Paracrine Interaction of Cancer Stem Cell Populations Is Regulated by the Senescence-Associated Secretory Phenotype (SASP)

Angelica M. Lagunas, Marybeth Francis, Nisha B. Maniar, Gergana Nikolova, Jianchun Wu, and David L. Crowe

Abstract

Dyskeratosis congenita is a telomere DNA damage syndrome characterized by defective telomere maintenance, bone marrow failure, and increased head and neck cancer risk. The Pot1b<sup>−/−</sup>;Terc<sup>−/−</sup> mouse exhibits some features of dyskeratosis congenita, but head and neck cancer was not reported in this model. To model the head and neck cancer phenotype, we created unique Pot1b- and p53-null-mutant models which allow genetic lineage tracing of two distinct stem cell populations. Loss of Pot1b expression depleted stem cells via ATR/Chk1/p53 signaling. Tumorigenesis was inhibited in Pot1b<sup>−/−</sup>;p53<sup>−/−</sup> mice due to cellular senescence. Pot1b<sup>−/−</sup>; p53<sup>−/−</sup> tumors also exhibited senescence, but proliferated and metastasized with expansion of Lgr6<sup>−/−</sup> stem cells indicative of senescence-associated secretory phenotype. Selective deletion of the small K15<sup>−/−</sup> stem cell fraction resulted in reduction of Lgr6<sup>−/−</sup> cells and inhibition of tumorigenesis via senescence. Gene expression studies revealed that K15<sup>−/−</sup> cancer stem cells regulate Lgr6<sup>−/−</sup> cancer stem cell expansion via chemokine signaling. Genetic ablation of the chemokine receptor Cxcr2 inhibited cancer stem cell expansion and tumorigenesis via senescence. The effects of chemokines were primarily mediated by PI3K signaling, which is a therapeutic target in head and neck cancer.

Implications: Paracrine interactions of cancer stem cell populations impact therapeutic options and patient outcomes.

Introduction

Chromosome ends are protected by telomeres that prevent DNA damage response (DDR) and degradation (1). Telomeres form a large duplex loop mediated by single-strand invasion of a G-rich overhang (2). When telomeres become critically short, the DDR is activated at chromosome ends, which induces cellular senescence or apoptosis (3). Cells can stabilize their telomeres and continue proliferation by upregulation of telomerase (4–6). Given the positive effects of telomerase on telomere length and cellular proliferation, telomerase activity is commonly upregulated in cancer cell lines and primary tumors (7, 8).

The telomeric shelterin complex contains the ssDNA-binding protein Pot1 (Pot1a/b in mice; ref. 9). Loss of Pot1b expression depleted cultured cells induces telomere DDR, ATR activation, senescence, and death (10–12). However, Pot1 mutations have been found in cancers (13, 14). These studies demonstrate the importance of Pot1b in regulating DDR at telomeres in cancer.

Cellular senescence was defined as a state of durable cell-cycle arrest (15). However senescent cells are metabolically active and can have both tumor suppressing and tumor-promoting effects on the tissue microenvironment by expression of growth factors and cytokines, which regulate proliferation of neighboring cells. This property of senescent cells is referred to as the senescence-associated secretory phenotype (SASP).

Dyskeratosis congenita is a human telomere DNA damage syndrome characterized by telomere shortening, early bone marrow failure, skin pigmentation, nail dystrophy, and increased risk of head and neck cancer (16, 17). It is believed that defective telomere maintenance is the cause of dyskeratosis congenita, although the reasons for specific tissues being more affected are less clear. Stem cell exhaustion due to telomere DDR may result not only in functional tissue failure but also pathologic repair processes such as lung and liver fibrosis observed in some patients with dyskeratosis congenita.

However, increased cancer risk in patients with dyskeratosis congenita is more difficult to explain by the stem cell depletion model. Approximately 80% of patients with dyskeratosis congenita exhibit oral intraepithelial neoplasia, which is one of the diagnostic features of the disease (16, 17). Hypothetically mucosal stem cell depletion should decrease cancer risk. Conversely a mucosal stem cell fraction may survive telomere DDR with resultant genomic instability. The Pot1b<sup>−/−</sup>;Terc<sup>−/−</sup> mouse exhibits some features of dyskeratosis congenita (18), but head and neck cancer was not reported in this model. The shortened lifespan of this mouse due to Terc heterozygosity may explain the absence of the cancer phenotype. In addition, most head and neck cancers exhibit loss of p53 tumor suppressor function (19), which inhibits DDR, cell-cycle arrest, and apoptosis. To address these issues, we created unique Pot1b- and p53-null-mutant models which allow genetic lineage tracing of two distinct stem cell populations in mucosal epithelium. Our results demonstrate that...
the telomere DDR regulates senescence-associated paracrine interactions between cancer stem cell populations, dramatically affecting tumor progression and metastasis.

Materials and Methods
Mouse breeding and procedures

Mouse strains and experimental procedures were approved by the institutional animal care committee. The B6.129S2-Tpr53tm1Tyj/J, C57Bl/6-Jr.Cdc25nbm1(ROSA)26Sortm1(rtTA,EGFP)Nagy/J, B6.Cg-Tg[KRT14-Cre]1Amc/J, and B6.SIL-Tg[Krt1-15-cre/PR2]22Co[1/J; Cd(ROSA)26Sortm1(tTA,EGFP)Nagy]/J, and B6.129P2-Lgr6tm2(creERT2)K502 J-mutant mouse strains were purchased from The Jackson Laboratory. Pot1b-null mice lacking exon 3 were kindly provided by Dr. Eros Lazzerini Denchi (The Scripps Research Institute, La Jolla, CA). Mice were crossed to create Pot1b+/−;K15CrePR;GFP, Pot1b+/−;p53+/−;Lgr6CreER;GFP, Pot1b+/−;K15CrePR;GFP, Pot1b+/−;p53+/−;Lgr6CreER;GFP, Pot1b−/−;K15CrePR;GFP, Pot1b−/−;p53−/−;Lgr6CreER;GFP, Pot1b+/−;K15CrePR;GFP, Pot1b+/−;p53+/−;Lgr6CreER;GFP, and Pot1b−/−;K15CrePR;GFP;DTA, Pot1b−/−;p53−/−;K14Cre;Ccsrc2+/−; and Pot1b−/−;p53−/−;K14Cre;Ccsrc2+/− offspring (n = 20 for each group). K15 lineage tracing mice were injected with 200 µg of the progesterone receptor antagonist RU486 to express GFP and the reverse tet activator in K15+ cells. Lgr6 lineage tracing mice were injected with 1 mg of the estrogen receptor antagonist tamoxifen to express GFP in Lgr6+ cells. Mice received 25 µg dimethylbenzanthracene (DMBA) in ethanol applied topically to oral mucosa twice weekly. Control mice received ethanol vehicle only. Pot1b+/−;p53+/−;K15CrePR;GFP;DTA mice received 2 mg/mL doxycycline in drinking water. The latency, number, and volume of tumors were recorded for each animal. In separate experiments, 103 Pot1b+/−;p53+/−;K15CrePR;GFP and Pot1b−/−;53−/−;Lgr6CreER;GFP SCC cells were injected subcutaneously in Matrigel into NU/J mice which were treated with 60 mg/kg buparlisib (PI3K inhibitor), 1 mg/kg tremetinib (MEK inhibitor), 240 mg/kg MK2206 (Akt inhibitor), or vehicle (n = 5/group). Skin, mucosa, and tumors from 9-month-old mice were fixed in 4% formaldehyde in PBS, flash frozen, and stored at −80°C, or trypsin dissociated and cryopreserved in liquid nitrogen.

