and 50 mM Tris-HCl pH 8.0) containing protease inhibitors for 20 min at 4°C. Lysates were sonicated to obtain chromatin fragments $<1$ kb and centrifuged for 15 min in a microfuge at room temperature. Chromatin was diluted 1:10 with dilution buffer (1.1% Triton X-100, 2 mM EDTA pH 8.0, 150 mM NaCl, 20 mM Tris-HCl pH 8.0) and precleared with 50 μl of protein A/G Plus-Agarose beads (sc-2003, Santa Cruz Biotechnology). After centrifugation, chromatin fragments were incubated at 4°C overnight on a rotating platform with rat monoclonal anti-POT1a (CNIO
homemade), rabbit polyclonal anti-TRF1 (CNIO homemade), rabbit polyclonal anti-TRF2 (1:1000, Novus Biologicals, NB110-57130,) or with rabbit IgG (sc-2025, Santa Cruz Biotechnology). Samples were then immunoprecipitated with 50 μl of protein A/G Plus-Agarose beads. The immunoprecipitated pellets were washed once with IP Wash A (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0), IP Wash B (150 mM NaCl, and then with 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0), IP Wash C (500 mM NaCl, 0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl pH 8.0) and finally with TE (10 mM Tris-HCl pH 8.0) two times. The chromatin was eluted from the beads twice by incubation with 250 μl 1% SDS and 0.05 M NaHCO₃ during 15 min at RT with rotation. After adding 20 μl of 5 M NaCl, the crosslink was reversed overnight at 65°C. Samples were supplemented with 20 μl of 1 M Tris-HCl pH 6.5, 10 μl of 0.5 M EDTA, 20 μg of RNase A, and 40 μg of proteinase K, and incubated for 1 h at 45°C. DNA was recovered by phenol–chloroform extraction and ethanol precipitation, denatured (0.3 M NaOH) at 50°C for 1 h, neutralized (1 M ammonium acetate), and transferred to a Hybond-N+ membrane (Amersham) on a dot blot. The membranes were hybridized with a telomeric probe obtained from a plasmid containing 1.6 kb of TTAGGG repeats (gift from T. de Lange, Rockefeller University). The signal was quantified with the ImageJ software. The amount of telomeric DNA immunoprecipitated was calculated in each ChIP based on the signal relative to the corresponding total telomeric DNA signal.

C-circle assay

One μg of genomic DNA was digested with HinfI and RsaI in the presence of RNase. Genomic DNA (200 ng) was diluted in phi29 reaction buffer (0.2 mg/ml BSA, 0.1% Tween, 1mM each dATP, dGTP and dTTP, 1xphi29 buffer) (total volume of 40 μl) and 10 units of 4BB QualiPhi DNA polymerase (4basebio) were added. The amplification reaction was performed at 30°C for 8 hours and inactivated at 65°C for 20 min. The reaction product was diluted with 2xSSC to 120 μl and dot-blotted on an N-Hybond (Amersham). DNA was UV-cross-linked onto the membrane which was hybridized at 57°C with 32P-(CCCTAA)₇ in Church and Gilbert hybridization buffer (1% BSA, 1mM EDTA, 7% SDS, 15% formamide and 200 mM sodium phosphate pH 7.2).

Supporting information

S1 Fig. Pot1a knock-in mouse generation. Genomic DNA was digested with EcoR1 restriction enzymes for southern analysis and the size of the fragments are indicated. A-C. Southern blot images from 5’- (A), 3’- (B) and neo (C) specific probes from two independent targeted stem cell clones (clone 47 and clone 52). D. Agarose gel of PCR product using primers F1 and R1 encompassing the distal LoxP511 site from the selected stem cell clones. These PCR products were further sequenced to assure the 3’-end of the targeted allele was not lost in the genome integration event. E. Southern blot image with the 3’-probe from EcoR1 digested genomic DNA from mice after excision of the Neo cassette by the FLP recombinase. These mice were used as the parental mice for generating the Pot1a⁺/flex colonies. F. Schematic representation of the PCR genotyping reaction for the Pot1a⁺ and Pot1a⁺⁺ alleles. A representative image of an agarose gel showing the PstI cleaved PCR amplification products using primers upstream and downstream E7 (F2 and R1, respectively) from genomic DNA from Pot1a⁺/+ and Pot1a⁺/+ mice after cross with the EIIa-Cre tool mouse. (TIFF)

S2 Fig. Expression levels of Pot1a alleles. A. Expected and observed number of mice of the offspring from Pot1a⁺/+ intercrosses. B-C. Expected and observed number of embryos of the
offspring from Pot1a+/flex (B) and from Pot1a+/ki p53−/− (C) intercrosses. The Fisher’s exact test was used to determine statistical significance. p-values are indicated.

