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ORIGINAL ARTICLE

Cooperation between p53 and the telomere-protecting shelterin component Pot1a in endometrial carcinogenesis

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Type II endometrial cancer (EMCA) represents only 10% of all EMCAs, but accounts for 40% of EMCA-related mortality. Previous studies of human tumors have shown an association between Type II tumors and damaged telomeres. We hypothesized that the lack of murine Type II EMCA models is due to the extremely long telomeres in laboratory mouse strains. We previously showed that telomerase-null mice with critically short telomeres developed endometrial lesions histologically resembling endometrial intraepithelial carcinoma (EIC), the accepted precursor for Type II EMCA. However, these mice did not develop invasive endometrial adenocarcinoma, and instead succumbed prematurely to multi-organ failure. Here, we modeled critical telomere attrition by conditionally inactivating *Pot1a*, a component of the shelterin complex that stabilizes telomeres, within endometrial epithelium. Inactivation of *Pot1a* by itself did not stimulate endometrial carcinogenesis, and did not result in detectable DNA damage or apoptosis in endometrium. However, simultaneous inactivation of *Pot1a* and *p53* resulted in EIC-like lesions by 9 months indistinguishable from those seen in late generation telomerase-null mice. These lesions progressed to invasive endometrial adenocarcinomas as early as 9 months of age with metastatic disease in 100% of the animals by 15 months. These tumors were poorly differentiated endometrial adenocarcinomas with prominent nuclear atypia, resembling human Type II cancers. Furthermore, these tumors were aneuploid with double-stranded DNA breaks and end-to-end telomere fusions and most were tetraploid or near-tetraploid. These studies lend further support to the hypothesis that telomeric instability has a critical role in Type II endometrial carcinogenesis and provides an intriguing *in-vivo* correlate to recent studies implicating telomere-dependent tetraploidization as an important mechanism in carcinogenesis.

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INTRODUCTION

Endometrial cancer (EMCA) is the most common type of gynecological malignancy with >40 K new cases and 7 K deaths each year in the United States.[1] It is not one disease: although all endometrial adenocarcinomas are believed to arise from the same cell type (endometrial epithelium), EMCA is categorized into two different subtypes based on their strikingly different epidemiologic and molecular features.[2] Type I EMCAs are strongly associated with estrogen-related risk factors such as obesity or treatment with estrogen. Type I EMCAs typically exhibit endometrioid histology and are well differentiated, and are categorized as low grade. In contrast, Type II EMCAs are not epidemiologically linked to estrogen-related risk factors. They occur in much older women and frequently arise in an otherwise atrophic endometrium, and are poorly differentiated.[3] They are characterized by greater nuclear atypia and are thus categorized as high nuclear grade,[4] likely a reflection of their aneuploidy and abnormal karyotypes. Type II cancers carry a much worse prognosis: they represent only 10% of all EMCAs but have a much greater propensity to metastasize and account for 40% of EMCA-related deaths.[5,6]

Type I and II EMCAs have largely distinct mutational spectra[3] and this knowledge has been exploited in the development of murine EMCA models. *Pten* is the most frequently mutated gene in Type I EMCAs[7] and $Pten^{+/-}$ mice spontaneously develop endometrial hyperplasias.[8] Models in which both alleles of *Pten* have been conditionally inactivated through the use of *PR-Cre* develop well-differentiated (Type I like) invasive endometrial adenocarcinomas.[9,10] Bi-allelic inactivation of the tumor suppressor *Lkb1* in endometrial epithelium with *Sprr2f-Cre* results in highly invasive adenocarcinomas that despite their aggressive behavior are well-differentiated endometrioid adenocarcinomas that thus also resemble Type I EMCAs.[11] *TP53* is the most commonly mutated gene in Type II EMCAs.[3,12] Over 90% of uterine serous carcinomas and >70% of endometrioid intraepithelial carcinomas (EICs), the precursor of invasive Type II EMCA, harbor *TP53* mutations.[13] *TP53* mutations are also detected in advanced, high-grade Type I cancers. Interestingly, $p53^{+/-}$ mice[9,14] or mice with conditional *p53* inactivation in the uterus[9] do not develop endometrial carcinomas by 5 months of age, suggesting that inactivation of *p53* alone is not sufficient for development of Type II EMCAs and that additional drivers are required.

Epithelial carcinogenesis is driven by genomic instability. Genetic and chromosomal changes such as aneuploidy, translocations, deletions and amplifications characterize most carcinomas.[15] Telomere shortening and ensuing dysfunction are believed to have an especially important role in the initiation and

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propagation of genomic instability in epithelial malignancies. Short telomeres function as a tumor suppressor mechanism, as cells with critically short telomeres undergo p53 or Rb-dependent senescence or apoptosis. However, when p53 has been inactivated, cancer cells with critically short telomeres bypass cell-cycle checkpoints,[16] and exhibit rampant genomic instability and an increased potential for progression.[17] Telomere shortening and p53 inactivation together drive a vicious cycle of genomic instability through breakage-fusion-bridge cycles.[18] More recently, telomere-dependent tetraploidization has been uncovered as an additional mechanism by which telomere dysfunction leads to genomic instability.[19]