qRT-PCR

RNA was extracted from mouse mucosa and reverse transcribed according to the manufacturer’s instructions (Invitrogen). cDNA was performed using Pot1b primers 5′-CGGCCCCACAGCACTCTTAC-3′ and 5′-TCTCTTGCTAAAATGCAGG-3′. p53 expression was determined using 5′-TGATGGAGAGTATTT-3′ and 5′-TGACATGGAGTATTTGACGGC-3′ primers. Cdx1 expression was determined using 5′-GTGAAGCCTCCAGGGAGG-3′ and 5′-CTCTTGGACGTCTCTCCCCAG-3′ primers. Cdx3 expression was determined using 5′-GATCCTACGCAAGCCGTCG-3′ and 5′-AGTGGCCACGGACGAAATGT-3′ primers. Probes were denatured with Cy3-labeled telomeric peptide nucleic acid probe (T′TAGGGG)3 in 70% formamide at 80°C for 10 minutes, followed by overnight incubation at room temperature. After washing, sections and cells were blocked with 10% normal serum, and incubated with anti-53BP1 antibody. After washing, sections were incubated with anti-IgG secondary antibody conjugated to AlexaFluor 488. After washing, colocalized DNA damage and telomere signals were visualized by fluorescence microscopy (Zeiss LSM 710 META). In separate experiments, replication protein A (RPA) was localized at telomeres using the same immunofluorescence/FISH protocol. Phospho-ATR, phospho-Chk1, p53, K15, Lgr6, and histone H3K9me3 proteins were localized in mouse mucosa and tumor tissue using the same immunofluorescence protocol. IHC analysis of proliferating cell nuclear antigen (PCNA) and p16INK4A expression was performed as described previously (20).

ssDNA analysis

Genomic DNA was extracted from SCC and analyzed for single-strand telomere overhangs as described previously (20).

Telomere length analysis of stem cells

We used a qPCR method to measure average telomere length ratios of sorted GFP+ and GFP− stem cells from mouse mucosa and tumors as described previously (20).

Cell death analysis

Mucosa and tumor tissue sections were incubated with terminal deoxynucleotidyl transferase and dUTP-fluorescein for 1 hour at 37°C according to the manufacturer’s recommendations (Roche Applied Sciences). After washing, apoptotic cells were visualized by fluorescence microscopy. The percent fluorescent cells was determined using quantitative image analysis software. Data were analyzed by ANOVA.

FACS

GFP+ and GFP− mucosal epithelial cells and SCCs were dissociated by trypsinization, washed in PBS, and sorted by flow cytometry (Beckman Coulter MoFlo). Data were analyzed by ANOVA.

Western blot analysis

Protein was extracted from mucosa and tumors in 1× Laemml buffer. Western blotting using antibodies was performed as described previously (20).

Senescence-associated β-galactosidase activity

Frozen tumor sections were processed for 5 minutes in phosphate buffered 2% formaldehyde and 0.2% glutaraldehyde at room temperature. Sections were incubated in staining solution containing 1 mg/mL X-gal. Sections were counterstained with nuclear fast red solution.

FISH, immunofluorescence, and IHC

Fixed mouse skin, mucosa, and tumor tissue was dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Sections were deparaffinned and stained with hematoxylin and eosin. For telomeric FISH, deparaffinned tissue sections or sorted cells were denatured with Cy3-labeled telomeric peptide nucleic acid probe (T′TAGGGG)3 in 70% formamide at 80°C for 10 minutes, followed by overnight incubation at room temperature. After washing, sections and cells were blocked with 10% normal serum, and incubated with anti-53BP1 antibody. After washing, sections were incubated with anti-IgG secondary antibody conjugated to AlexaFluor 488. After washing, colocalized DNA damage and telomere signals were visualized by fluorescence microscopy. Senescence-associated β-galactosidase activity was determined using 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) for 1 minute. qPCR was performed using the StepOnePlus System (Thermo Fisher Scientific).
Antibodies
Novus 53BP1 NB100-304, keratin 15 NBPF2-50461, Abcam RPA AAB177, Chkl1 AB47754, pChkl1 AB47318, Cxc2 AB14935, pLiNKAA AB54210, H3K9me3 AB88898, Cell Signaling Akt2 2938, pAkt1 13038T, ERK1 4695, pERK1 4377T, P73K 4257, pPI3K 4228, PKC 2056, PKPC 9375T, PLCγ 2822, pPLCγ 2821T, β-actin 4970T, phospho-p53 9284, p21 2947, Becton Dickinson p53 BD5544147, Santa Cruz ATR SC-1887, pATR SC-109912, PCNA SC-7907, and Lgr6 SC-48236.