D. Schematic representation of Pot1a, Pot1aki and Pot1aflex mRNA. The R117C substitution is within exon 7 (E7∗).

Five different primers pairs were used to quantify transcripts levels corresponding to E3-E5 (green arrows), E7 (black and red arrows for E7 wildtype and E7∗, respectively), E8-E9 (purple arrows) and E9-E10 (brown arrows). The scheme is not drawn to scale. E. Quantification of expression levels by qRT-PCR of different exons in Pot1a, Pot1a ki and Pot1a flex alleles in tail tissue of Pot1a+/+, Pot1a+/ki and Pot1a+/flex mice. A t-test two tailed was used for statistical analysis. The p-value is indicated. Mean values +/- SEM are represented. N = number of mice analyzed per genotype.

(TIFF)

S3 Fig. The Pot1a ki allele is properly transcribed. A. Nucleotide BLAST alignment of Pot1a wild type and mutant knock-in exon 7 sequences. The mutant exon 7 contains several silent substitutions and one missense mutation that results in an amino acid change from arginine to cysteine at position 117. B. Sequencing spectrum of wild type (upper panel) and mutant (lower panel) Pot1a-exon 7. The cDNA from Pot1a+/+ and Pot1a+/ki MEFs was PCR amplified using primers annealing within exon 3 (Pot1a E3-E5-F) and within exon9 (Pot1a E8-E9-R) (S1 Table). The PCR products were subjected to Sanger sequencing. The 5’ and 3’ ends of exon 7 and are indicated.

(TIFF)

S4 Fig. POT1a mutant protein binds telomeric DNA in vivo. A-C. Quantification of telomeric DNA pulled down with anti-TRF1(A), with anti-TRF2 (B) and with anti-POT1a (C) of MEFs of the indicated genotype. DNA input signal is also shown. D. Representative images of chromatin immunoprecipitation (ChIP) of telomeric DNA. Low and high exposure images are shown. ChIP values are normalized by the input of each individual sample. E-F. Quantification of TRF1 fluorescence intensity levels in intestines (E) and in liver (F) of mice of the indicated genotype. Representative images of TRF1 immunofluorescence are shown below. Bars and error bars represent mean values ± SE. N = number of independent experiments. Student’s t-test was used for the statistical analysis. P-values are indicated.

(TIFF)

S5 Fig. Generation of induced pluripotent stem (iPS) cells. A. Quantification and representative western blot images of nuclear extracts of TRF1 and Nanog protein levels in Pot1a+/+ p53−/− and Pot1a+/ki p53−/− MEFs and iPS cells. SMC1 was used as loading control. IPS cells were generated from two independent MEFs from each genotype. The protein level quantification is represented in the bar plot. B. Quantification of Tert expression levels by q-RT-PCR in MEFs and IPs of the indicated genotype. Two MEFs and two iPS cells from each genotype were used for the analysis. The analysis was performed four times for each cell line. n = replicates. Two tailed Student’s t-test was used for the statistical analysis. P-values are indicated.

(TIFF)

S6 Fig. POT1a p.R117C expression does not lead to ATR activation. A,B. Representative western blot images of total cellular extracts of phosphor-CHK1, total CHK1, phospho-RPA, total RPA protein levels in Pot1a+/+ p53−/− and Pot1a+/ki p53−/− MEFs (A) and IPSs (B). SMC1 was used as loading control. Cells were treated with hydroxyurea (2 mM) for 3 hours as positive controls for replicative stress. C. C-circle quantification and representative dot-blot images of MEFs of the indicated genotype. U2OS and HEK293T cells were used as positive and negative controls, respectively. A negative control without Phi29 polymerase is also shown. The C-
circle score is calculated as the percentage of the signal relative to that of ALT positive U2OS cell line. \( n = \) number of independent experiments. D. Number of ALT-associated PML bodies (APBs) in PML positive cells (E) in MEFs of the indicated genotype. \( n = \) number of cells. Representative Immune-Fish images of PML and a telomeric probe. APBs were detected by PML and Telomere co-localizing foci (white arrowheads). A t-test two tailed was used for statistical analysis. Error bars represent standard error. The p-value is indicated.