Eukaryotic cells maintain their telomeres through the action of the shelterin complex, an assembly of proteins that specifically binds to telomeres, helps form the T loop, suppresses DNA repair machinery[20] and regulates telomere length.[21] In functional human telomeres, the shelterin component POT1 suppresses ATR kinase signaling, while TRF2 suppresses ATM signaling.[22–24] In the absence of shelterin proteins, telomeres are unprotected and uncapped, inducing ATM- or ATR-dependent cell-cycle arrest, chromosome fusions through non-homologous end joining, or other genetic alterations through homologous DNA recombination.[25] Shelterin is composed of at least six core subunits and of these POT1 is the most conserved, being present from yeast to human.[26] Whereas in humans POT1 is encoded by a single locus, in mice two distinct genes encode two isoforms (Pot1a and Pot1b) that serve distinct functions.[27–29] *Pot1b* inactivation in a telomerase heterozygous background results in accelerated telomere shortening in highly proliferative organs (in the first generation of knockout animals) and these animals develop an accelerated aging phenotype similarly to dyskeratosis congenita, a human disease caused by mutations in the RNA subunit of the telomerase holoenzyme (Telomerase RNA Component, or TERC).[30,31] In contrast, *Pot1a* inactivation results in telomere elongation, aberrant homologous recombination and p53-dependent replicative senescence.[24,27,28,32]

We recently proposed a model where Type II endometrial carcinogenesis is driven by telomeric dysfunction and obtained some data in support of this model from human and mouse studies.[12] Mismatch repair defects are common in Type I cancers but rare in Type II, further supporting the notion that these tumor subtypes are distinct and driven by different patterns of genomic instability.[33–35] Telomerase RNA component knockout (mTERC) mice with critically short telomeres develop lymphomas and sarcomas by the fifth generation (G5i),[36] and we showed that these animals also develop EICs by 15 months.[12] However, these mice did not readily permit the study of EMCA progression because of their premature aging and death.[12] Of the many genetically engineered murine models of EMCA generated to date,[37] all exhibit Type I features. We hypothesized that the absence of Type II-like endometrial carcinogenesis in the mouse is a reflection of the long telomeres in most laboratory strains of mice, preventing telomere dysfunction during normal aging.[38] To test this hypothesis and to develop Type II-like models useful to study this clinically important subtype, we conditionally deleted the shelterin component *Pot1a* by itself and in combination with *p53* using the endometrial epithelium deletor *Sprr2f-Cre*. *Sprr2f-Cre; Pot1a*$^{L/L}$animals did not develop any EMCAs or precancers up to 20 months. In contrast, *Sprr2f-Cre*; *Pot1a*$^{L/L}$; *p53*$^{L/L}$mice developed EICs by 9 months and aggressive invasive endometrial adenocarcinomas with Type II-like features and 100% penetrance by 15 months of age. Further studies of these tumors including ploidy analyses and spectral karyotyping (SKY) lent further support to the hypothesis that telomeric instability drives Type II neoplasia. Thus, inactivation of components of the shelterin complex can be used to model telomeric instability and to study its role in Type II endometrial carcinogenesis.

RESULTS

Cooperation of Pot1a and p53 in endometrial carcinogenesis

Our objective was to study the contribution of dysfunctional telomeres to endometrial carcinogenesis. To perform conditional genetic analyses, we employed the endometrial epithelial-specific deletor line *Sprr2f-Cre*. In *Sprr2f-Cre* mice, Cre is specifically expressed in the epithelial compartment of the uterus by 3 weeks of age in both glandular and surface endometrial epithelial cells (Figures 1a and b).[11] The ability of *Sprr2f-Cre* to mediate deletion of *Pot1a* in the uterus was first confirmed by PCR. The null *Pot1a* allele was detected in the uterus but not in a control tissue, as expected (Figure 1c).

We generated (1) *Sprr2f-Cre; Pot1a*$^{L/L}$, (2) *Sprr2f-Cre; p53*$^{L/L}$, (3) *Sprr2f-Cre; Pot1a*$^{L/L}$; *p53*$^{L/L}$ and (4) sibling control *Pot1a*$^{L/L}$ mice ($n = 20$ per genotype). *Sprr2f-Cre; Pot1a*$^{L/L}$mice did not develop any tumors up to 15 months of age (Figure 1d), while *Sprr2f-Cre; p53*$^{L/L}$ mice developed uterine cancer only with very long latency and incomplete penetrance. In contrast, *Sprr2f-Cre; Pot1a*$^{L/L}$; *p53*$^{L/L}$ mice developed endometrial carcinomas with obvious myometrial invasion by 9 months of age. *Sprr2f-Cre; Pot1a*$^{L/L}$; *p53*$^{L/L}$mice started dying due to metastatic tumors by 12 months and 100% of the mice died or reached tumor burden euthanasia criteria before 18 months ($P < 0.0001$; Figure 1e). Concordantly, tumor burden was greatly increased in the double knockouts relative to the single *Pot1a* or *p53* knockouts (Figures 1f and g). Given that *p53* inactivation alone was a relatively weak endometrial tumor suppressor here and in previous studies,[9] these results revealed a potent *in-vivo* cooperation between *Pot1a* and *p53* in endometrial carcinogenesis.