Results
The Pot1b−/−;Terc−/− mouse exhibits features of dyskeratosis congenita but lacked the cancer phenotype observed in these patients. We reasoned that the shortened lifespan of this mouse due to Terc heterozygosity may explain the absence of the cancer phenotype. In addition, most head and neck cancers exhibit loss of p53 tumor suppressor function, which inhibits DDR, cell-cycle arrest, and apoptosis. To address these issues we created Pot1b−/−;p53−/−, Pot1b−/−;p53+/−, and Pot1b−/−;p53+/+ mice with K15+ and Lgr6+ stem cell lineage tracing capability. All mice were born at the expected Mendelian ratios. Pot1b and p53 mRNA expression mucosal epithelium of all genotypes is shown in Fig. 1A. Only Pot1b−/−;p53−/− mice exhibited skin pigmentation similar to that observed in dyskeratosis congenita (Fig. 1B–E). Pot1b−/−;p53−/− epidermis exhibited increased numbers of melanocytes which was not observed in other genotypes (Fig. 1F–J). Bone marrow failure and wait dystrophy were not observed in any genotype. Pot1b−/−;p53−/−/C0 mice developed subcutaneous nodules with mean latency of 117 days which were histopathologically classified as undifferentiated sarcoma (Fig. 1f; ref. 21). However no spontaneous sarcomas or carcinomas were observed in 2-year-old Pot1b−/−;p53+/− mice following necropsy, indicating that Pot1b was required for cancer development even in the tumor prone p53-deficient background. Histopathologic evaluation of mucosal epithelium revealed no evidence of squamous cell carcinoma in mice ranging from 2 months (Fig. 1K–N) to 2 years old (Fig. 1O–R). These results indicate that Pot1b does not exhibit tumor suppressor function in the context of epithelial tumorigenesis.

To determine the mechanisms of Pot1b function in mucosal epithelium, we first examined the telomere DDR. Pot1b−/−;p53−/− and Pot1b−/−;p53−/−;epithelial cells exhibited increased telomere DNA damage 53BP1 foci (39% and 38%; P < 0.00001) compared with Pot1b−/−;p53+/− and Pot1b−/−;p53+/+ epithelium (0.4% and 0.1%; Supplementary Fig. S1A–S1D). Pot1b−/−;p53−/− and Pot1b−/−;p53−/−/C0 epithelial cells exhibited increased pATR expression (44% and 40%; P < 0.00003) compared with Pot1b−/−;p53+/− and Pot1b−/−;p53+/+ epithelium (0.5% and 0.3%; Supplementary Fig. S1E–S1H). Pot1b−/−;p53+/− and Pot1b−/−;p53+/+ epithelial cells exhibited increased pCHK1 expression (42% and 31%; P < 0.0007) compared with Pot1b−/−;p53−/− and Pot1b−/−;p53−/−/C0 epithelium (0.7% and 0.2%; Supplementary Fig. S1I–S1L). Pot1b−/−;p53−/−/C0 epithelial cells exhibited increased p53 expression (25%; P < 0.005) compared with Pot1b−/−;p53+/− (0.2%), Pot1b−/−;p53−/− (0.1%), and Pot1b−/−;p53−/−/C0 epithelium (0.1%; Supplementary Fig. S1M–S1P). Pot1b deficiency reduced ATR in K15+ cancer stem cells (1.3 vs. 1.7; P < 0.02), Lgr6+ stem cells (1.1 vs. 1.4; P < 0.04), and basal cells (0.5 vs. 0.9; P < 0.03) which was independent of p53 expression (Supplementary Fig. S1Q). These results indicate that Pot1b deficiency induces telomere DDR and telomere shortening in mucosal epithelium.

Despite Pot1b mediated telomere DDR in mucosal epithelium, no significant increases in apoptotic cells were observed by TUNEL analysis (Supplementary Fig. S2A–S2D). Decreased proliferating cell fraction was detected in Pot1b−/−;p53−/+ epithelium (25% vs. 50%; P < 0.01) compared with Pot1b−/−;p53−/− tissue, but increased proliferating cell fraction was detected in p53-deficient backgrounds (68% and 87%; P < 0.009; Supplementary Fig. S2E–S2I). K15+ and Lgr6+ stem cell fractions were reduced in Pot1b−/−;p53−/+ (1.0% and 2.6%; P < 0.02) epithelium compared with Pot1b−/−;p53−/− (1.8% and 4.9%), but K15+ and Lgr6+ stem cell fractions were increased in Pot1b−/−;p53−/− (2.7% and 7.3%; P < 0.04) and Pot1b−/−;p53−/− (3.4% and 8.1%; P < 0.03; Supplementary Fig. S2J–S2Q) epithelium. These results indicate that loss of Pot1b expression inhibits cell proliferation and stem cell expansion in mucosal epithelium.

Given that spontaneous head and neck cancer was not observed in Pot1b−/−;p53−/− mice, we used our published DMBA carcinogenesis protocol to induce SCC in these animals (22). Pot1b−/−;p53−/+ SCC exhibited increased latency (175 vs. 153 days; P < 0.05; Fig. 2A) compared with Pot1b−/−;p53−/− tumors. The tumor volume of Pot1b−/−;p53−/+ SCC was dramatically reduced at 100 days after detection (35 mm3 vs. 453 mm3; P < 0.0008; Fig. 2B) compared with Pot1b−/−;p53−/− tumors. Pot1b−/−;p53−/+ and Pot1b−/−;p53−/− SCC exhibited higher latency (124 and 104 days; P < 0.02) and increased growth rate (maximum 450 mm3 tumor volume at 81 and 60 days; P < 0.001) Pot1b−/−;p53−/− SCC exhibited terminal differentiation compared with tumors of other genotypes (45% vs. 8%; P < 0.003; Fig. 2C–F). Decreased tumor volume in Pot1b−/−;p53−/+ mice is due to p53-dependent telomere DDR. Surprisingly Pot1b−/−;p53−/+ SCC failed to develop metastatic tumors compared with other genotypes, and 20% of primary tumors underwent complete regression (P < 10−12; Fig. 2G–L). These results indicate that loss of Pot1b expression inhibits development of primary and metastatic SCC.