(S7 Fig) Pot1a knock-in mice do not present higher incidence of benign, hematopoietic and sarcoma tumors. A. Kaplan-Meier survival curves of mouse cohorts of the indicated genotypes. Median survival values are indicated. A Log-rank (Mantel-Cox) test was performed for statistical analysis. P- values are indicated. \( n = \) number of mice. B. Percentage of mice presenting carcinomas at death. Representative H&E images of a hypophysis carcinoma are shown to the right. The neoplasm invades hypothalamus and the thalamus (asterisk). Hemorrhagic areas were present (red arrow). Atypical polygonal cells arranged in solid sheets with fibrovascular stroma. The tumor cells showed pleomorphic nuclei, large nucleoli (black arrow) and high number of mitosis (green arrows) were observed. Chi squared test was performed for statistical analysis. The number of mice and the p values are indicated. C-E. Percentage of mice of the indicated genotype presenting benign (C), hematopoietic (D) and sarcoma (E) tumors at death. The types of tumors are indicated in the legend. Chi squared test was performed for statistical analysis. None of the comparisons were statistically significant (ns). \( N = \) number of mice.

(S8 Fig) Shorter telomeres in tumors than in surrounding healthy tissue. A. Telomere mean spot intensity in hypophysis tumors and in surrounding non-tumoral hypophysis tissue. Three adenomas from Pot1a\(^{+/ki}\)p53\(^{+/+}\) and one carcinoma from Pot1a\(^{+/ki}\)p53\(^{+/−}\) mice at death were analyzed. B. Telomere mean spot intensity in lung tumors and in surrounding non-tumoral lung tissue. One lung adenoma and two bronchoalveolar carcinomas from Pot1a\(^{+/ki}\)p53\(^{+/+}\) mice at death were analyzed. C. Telomere mean spot intensity in stomach carcinomas and in surrounding non-tumoral stomach tissue. Two Pot1a\(^{+/ki}\)p53\(^{+/−}\) stomach carcinomas were analyzed. The box-and-whisker graph shows the values lower and greater than first and 99\(^{th}\) percentile for each group. Mean spot intensity is indicated in each case. \( n = \) number of cells. A t-test two tailed was used for statistical analysis. The p-values are indicated. D. Representative Immune-Fish images of PML and a telomeric probe in carcinomas from hypophysis, lung and stomach. Bone marrow sections were used as staining positive control. Insets correspond to higher magnification images.

(S9 Fig) Higher incidence of ALT-associated PML bodies in Pot1a\(^{+/ki}\) angiosarcomas. A-B. Percentage of PML positive cells (A) and number of ALT-associated PML bodies (APBs) per cell (B) in healthy vascular endothelium (VE) and in angiosarcomas (AS) from mice of the indicated genotype at death point. A PML positive cell was defined as having >2 foci. Two Pot1a\(^{+/+}\) tumors were analyzed, a utero angiosarcoma (Pot1a\(^{+/+}\)p53\(^{+/−}\)) and a cardiac angiosarcoma (Pot1a\(^{+/+}\)p53\(^{+/−}\)). Four Pot1a\(^{+/ki}\) thoracic angiosarcomas were analyzed, three Pot1a\(^{+/ki}\)p53\(^{+/−}\) and one Pot1a\(^{+/ki}\)p53\(^{+/−}\). Non-tumoral vascular endothelium from the same mice were also analyzed. F. Representative Immune-Fish images of PML and a telomeric probe in vascular endothelium and in cardiac angiosarcomas of the indicated genotypes. Vascular endothelium was identified by autofluorescence of red blood cells within the blood vessels. APBs were detected by PML and Telomere co-localizing foci (white arrowheads). D-E. Percentage of
γH2AX positive cells (D) and number of Telomere-Induced Foci (TIF) per cell (E) in AS from mice of the indicated genotype at death point. The samples analyzed were the same as in A. A representative image of γH2AX and TRF1 staining is shown. TIFs were detected by γH2AX and TRF1 co-localizing foci. A t-test two tailed was used for statistical analysis. The p-values are indicated.

(SIFF)

S1 Table. Primers used in this study.

(DoCX)

S1 Data. Numerical data underlying graphs.

(XLSX)

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