Type II-like histologic features of Pot1a p53 tumors

We next analyzed *Sprr2f-Cre; Pot1a*$^{L/L}$; *p53*$^{L/L}$uterine tumors histologically. For comparison, *Sprr2f-Cre; Pot1a*$^{L/L}$ endometrium was histologically normal (Figure 2c). *Sprr2f-Cre; p53*$^{L/L}$ tumors were surprisingly well differentiated, exhibited typical endometrioid histology and modest nuclear atypia (Figure 2d), and thus closely resembled human Type I endometrioid adenocarcinomas (Figure 2a).[11] In contrast, *Sprr2f-Cre; Pot1a*$^{L/L}$; *p53*$^{L/L}$tumors uniformly exhibited striking nuclear atypia and were thus all high grade, a feature of Type II cancers (Figures 2b, f, g and k). Many tumors exhibited architectural features characteristic of Type II tumors, including at least focal areas of papillary differentiation (Figures 2h and i). Other tumors also exhibited areas where the tumor cell cytoplasm protruded into the lumen ('hobnailing'), a feature also seen in some Type II cancers (Figure 2j). However, no tumors exhibited obvious clear cell differentiation (a histologic pattern associated with Type II neoplasia). We also observed *in-situ* lesions characterized by severe nuclear atypia that closely resembled human EICs in 100% of *Sprr2f-Cre; Pot1a*$^{L/L}$; *p53*$^{L/L}$mice by 9 months of age (Figure 2e), demonstrating that tumorigenesis in this model proceeds through the recognized morphologic intermediates associated with Type II neoplasia. Similar lesions were previously observed in G5i mice with critically short telomeres.[12] *Sprr2f-Cre; Pot1a*$^{L/L}$; *p53*$^{L/L}$tumors also displayed tripolar mitoses (Figure 2k) and anaphase bridges (Figure 2l), evidence of genomic instability and telomere dysfunction.[39]

We also note that neoplastic foci in *Sprr2f-Cre; Pot1a*$^{L/L}$; *p53*$^{L/L}$ tumors were somewhat heterogenous histologically, with foci of poorly differentiated carcinoma present in many tumors. In some of these foci, the tumor cells adopted a distinctive spindle-cell morphology indicative of sarcomatous differentiation (Figure 2m), which in human EMCAs is associated with advanced tumors and extremely aggressive behavior. Concordantly, *Sprr2f-Cre; Pot1a*$^{L/L}$; *p53*$^{L/L}$tumors were highly aggressive, exhibited prominent lymphovascular invasion and metastasized in most animals to nearby and distant organs within the peritoneal cavity, such as bladder,

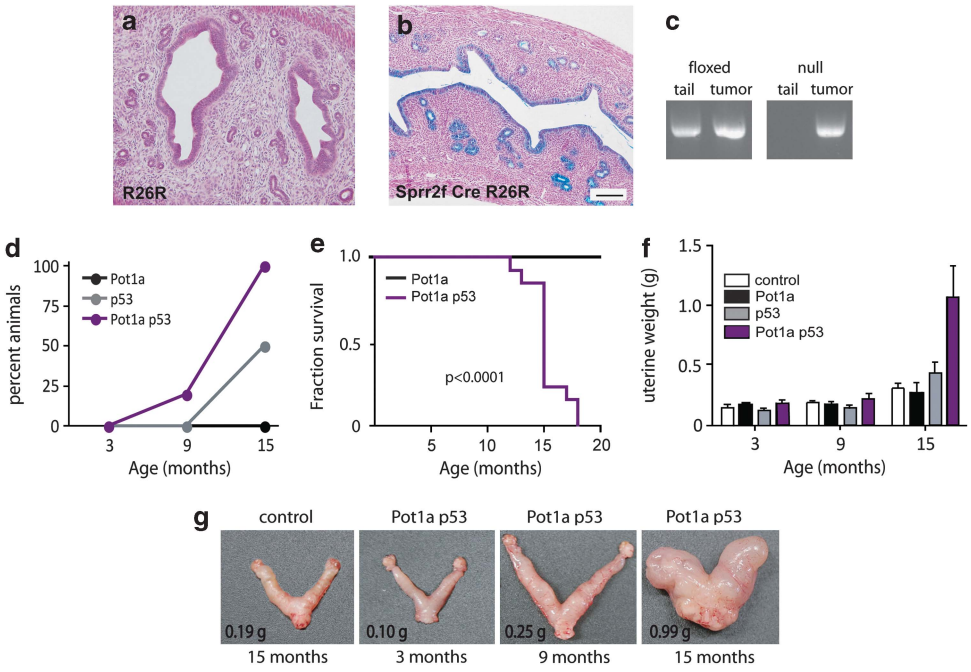

Figure 1. *Pot1a* and *p53* cooperate in endometrial carcinogenesis. X-gal-stained uterine sections from 12-week-old mice. (**a**) *Rosa26* LacZ reporter (*R26R*). (**b**) *Sprr2f-Cre; R26R*, showing Cre activity in both surface and deep endometrial glands. Scale bar = 100 μm for (**a**) and (**b**). (**c**) PCR for floxed (left) and null (right) *Pot1a* alleles from *Sprr2f-Cre; Pot1a*$^{L/L}$*; p53*$^{L/L}$ mouse tail and uterine tumor. As expected, both control (tail) and tumor samples harbor floxed alleles, since tumor stroma does not undergo gene deletion. However, only tumor DNA harbors the null allele, consistent with endometrial-specific Cre activity. (**d**) Tumor incidence in aging cohorts at 3, 9 and 15 months. (**e**) Kaplan–Meier survival analysis for aging cohorts of *Sprr2f-Cre; Pot1a*$^{L/L}$ vs. *Sprr2f-Cre; Pot1a*$^{L/L}$*; p53*$^{L/L}$ ($P < 0.0001$ by log-rank test). (**f**) Uterine weights at 3, 9 and 15 months. (**g**) Gross uterine pictures representative of $n = 5$ animals analyzed per time point. Weight of uterus shown is indicated in the left lower corner.