To understand how loss of Pot1b inhibits SCC, we examined telomere DDR in tumors from each genotype. Pot1b−/−;p53−/+ and Pot1b−/−;p53−/− SCC exhibited 8-fold increased ssDNA at telomeres compared with Pot1b−/−;p53+/− and Pot1b−/−;p53+/+ tumors as determined by Southern blotting of native and denatured genomic DNA (P < 0.005; Fig. 3A; Supplementary Fig. S3A). Similarly Pot1b−/−;p53+/+ and Pot1b−/−;p53−/− SCC exhibited increased RPA foci at telomeres, indicating increased single-stranded telomeric DNA in the absence of Pot1b (56% and 51%; P < 0.0004) compared with Pot1b−/−;p53+/− and Pot1b−/−;p53+/+ tumors (0.2%; Fig. S3B–E). Pot1b−/−;p53−/+ and Pot1b−/−;p53−/− SCC exhibited increased 53BP1 DNA damage foci at telomeres (41% and 39%; P < 0.0006) compared with Pot1b−/−;p53−/+ and Pot1b−/−;p53−/− SCC (0.9 and 0.8%; Fig. 3F–I). Pot1b deficiency reduced ATR in K15+ cancer stem cells (1.3 vs. 1.7; P < 0.02), Lgr6+ stem cells (1.1 vs. 1.4; P < 0.04), and basal cells (0.5 vs. 0.9; P < 0.03) which was independent of p53 expression (Fig. 3I). Given increased single-stranded telomere DNA in Pot1b−/−;p53−/− and Pot1b−/−;p53−/− SCC, we examined activation of the ATR/Chkl1/p53 signaling pathway in these tumors by Western blot analysis. pATR expression increased 4-32-fold in Pot1b−/−;p53−/+ and Pot1b−/−;p53−/− SCC (P < 0.0007; Fig. 3K; Supplementary Fig. S3C). pChkl1 expression increased 8-30-fold in Pot1b−/−;p53−/+ and Pot1b−/−;p53−/−.
SCC (P < 0.001; Fig. 3K; Supplementary Fig. S3C). p53 expression was detected only in Pot1b−/−;p53+/− SCC (4-fold induction compared with Pot1b+/+;p53+/− tumors; P < 0.008; Fig. 3K, Supplementary Fig. S3C). Expression of phospho-p53 was increased by 4–6-fold in Pot1b−/−;p53+/− SCC (Supplementary Fig. S3B and S3C). Expression of the p53 target gene p21WAF1/Cip1

Figure 1.
No spontaneous SCC development in Pot1b−/−;p53−/− mice. A, Pot1b and p53 expression in mucosal epithelium from Pot1b+/+;p53+/+, Pot1b−/−;p53−/−, and Pot1b−/−;p53−/− mice is shown by quantitative reverse transcription-PCR. Error bars, SEM. Epidermis from Pot1b−/−;p53−/− (B), Pot1b−/−;p53−/− (C), Pot1b+/−;p53+/− (D), and Pot1b−/−;p53+/− (E) mice. Histopathology of epidermis from Pot1b−/−;p53−/− (F), Pot1b−/−;p53−/− (G), Pot1b−/−;p53−/− (H), and Pot1b−/−;p53−/− (I) mice is shown by hematoxylin and eosin staining. L, Undifferentiated sarcoma from p53−/− mouse dermis is shown by hematoxylin and eosin staining. Histopathology of mucosal epithelium in 2 months old Pot1b−/−;p53−/− (K), Pot1b−/−;p53−/− (L), Pot1b−/−;p53−/− (M), and Pot1b−/−;p53−/− (N) mice is shown by hematoxylin and eosin staining. Histopathology of mucosal epithelium in 2 years old Pot1b+/−;p53+/− (O), Pot1b−/−;p53−/− (P), Pot1b−/−;p53−/− (Q), and Pot1b−/−;p53−/− (R) mice is shown by hematoxylin and eosin staining. Representative photomicrographs are shown.
was increased 12-fold in Pot1b<sup>−/−</sup>;p53<sup>+/+</sup> mice. We were surprised that few apoptotic cells were detected in these SCC (Fig. 4A–D). We examined expression of senescence markers in SCC from all genotypes. Senescence was induced in SCC of Pot1b<sup>−/−</sup>;p53<sup>−/−</sup> mice, but not in mice with reduced Pot1b expression. These results indicate that loss of Pot1b expression in SCC induces telomere DNA damage signaling and telomere shortening. Given the dramatic inhibition of tumorigenesis in Pot1b<sup>−/−</sup>;p53<sup>−/−</sup> mice, we were surprised that few apoptotic cells were detected in these SCC (Fig. 4A–D). We examined expression of senescence markers in SCC from all genotypes. Senescence markers were induced in SCC of Pot1b<sup>−/−</sup>;p53<sup>−/−</sup> mice, but not in mice with reduced Pot1b expression. These results indicate that loss of Pot1b expression in SCC induces telomere DNA damage signaling and telomere shortening.
associated heterochromatin foci were increased in Pot1b−/−; p53+/+ and Pot1b−/−; p53−/− SCC (49% and 24%; P < 0.004) compared with tumors from other genotypes (Fig. 4E–H). Expression of the senescence marker p16INK4A was increased in cells from Pot1b−/−; p53+/+ and Pot1b−/−; p53−/− SCC (63% and 31%; P < 0.001) compared with tumors from other genotypes (Fig. 4I–L). Senescence-associated β-galactosidase activity was increased in Pot1b−/−; p53+/+ and Pot1b−/−; p53−/− SCC (57% and 23%; P < 0.0003) compared with tumors from other genotypes (Fig. 4M–P). We also examined cell

Figure 3. Telomere DDR in Pot1b−/−; p53+/+ and Pot1b−/−; p53−/− SCC. A, Single-stranded telomeric DNA in Pot1b+/+; p53+/+, Pot1b−/−; p53+/+, Pot1b+/+; p53−/−, and Pot1b−/−; p53−/− SCC was determined by hybridization of radiolabeled telomere probe to native and denatured genomic DNA. ssDNA at telomeres in Pot1b+/+; p53+/+ (B), Pot1b−/−; p53+/+ (C), Pot1b−/−; p53−/− (D), and Pot1b−/−; p53−/− (E) SCC was determined by immunofluorescent localization of the ssDNA binding protein RPA at telomeres (FISH). Scale bar, 2 μm. Telomere DDR in Pot1b−/−; p53+/+ (F), Pot1b−/−; p53−/− (G), Pot1b+/+; p53−/− (H), and Pot1b−/−; p53−/− (I) SCC was determined by immunofluorescent localization of 53BP1 at telomeres (FISH). Scale bar, 10 μm. Nuclei were counterstained with DAPI. Representative photomicrographs are shown. J, Average telomere length ratios in K15+ stem, Lgr6+ stem, and basal cells from SCC of Pot1b−/−; p53+/+, Pot1b−/−; p53−/−, Pot1b+/+; p53−/−, and Pot1b−/−; p53−/− mice. Error bars, SEM. K, Telomere DNA damage signaling in SCC from Pot1b−/−; p53+/+, Pot1b−/−; p53−/−, Pot1b+/+; p53−/−, and Pot1b−/−; p53−/− mice was determined by Western blot analysis using antibodies indicated at left. β-Actin protein expression was used to control for equal loading of each lane. Representative blots are shown.
proliferation in SCC from all genotypes. The proliferating cell fraction was reduced in Pot1b−/−;p53−/+ SCC (5% vs. 29%; P < 0.03; Fig. 4Q and R) compared with Pot1b+/+;p53−/+ SCC. The proliferating cell fraction was increased in Pot1b−/−;p53+/− and Pot1b+/−;p53+/− SCC (45% and 78%; P < 0.008; Fig. 4S and T). These results indicate that loss of Pot1b expression inhibits cell proliferation and induces senescence in SCC.