peri-intestinal adipose tissue (Figures 2o and p) and also liver capsule and pancreas (not shown). Most of these mice did not harbor distant metastases, with the exception of two mice with subcutaneous nodules of metastatic carcinoma far from the primary site (that is, the back). Thus, simultaneous inactivation of Pot1a and p53 in mouse endometrium resulted in tumors with histologic features associated with aggressive behavior and that resemble in some respects Type II tumors. Furthermore, these *Pot1a p53* tumors displayed a marked propensity to spread and metastasize within the peritoneum, unlike most mouse models of EMCA generated to date.[37]

Double-strand breaks characterize Pot1a p53 EMCAs

Shelterin protects telomeres from being recognized as DNA double-stranded breaks and thereby maintains telomere length homeostasis.[26] When dysfunctional telomeres are recognized as DNA damage,[40,41] 3BP1, γ-H2AX, the Mre11 complex, Rif1 and the phosphorylated form of ATM, ATM S1981-P, accumulate at the telomeres.[26] We had previously observed significantly increased DNA double-stranded breaks in Type II EMCAs relative to Type I.[12] We thus wanted to determine if *Sprr2f-Cre; Pot1a*$^{L/L}$*; p53*$^{L/L}$tumors also exhibited patterns of genomic instability associated with dysfunctional telomeres. *Sprr2f-Cre; Pot1a*$^{L/L}$*; p53*$^{L/L}$tumors showed a dramatic increase of γ-H2AX foci starting at 3 months of age (Figures 3a–d and i). By 15 months, nearly 50% of tumor cells were positive for γ-H2AX (Figure 3i; $P < 0.0001$). In contrast, *Sprr2f-Cre; Pot1a*$^{L/L}$ endometrial cells did not exhibit increased numbers of γ-H2AX foci relative to controls (Figure 3c).

In some experimental systems, Pot1a inactivation results in p53-dependent replicative senescence *in vitro*.[28] Consistent with this idea, the Ki67 index was markedly increased in *Sprr2f-Cre; Pot1a*$^{L/L}$*; p53*$^{L/L}$tumors by 9 months of age (Figures 3e–h and j; $P = 0.0003$). Although a slight decrease in the Ki67 index in the endometrium of *Sprr2f-Cre; Pot1a*$^{L/L}$mice was observed (Figure 3j), this difference was not statistically significant ($P = 0.43$). Interestingly, fewer cells from *Pot1a* uteri exhibited γ-H2AX foci at 15 weeks (Figure 3i;$P = 0.08$), suggesting that telomere dysfunction may promote apoptosis in the presence of wild-type p53; that is, p53 serves as an active tumor suppressor mechanism that eliminates endometrial cells with dysfunctional telomeres.

Normal telomere lengths in Pot1a p53 EMCAs

Interaction of POT1 with TRF2 and single-stranded telomeres inhibits telomere elongation by telomerase.[42–44] We thus assessed telomere length in endometrial tumor cells with telomere chromogenic *in-situ* hybridization (Telo-CISH), a chromogenic assay we previously developed that permits semiquantitative analysis of telomere lengths in the context of normal tissue architecture by standard light microscopy.[12] Significant telomere length differences were not detected in the single or double knockouts compared with age matched controls (Figure 4a). These findings are consistent with the idea that the *Pot1a; p53* EMCA phenotype is related to telomere dysfunction independent of telomere length. However, since Telo-CISH may be unable to detect small differences in telomere length, these results did not entirely exclude the possibility that some telomere length alterations have occurred, as has been observed in the context of Pot1a deficiency in cultured cells.[28] To further explore this point, quantitative telomere determinations (quantitative fluorescence *in-situ* hybridization—Q-FISH) were conducted with three independently derived *Pot1a; p53* EMCA cell lines and a control *Lkb1* EMCA cell line derived from the extremely well-differentiated *Sprr2f-Cre; Lkb1*$^{L/L}$ EMCA model.[11] Q-FISH confirmed similar telomere lengths in *Pot1a; p53* and *Lkb1* cell lines (Figures 4b and c). We also did not