Figure 4.
Loss of Pot1b expression induces p53-dependent senescence and reduced proliferation in SCC. Apoptosis in SCC from Pot1b−/−;p53−/+ (A), Pot1b−/−;p53−/+ (B), Pot1b−/−;p53−/+ (C), and Pot1b−/−;p53−/+ (D) mice was determined by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) analysis. Nuclei were counterstained with DAPI. Senescence-associated heterochromatin foci in SCC from Pot1b−/−;p53−/+ (E), Pot1b−/−;p53+/− (F), Pot1b+/+;p53+/− (G), and Pot1b−/−;p53−/+ (H) mice was determined by histone H3K9me3 immunofluorescence microscopy. Nuclei were counterstained with DAPI. Cellular senescence in SCC from Pot1b−/−;p53−/+ (I), Pot1b−/−;p53−/+ (J), Pot1b−/−;p53−/+ (K), and Pot1b−/−;p53−/+ (L) mice was determined by p16INK4A IHC. Nuclei were counterstained with hematoxylin. Senescence-associated β-galactosidase (SAβgal) activity in SCC from Pot1b−/−;p53−/+ (M), Pot1b−/−;p53−/+ (N), Pot1b−/−;p53−/+ (O), and Pot1b−/−;p53−/+ (P) mice. Nuclei were counterstained with nuclear fast red. Proliferating cells in SCC from Pot1b+/+;p53−/+ (Q), Pot1b+/+;p53−/+ (R), Pot1b−/−;p53−/+ (S), and Pot1b−/−;p53−/+ (T) mice was determined by PCNA IHC. Nuclei were counterstained with hematoxylin. Representative photomicrographs are shown. Scale bar, 20 μm; F and H, 10 μm.
K15⁺ and Lgr6⁺ cancer stem cell fractions were reduced in Pot1b⁻/⁻;p53⁻/⁻ (0.6% and 3.5%; P < 0.001) SCC compared with Pot1b⁺/++;p53⁺/⁺ (1.9% and 8.6%) as determined by flow cytometry of GFP⁺ cells, but K15⁺ and Lgr6⁺ stem cell fractions were increased in Pot1b⁺/++;p53⁻/⁻ (2.7% and 19.6%; P < 0.005) and Pot1b⁻/⁻;p53⁻/⁻ (2.5% and 57.3%; P < 0.0003; Fig. 5A–H) SCC. K15⁺ and Lgr6⁺ cancer stem cells were localized in tumor tissue sections using immunofluorescence microscopy (Fig. 5I and J). These results indicate that loss of Pot1b expression inhibits cancer stem cell expansion in a p53-dependent manner.

Although both Pot1b⁻/--;p53⁺/+ and Pot1b⁻/--;p53⁻/⁻ SCC exhibited increased senescent cell fractions, the former exhibited reduced Lgr6⁻ cells and were indolent, whereas the latter showed dramatic Lgr6⁻ cell expansion, rapid proliferation, and cervical lymph node metastasis. These data suggest that the telomere DDR may differentially regulate SASP. The dramatic expansion of Lgr6⁻ may not be due to Lgr6⁻ cell proliferation but rather the Lgr6⁻ population in this context. We selectively ablated K15⁺ cancer stem cells in Pot1b⁻/--;p53⁻/⁻ SCC by crossing the Pot1b⁻/--;p53⁻/⁻;K15CrePR;GFP mouse with tetO-DTA animals. Following RU486 injection, K15⁺ cells in this mouse express the reverse tetracycline activator, which drives diphtheria toxin A expression in this fraction when doxycycline is added to drinking water. SCC with depletion of K15⁺ cancer stem cells failed to grow (93 mm³ vs. 460 mm³; P < 0.002; Fig. 6A) compared with control tumors. The number of metastatic lymph nodes was reduced in cancer stem cell–depleted tumors (21% vs. 59%; P < 0.006; Fig. 6B), and cancer stem cell depletion–induced marked terminal differentiation and regression of SCC (31%; P < 0.001; Fig. 6C–F) compared with control tumors as determined by histopathology. Senescence-associated β-galactosidase activity was increased in cancer stem cell–depleted SCC (66% vs. 0.9%; P < 0.00002) compared with control tumors, and expression of the senescence marker p16INK4A was increased in cells from cancer stem cell–depleted SCC (49% vs. 2.3%; P < 0.0001; Fig. 6G–J) compared with control tumors. Doxycycline-induced ablation reduced the K15⁺ cancer stem cell population to 0.01% (P < 10⁻⁷; Fig. 6K and L). The Lgr6⁻ cancer stem cell population was reduced to 0.04% (P < 10⁻⁷; Fig. 6M and N). These results indicate that K15⁺ cancer stem cells are responsible for the dramatic expansion of Lgr6⁻ cancer stem cells observed in Pot1b⁻/--;p53⁻/⁻ SCC.

Gene expression profiling studies of K15⁺ and Lgr6⁺ cancer stem cells from the Pot1b⁻/--;p53⁻/⁻ background revealed

---

Figure 5.
Quantitation of K15/GFP⁺ cell fraction from SCC of Pot1b⁻/--;p53⁻/⁻ (A), Pot1b⁻/--;p53⁺/+ (B), Pot1b⁺/++;p53⁻/⁻ (C), and Pot1b⁺/++;p53⁻/⁻ (D) mice was determined by flow cytometry. Quantitation of Lgr6/GFP⁺ cell fraction from SCC of Pot1b⁻/--;p53⁻/⁻ (E), Pot1b⁺/++;p53⁻/⁻ (F), Pot1b⁻/--;p53⁻/⁻ (G), and Pot1b⁻/--;p53⁻/⁻ (H) mice was determined by flow cytometry. GFP log and forward scatter linear scales are shown. K15⁺ (I) and Lgr6⁺ (J) cancer stem cells are shown in SCC histopathologic sections by immunofluorescence microscopy. Nuclei were counterstained with DAPI. Scale bar, 10 μm.
Figure 6. Selective depletion of K15⁺ cancer stem cells blocks Lgr6⁺ cancer stem cell expansion and inhibits SCC tumorigenesis. A, Tumor volume in K15⁺ (dox−) and K15⁻ (dox+) SCC is shown by weeks following primary cancer detection. Percent metastatic lymph nodes in K15⁺ (dox−) and K15⁻ (dox+) SCC is shown 12 weeks after primary cancer detection. Histopathology of primary (C) and metastatic (D) K15⁺ SCC is shown by hematoxylin and eosin staining. Scale bar, 10 μm. Histopathology of primary (E) and metastatic (F) K15⁻ SCC is shown by hematoxylin and eosin staining. Senescence-associated β-galactosidase (SAβgal) activity in K15⁺ (G) and K15⁻ (H) SCC. Nuclei were counterstained with nuclear fast red. Cellular senescence in K15⁺ (I) and K15⁻ (J) SCC was determined by p16INK4A IHC. Nuclei were counterstained with hematoxylin. Quantitation of K15⁺ cells from K15⁻ SCC was determined by flow cytometry. K15 log and forward scatter linear scales are shown. K, Absence of K15⁺ cancer stem cells in K15⁻ SCC is shown by immunofluorescence microscopy. Nuclei were counterstained with DAPI. M, Quantitation of Lgr6⁺ cell fraction from K15⁻ SCC was determined by flow cytometry. N, Absence of Lgr6⁻ cancer stem cells in K15⁻ SCC is shown by immunofluorescence microscopy. Scale bar, 2 μm.
increased expression of chemokines Cxcl1 (16-fold), Cxcl3 (5-fold), and Cxcl5 (3-fold) in K15<sup>+</sup> cancer stem cells compared with Lgr6<sup>+</sup> cells (P < 0.001; Fig. 7A). These three chemokines bind to the Cxcr2 receptor which was expressed at 9-fold higher levels in Lgr6<sup>+</sup> cancer stem cells (P < 0.004). Increased chemokine expression was not detected in Pot1b<sup>−/−</sup>;p53<sup>+/+</sup> K15<sup>+</sup> cancer stem cells compared with Lgr6<sup>+</sup> cells (P < 0.001; Fig. 7A).
stem cells which undergo senescence. These results suggest that paracrine chemokine signaling from K15\(^+\) cancer stem cells drives expansion of the Lgr6\(^+\) population. To test this hypothesis, we genetically ablated Cxcr2 expression in mucosal epithelium using K14Cre;Cxcr2\(^{-}\)/Pot1b\(^{-}\)/p53\(^{-}\) mice to selectively target stratified epithelium. Mice with Cxcr2 ablation in mucosal epithelium largely failed to develop SCC using our DMBA carcinogenesis protocol (23 mm\(^3\) vs. 443 mm\(^3\) by 10 weeks after tumor detection; \(P < 10^{-7}\); Fig. 7B) compared with control tumors. These SCC lacking Cxcr2 expression also largely failed to develop lymph node metastases (65% vs. 6%; \(P < 10^{-4}\)) compared with control tumors, and SCC lacking Cxcr2 expression also exhibited terminal differentiation by histopathologic analysis (59% vs. 5%; \(P < 0.0001\); Fig. 7C–G) compared with control tumors. Senescence-associated \(\beta\)-galactosidase activity was increased in SCC without Cxcr2 expression (53% vs. 1.7%; \(P < 0.000009\); Fig. 7H and I) compared with control tumors. These results indicate that control of Lgr6\(^+\) cell expansion by K15\(^+\) cancer stem cells in SCC is mediated by chemokine signaling.

We examined signaling pathways downstream of Cxcr2 in control and Cxcr2-null SCC by Western blot analysis. Cxcr2 protein expression was not detected in Cxcr2 null SCC, nor was p53 detected in p53-null samples (Supplementary Fig. S4). pAkt1 and total Akt1 protein expression was reduced by 2-fold in Cxcr2-null SCC. pERK1 expression was reduced by 2-3-fold, and total ERK1 was reduced by 2-8-fold in Cxcr2 null SCC. pPI3K expression was reduced 2-fold, and total PI3K levels were reduced by 2-4 in Cxcr2-null SCC. pPKC expression was reduced to undetectable levels in Cxcr2-null SCC, as was total PKC levels in some Cxcr2-null samples. pPLC\(\gamma\) expression was undetectable in most samples, and total PLC\(\gamma\) expression was reduced by up to 32-fold in Cxcr2-null SCC. These results indicate that loss of Cxcr2 expression inhibits multiple downstream signaling pathways in SCC.

To determine which of multiple signaling pathways was most important in regulating chemokine signaling in SCC, we treated Pot1b\(^{-}\)/p53\(^{-}\)/p53\(^{-}\) SCC transplanted to NUI/J mice separately with three small-molecule inhibitors currently in cancer clinical trials. After 4-weeks of treatment, the PI3K inhibitor buparlisib produced a 64% reduction in tumor volume (\(P < 0.007\); Supplementary Fig. S5A) compared with vehicle-treated control tumors. The MEK inhibitor trametinib resulted in a 64% reduction in tumor volume (\(P < 0.04\); Supplementary Fig. S5B and S5C). The Akt inhibitor MK2206 created a 23% reduction. Histopathologic sections of SCC treated with buparlisib or vehicle are shown by hematoxylin and eosin staining in Supplementary Fig. S5B and S5C.

Although senescence can lead to a tumor promoting inflammatory microenvironment, inflammation may be difficult to sustain when bone marrow failure is one of the pathognomonic features of dyskeratosis congenita. However, the SASP secretome is complex and may trigger tumor cell proliferation in the absence of inflammatory signals.