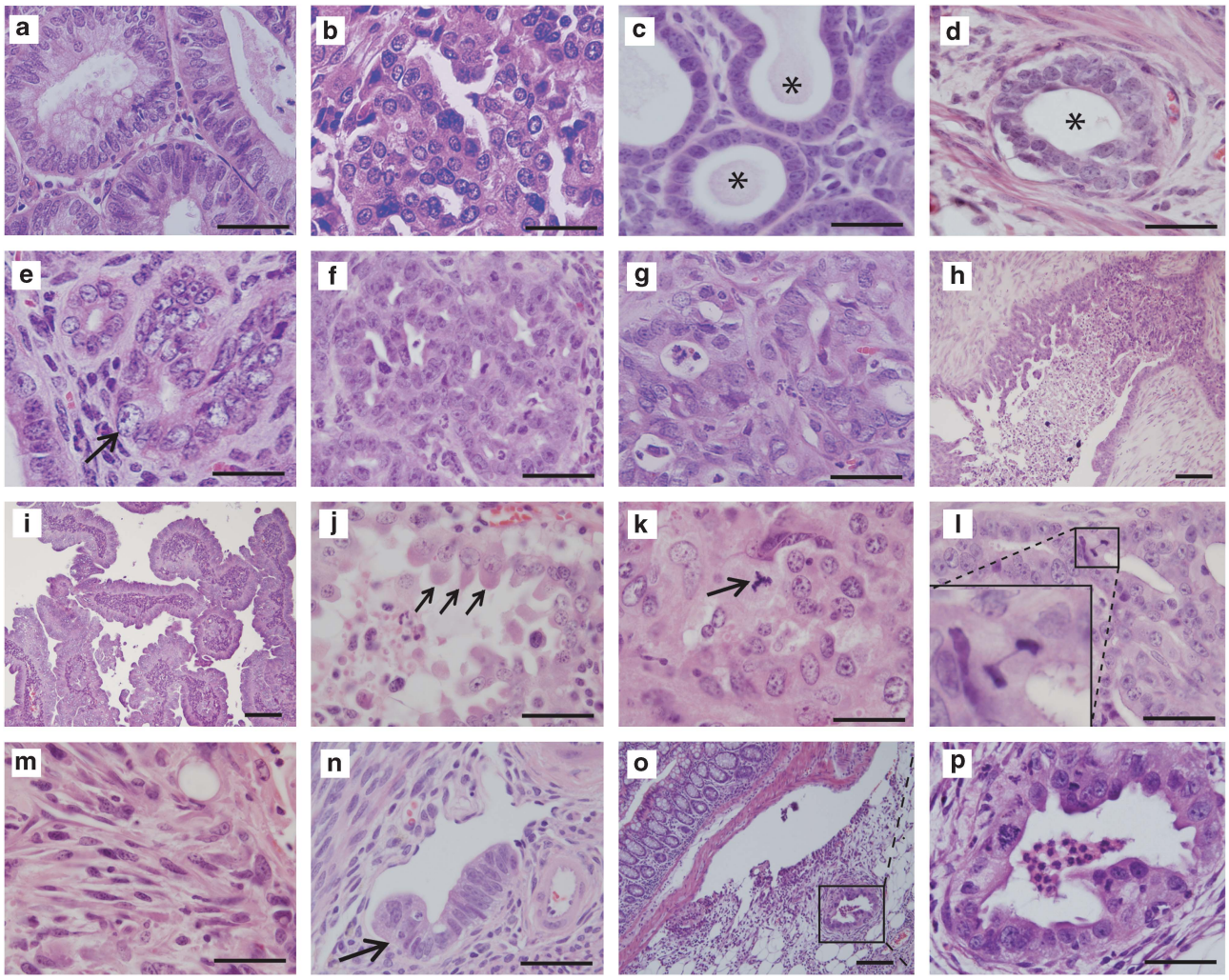

Figure 2. *Pot1a p53* tumors resemble human Type II EMCAs. H&E-stained tissue sections. (**a**) Human Type I endometrioid adenocarcinoma. (**b**) Human Type II serous carcinoma. (**c**) *Sprr2f-Cre; Pot1a*$^{L/L}$ mouse, histologically normal endometrial glands at 9 months of age (asterisks). (**d**) *Sprr2f-Cre; p53*$^{L/L}$ invasive EMCA, uterus. Tumor is composed of well-differentiated invasive glands with relatively minor atypia (asterisk). (**e**) EIC-like lesion with severe atypia in uterus from 9-month-old *Sprr2f-Cre; Pot1a*$^{L/L}$*; p53*$^{L/L}$ mouse. Arrow points to highly atypical cell with large nucleus; other cells in field display varying degrees of pleomorphism and atypia. (**f**–**p**) Representative histologies from different *Sprr2f-Cre; Pot1a*$^{L/L}$*; p53*$^{L/L}$ animals. (**f**, **g**) Representative areas of tumor showing high-grade features. (**h**) Tumor with focal micropapillary features. (**i**) Tumor with fibrovascular papillary architecture. (**j**) Hobnailing of tumor cells (arrows). (**k**) Representative high-grade tumor with tripolar mitosis consistent with severe aneuploidy (arrow). (**l**) Anaphase bridge consistent with presence of telomeric end-to-end fusions. (**m**) Focal sarcomatous differentiation, an example of extreme de-differentiation strongly associated with aggressive behavior. (**n**) Lymphovascular invasion by tumor away from the main tumor mass (arrow). (**o**) Colon with metastasis to pericolonic fat. (**p**) Higher magnification of metastatic gland in (**o**). Scale bars = 20 μm for (**c**) and (**e**), 100 μm for (**h**), (**i**) and (**o**) and 50 μm for all other panels.

observe a reduction in the amount of telomeric DNA compared with genetically wild-type control cells (not shown).