Chemokines mediate numerous functions in tumorigenesis, including proliferation, survival, angiogenesis, epithelial–mesenchymal transition, and metastasis (24). Cxcl1 expression was induced in stratified epithelia treated with phorbol ester (25), and Cxcl1 itself induced epithelial proliferation (26). Cxcl1 and Cxcl3 were expressed in human head and neck squamous cell carcinoma lines and patient tissue (27–29). Cxcr2 regulated head and neck cancer cell proliferation and migration in cell lines (30). Cxcr2 expression was associated with cervical lymph node metastasis in human head and neck cancer (31). Ras transformation of mouse stratified epithelial cells induced Cxcr2 ligands, and these cells failed to form tumors when Cxcr2 expression was inhibited (32). While paracrine signaling from stromal cells to epithelial tumors has been extensively studied (33), our paper provides a unique example of high level Cxcl secretion by one stem cell population (K15\(^+\)) which regulates expansion of a second stem cell fraction (Lgr6\(^+\)) expressing high levels of the receptor for these ligands (Cxcr2). Depletion of the K15\(^+\) tumor population or deletion of Cxcr2 receptor expression in Lgr6\(^+\) cells dramatically inhibits expansion of the latter population, and blocks tumor growth and metastasis. These studies indicate an important role for Cxcl chemokines and Cxcr2 in human head and neck cancer.

A striking result of our study was marked inhibition of metastasis in Pot1b\(^{-}\)/p53\(^{-}\)/p53\(^{-}\) SCC. Given low levels of apoptosis in these tumors, the metastatic phenotype is difficult to attribute to increased programmed cell death, and our previous studies indicate that apoptosis in primary SCC does not correlate with metastatic phenotype (20). In contrast, increased senescence and terminal differentiation of SCC provides a likely mechanism for inhibiting metastasis. Reduction in the proliferating cell fraction likely inhibits genomic instability and therefore creation of potentially metastatic clones. This mechanism has therapeutic implications particularly for late evolving metastatic clones.

Another key finding of our study is that Pot1b deficiency does not induce head and neck squamous cell carcinoma, even in the p53\(^{-}\)/tumor prone background. Our previous studies on TRF2- and Terc-mediated telomereDDR in stratified epithelium did not reveal spontaneous carcinoma formation (20, 34). In fact reduced TRF2 expression and to lesser extent
Terc deficiency induce a powerful telomere DDR which results in high apoptotic cell fraction, although neither of these mutants exhibit dyskeratosis congenita features. Telomere DNA damage signaling has been associated with chemokine and cytokine expression in other experimental models. Reduced TRF1 expression in endothelial cells correlated with increased expression of CCL2 and GM-CSF (35). IL8 induced telomerase activity and prevented senescence of human endothelial cells (36). Stromal cell–derived factor 1α inhibited endothelial cell senescence via telomerase activation (37). Telomerase was required for TNFα-mediated targeted gene expression in airway smooth muscle cells (38). Telomerase deficiency was associated with inflammatory chemokine expression and alveolar cell stem senescence in the mouse lung (39). Cxcr2 inhibition was associated with reduced TERT expression and differentiation of human pluripotent stem cells (40). A more limited telomere DDR may be required for dyskeratosis congenita. Alternatively immunosuppression (a feature of dyskeratosis congenita) is a known risk factor for head and neck cancer (41). The increased risk of head and neck cancer in dyskeratosis congenita may therefore be due to progressive bone marrow suppression (42). However, genetically unstable epithelial cells due to dyskeratosis congenita-associated telomere DDR may escape immune surveillance in the context of dyskeratosis congenita–induced bone marrow suppression. A previous study reported that p53 deletion inhibited bone marrow suppression in Pot1b−/−; Terc−−/− mice (43). Interestingly Pot1b:p16INK4a deletion accelerated bone marrow failure with increased ATR activation, telomere shortening, chromosomal fusions, telomere replication defects, and p53-dependent apoptosis (44).

The mouse genome has a Pot1a gene whose conditional deletion also results in telomere DNA damage and p53-dependent cellular senescence (45, 46). Pot1a depletion at telomeres induces an ATR-dependent DDR similar to that observed in Pot1b null cells (47). Pot1a and Pot1b did not differ in their ability to repress telomere recombination (48). Reduced Pot1a expression inhibits renewal of hematopoietic stem cells (HSC; 49), but Pot1a and p53 depletion resulted in T-cell lymphoma, consistent with p53-deficient HSC being prone to lymphoma development (50). These studies indicate that Pot1a and Pot1b have functional similarities.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: D.L. Crowe
Development of methodology: A.M. Lagunas, M. Francis, N.B. Maniar, G. Nikolova, J. Wu, D.L. Crowe
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.M. Lagunas, M. Francis, N.B. Maniar, G. Nikolova, J. Wu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.M. Lagunas, M. Francis, N.B. Maniar, G. Nikolova, J. Wu, D.L. Crowe
Writing, review, and/or revision of the manuscript: A.M. Lagunas, M. Francis, N.B. Maniar, G. Nikolova, J. Wu, D.L. Crowe
Study supervision: D.L. Crowe

Acknowledgments
We thank Dr. Eros Lazzerini Denchi (The Scripps Research Institute, La Jolla, CA) for Pot1b null mutant mice. We thank Dr. Ke Ma and Jewell Graves (University of Illinois Research Resources Center) for assistance with microscopy and flow cytometry. A.M. Lagunas was supported by NIH National Research Service Award DE18381. M. Francis was supported by NIH Individual Predoctoral Fellowship DE24352. This study was supported by NIH grant DE14283 to D.L. Crowe.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 22, 2018; revised March 25, 2019; accepted April 25, 2019; published first May 1, 2019.
21. Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA, Butel JS, et al. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumors. Nature 1992;356:215–21.

22. Bojovic B, Crowe DL. Telomere dysfunction promotes metastasis in a TERC null mouse model of head and neck cancer. Mol Cancer Res 2011;9: 1131–13.

23. Ouchi R, Okabe S, Migita T, Nakano I, Seimiya H. Senescence from glioma stem cell differentiation promotes tumor growth. Biochem Biophys Res Commun 2016;470:275–81.

24. Albert S, Riveiro ME, Halimi C, Hourseau M, Couvelard A, Serova M, et al. Focus on the role of the CXCL12/CXCR4 chemokine axis in head and neck squamous cell carcinoma. Head Neck 2013;35:1819–28.

25. Shintani S, Ishikawa T, Nonaka T, Li C, Nakashiro K, Wong DT, et al. Growth factor regulated oncogene 1 expression is associated with angiogenesis and lymph node metastasis in human oral cancer. Oncology 2004;66:316–22.