Aneuploidy and near-tetraploidy in Pot1a p53 EMCAs

Type I and II EMCAs are associated with distinct patterns of genetic instability. Type I EMCAs are strongly associated with defects in mismatch repair (at least 30% of cases).[33–45] For example, EMCA is the most common cancer in women with Lynch Syndrome, a hereditary cancer-predisposition syndrome due to mutations in various mismatch repair genes. Furthermore, spontaneous (that is, non-hereditary) EMCAs often have mutation or epigenetic downregulation of mismatch repair factors. Thus, while Type II EMCAs are associated with telomeric instability and have highly rearranged chromosomes and numerous copy-number alterations, Type I EMCAs are often diploid or nearly so.[12,46–48] To assess ploidy of tumor cells *in situ*, we performed image analysis of interphase nuclei in Feulgen-stained tissue sections. As a baseline for Type I tumors, we first analyzed the extremely well-differentiated tumors that arise in the *Sprr2f-Cre; Lkb1*$^{L/L}$murine EMCA model. All three of these tumors had GO/G1 DNA indices of ~1 (2*n*), similarly to *Pot1a*$^{L/L}$ controls (Figures 5a and b; Table 1). Thus, invasive tumors from this Type I model were diploid or near-diploid. In contrast, invasive EMCA cells from *Sprr2f-Cre; Pot1a*$^{L/L}$*; p53*$^{L/L}$mice had a much more heterogeneous DNA distribution (Figure 5c). All (10/10) *Pot1a p53* tumors were in the aneuploid range (Table 1) with GO/G1 DNA indices >1 (2*n*) ($P = 0.002$ *Pot1a p53* vs *Lkb1*). Four of the ten tumors had DNA contents in the near-tetraploid range (1.8–2.2). From these results, we conclude that simultaneous *Pot1a* and *p53* inactivation in endometrium promotes tumors characterized by severe genomic instability and aneuploidy.

We next analyzed chromosomal abnormalities by standard methods. Metaphases from early passage cells from *Pot1a p53*

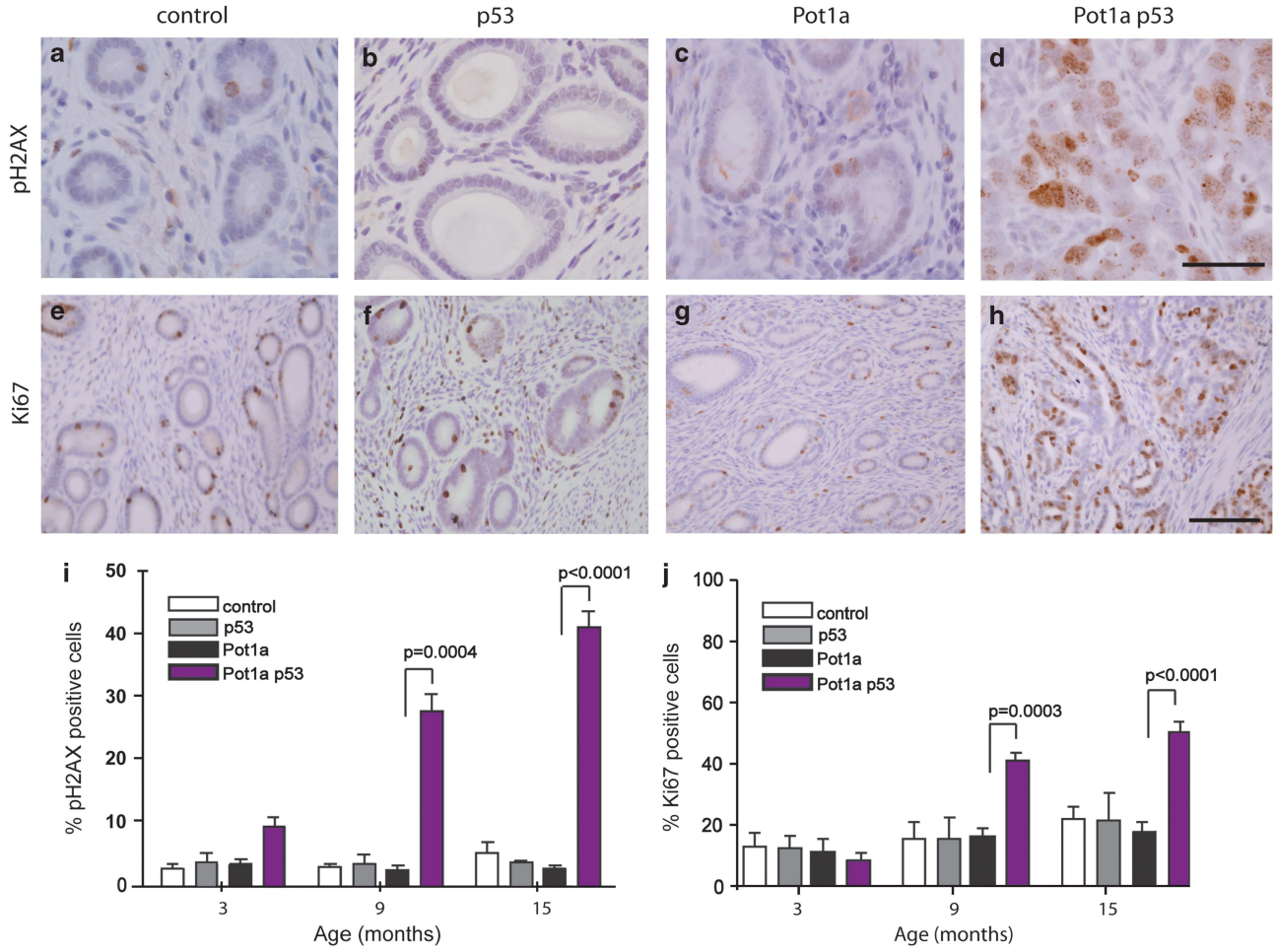

Figure 3. Double-strand DNA breaks in *Pot1a p53* tumors. (**a**–**d**), γ-H2AX immunohistochemistry, 15-month-old animals. (**a**) *Pot1a*$^{L/L}$ control. (**b**) *Sprr2f-Cre; p53*$^{L/L}$. (**c**) *Sprr2f-Cre; Pot1a*$^{L/L}$. (**d**) *Sprr2f-Cre; Pot1a*$^{L/L}$*; p53*$^{L/L}$. (**e**–**h**) Ki67 immunohistochemistry, 15-month-old animals. (**e**) *Pot1a*$^{L/L}$ controls. (**f**) *Sprr2f-Cre; p53*$^{L/L}$. (**g**) *Sprr2f-Cre; Pot1a*$^{L/L}$. (**h**) *Sprr2f-Cre; Pot1a*$^{L/L}$*; p53*$^{L/L}$. (**i**, **j**) Percentage of immunopositive nuclei plotted for each genotype at 3, 9 and 15 months ($n = 5$ for each time point). (**i**) γ-H2AX. (**j**) Ki67. Size bars = 50 μm for (**a**–**d**) and 100 μm for (**e**–**h**).

tumors ($n = 4$) showed abnormal chromosomal patterns including fusions forming bi-armed chromosomes as well as other chromosomal rearrangements (Figure 5e; Table 2). Some of the tumors also contained multiple abnormal, minute chromosomes (not shown). The chromosomal modal numbers for the four tumors were 70–80, 70–80, 76 and 83. Thus, all of the four mouse tumors appeared to be near-tetraploid (the *Mus musculus* diploid chromosome number is 40). To characterize these abnormal chromosomes in greater detail, SKY was performed on two of the four cell lines. SKY confirmed that the tumors were highly aneuploid, with most chromosomes being present in 3–7 copies (Figure 5f). Many of the chromosomes were tetrasomic, with most others present in 3–5 copies, consistent with near-tetraploidy. Several recurrent chromosomal translocations were identified in one of the tumors, including t(3:16), present in 2 copies in 8/8 cells (Figure 5f) and t(11:18) in 3/8 cells (not shown). t(3:16) was found in both of the cell lines karyotyped. SKY thus further confirmed that *Pot1a p53* tumors are severely aneuploid and characterized by abnormal chromosomes that likely result from defective, functionally uncapped telomeres.

DISCUSSION

This study provides further experimental evidence for the hypothesis that 'telonomic' instability has a significant role in Type II endometrial carcinogenesis. While inactivation of Pot1a was insufficient to drive endometrial carcinogenesis in the mouse, combined inactivation of Pot1a and p53 revealed a potent synergistic interaction in endometrial carcinogenesis. Furthermore, these tumors exhibited several cytologic and architectural histologic features classically associated with Type II uterine cancers including severe nuclear atypia and papillary growth patterns. Interestingly, but in accordance with previous studies, p53 inactivation resulted in EMCAs but only with incomplete penetrance and long latency. These p53-only tumors exhibited classic endometrioid histology and were surprisingly well differentiated and thus resembled Type I tumors. This may be somewhat at odds with p53-associated EMCA phenotypes in women, where p53 mutations are frequent either in Type II tumors or in more advanced, poorly differentiated Type I tumors. These differences may be due to much longer telomeres in laboratory strains of mice (30–150 kb) relative to humans (in the range of 10 kb) a notion consistent with substantial evidence that p53 loss can promote tumorigenesis through both telomere-dependent and -independent mechanisms.[49]

The longer telomeres of mice represent a variable that may potentially limit the utility and faithfulness of some mouse models of human malignancies, particularly those of epithelial origin (carcinomas). Mice have a marked propensity to develop sarcomas and lymphomas in both spontaneous and induced models of carcinogenesis, but develop carcinomas much less frequently. Indeed, previous studies have shown that experimental