26. Ye H, Yu T, Temsam M, Ziober BL, Wang J, Schwartz JL, et al. Transcriptomic dissection of tongue squamous cell carcinoma. BMC Genomics 2008;9:69.

27. Ku TK, Crowe DL. Impaired T lymphocyte function increases tumorigenicity and decreases tumor latency in a mouse model of head and neck cancer. Int J Oral Oncol 2009;35:1211–21.

28. Wang Y, Shen MF, Chang S. Essential roles for Pot1b in HSC self-renewal and survival. Blood. 2011;118:6066–77.

29. Ye H, Yu T, Temsam M, Ziober BL, Wang J, Schwartz JL, et al. Transcriptomic dissection of tongue squamous cell carcinoma. BMC Genomics 2008;9:69.

30. Khurram SA, Bingle L, McCabe BM, Farthing PM, Whawell SA. The chemokine receptors CXCR1 and CXCR2 regulate oral cancer cell behavior. J Oral Pathol Med 2011;50:145–54.

31. Feller L, Kulke R, Christopher E. Interleukin 1 stimulated secretion of interleukin 8 and growth related oncogene alpha demonstrates greatly enhanced keratinocyte growth in human raft cultured epidermis. J Invest Dermatol 2002;119:1254–60.

32. Catalsson C, Ohlman R, Patel G, Pearson A, Tisen M, Jäsy S, et al. Inducible cutaneous inflammation reveals a protumorigenic role for keratinocyte CXCR2 in skin carcinogenesis. Cancer Res 2009;69:319–28.

33. Ku TK, Crowe DL. Impaired T lymphocyte function increases tumorigenicity and decreases tumor latency in a mouse model of head and neck cancer. Int J Oral Oncol 2009;35:1211–21.

34. Wang Y, Shen MF, Chang S. Essential roles for Pot1b in HSC self-renewal and survival. Blood. 2011;118:6066–77.

35. Wu L, Multani AS, He H, Cosme-Blanco W, Deng Y, Bachilo O, et al. Pot1 deficiency initiates DNA damage checkpoint activation and aberrant homologous recombination at telomeres. Cell 2006;126:49–66.

36. Shen XH, Xu SJ, Jin CY, Ding E, Zhou YC, Fu GS. Interleukin 8 prevents oxidative stress induced human endothelial cell senescence via telomerase activation. Int Immunopharmacol 2013;16:261–7.

37. Zheng H, Shen CJ, Qiu FY, Zhao YB, Fu GS. Stromal cell-derived factor 1alpha reduces senescence of endothelial progenitor subpopulation in lesion binding and DHL uptaking cell through telomerase activation and telomere elongation. J Cell Physiol 2010;223:757–63.

38. Deacon K, Knox AJ. PINX1 and TERT are required for TNFα induced airway smooth muscle chemokine gene expression. J Immunol 2018;200:1283–94.

39. Chen R, Zhang K, Chen H, Zhao X, Wang J, Li L, et al. Telomerase deficiency causes alveolar stem cell senescence associated low grade inflammation in lungs. J Biol Chem 2015;290:30813–29.

40. Jung JH, Kang KW, Kim J, Hong SC, Park Y, Kim BS. CXCR2 inhibition in human pluripotent stem cells induces predominant differentiation to mesoderm and endoderm through repression of mTOR, β-catenin, and HNTP activities. Stem Cells Dev 2016;25:1006–19.

41. Preciado DA, Matas A, Adams GL. Squamous cell carcinoma of the head and neck in solid organ transplant recipients. Head Neck 2002;24:319–25.

42. Shintani S, Ishikawa T, Nonaka T, Li C, Nakashiro K, Wong DT, et al. Growth factor regulated oncogene 1 expression is associated with angiogenesis and lymph node metastasis in human oral cancer. Oncology 2004;66:316–22.

43. Wang Y, Sharpless N, Chang S. Essential roles for Pot1b in HSC self-renewal and survival. Blood. 2011;118:6066–77.

44. Ku TK, Crowe DL. Impaired T lymphocyte function increases tumorigenicity and decreases tumor latency in a mouse model of head and neck cancer. Int J Oral Oncol 2009;35:1211–21.

45. Wang Y, Shen MF, Chang S. Essential roles for Pot1b in HSC self-renewal and survival. Blood. 2011;118:6066–77.

46. Wu L, Multani AS, He H, Cosme-Blanco W, Deng Y, Bachilo O, et al. Pot1 deficiency initiates DNA damage checkpoint activation and aberrant homologous recombination at telomeres. Cell 2006;126:49–66.

47. Wang Y, Sharpless N, Chang S. p16INK4A protects against dysfunctional telomeres in cancer. Oncogene 2006;25:63–73.

48. Wu L, Multani AS, He H, Cosme-Blanco W, Deng Y, Bachilo O, et al. Pot1 deficiency initiates DNA damage checkpoint activation and aberrant homologous recombination at telomeres. Cell 2006;126:49–66.

49. Wu L, Multani AS, He H, Cosme-Blanco W, Deng Y, Bachilo O, et al. Pot1 deficiency initiates DNA damage checkpoint activation and aberrant homologous recombination at telomeres. Cell 2006;126:49–66.

50. Wu L, Multani AS, He H, Cosme-Blanco W, Deng Y, Bachilo O, et al. Pot1 deficiency initiates DNA damage checkpoint activation and aberrant homologous recombination at telomeres. Cell 2006;126:49–66.

51. Wu L, Multani AS, He H, Cosme-Blanco W, Deng Y, Bachilo O, et al. Pot1 deficiency initiates DNA damage checkpoint activation and aberrant homologous recombination at telomeres. Cell 2006;126:49–66.

52. Wu L, Multani AS, He H, Cosme-Blanco W, Deng Y, Bachilo O, et al. Pot1 deficiency initiates DNA damage checkpoint activation and aberrant homologous recombination at telomeres. Cell 2006;126:49–66.

53. Wu L, Multani AS, He H, Cosme-Blanco W, Deng Y, Bachilo O, et al. Pot1 deficiency initiates DNA damage checkpoint activation and aberrant homologous recombination at telomeres. Cell 2006;126:49–66.

54. Wu L, Multani AS, He H, Cosme-Blanco W, Deng Y, Bachilo O, et al. Pot1 deficiency initiates DNA damage checkpoint activation and aberrant homologous recombination at telomeres. Cell 2006;126:49–66.