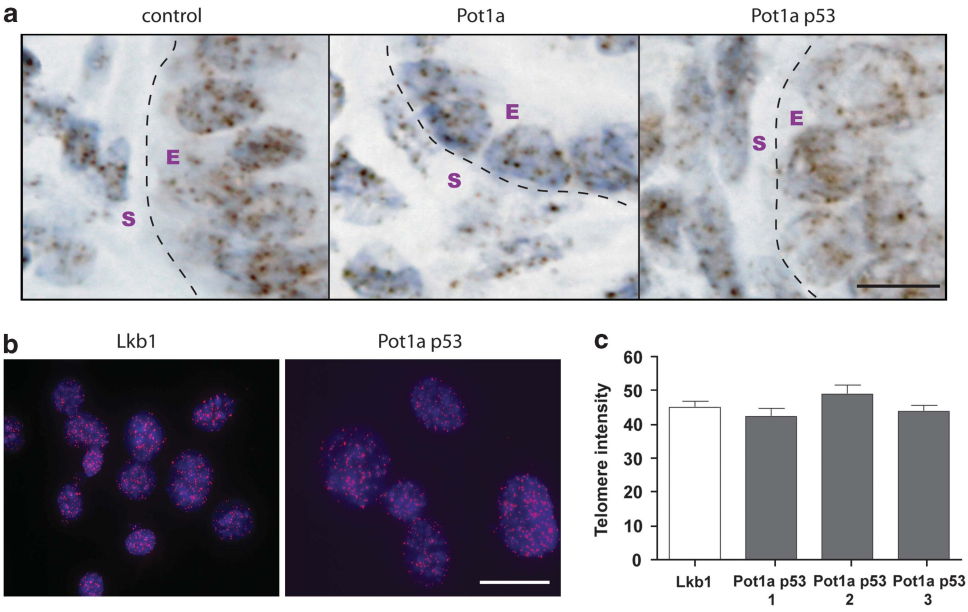

Figure 4. Inactivation of *Pot1a* and *p53* does not result in detectable telomere shortening. (**a**) Telo-CISH on uterine tumor sections from 15-month-old animals (genotypes as shown); S = stroma, E = epithelium. Size bar = 10 μm for all panels. (**b**) Q-FISH on cultured cells derived from *Sprr2f-Cre; Lkb1*$^{L/L}$ and representative *Sprr2f-Cre; Pot1a*$^{L/L}$*; p53*$^{L/L}$ EMCAs (DAPI counterstain). (**c**) Average telomere intensity measurements of the four primary cultures analyzed.

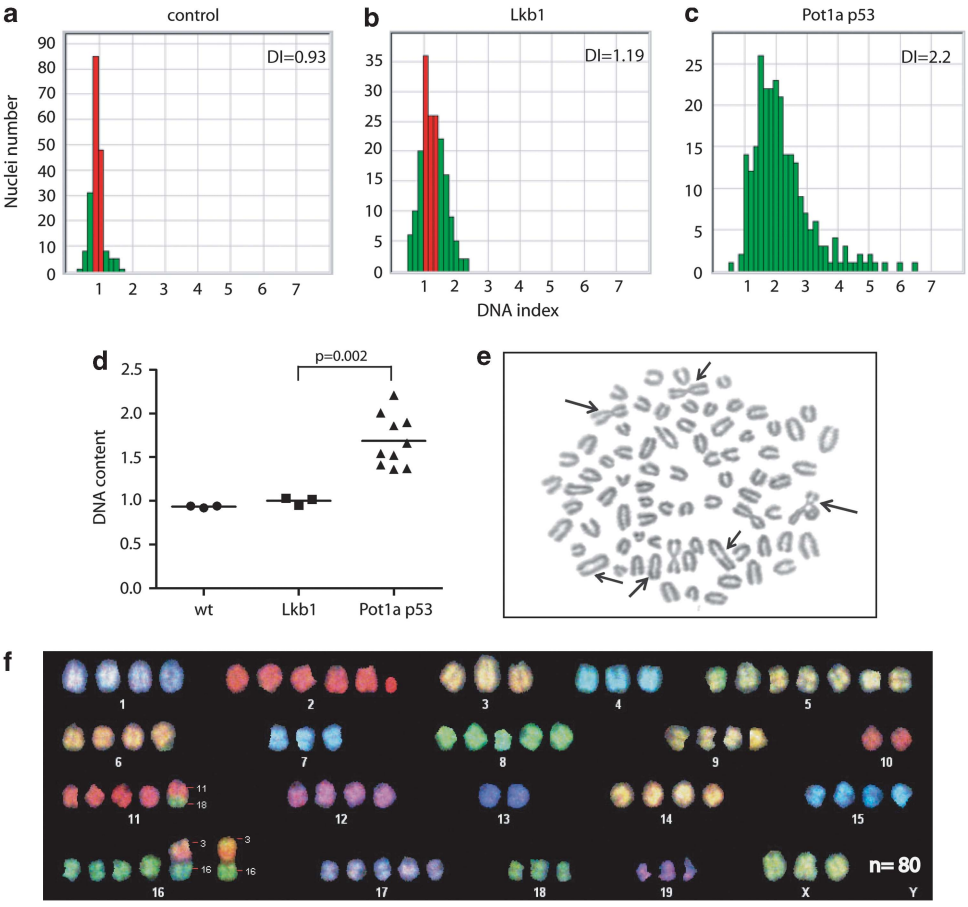

Figure 5. *Pot1a p53* tumors exhibit chromosomal instability. (**a**–**c**) Ploidy determinations of interphase nuclei by image analyses of Feulgen-stained tissue sections. (**a**) Uterus from 20-week-old *Pot1a*$^{L/L}$ control mouse. (**b**) Uterine tumor from 20-week-old *Sprr2f-Cre; Lkb1*$^{L/L}$ mouse. (**c**) Uterine tumor from *Sprr2f-Cre; Pot1a*$^{L/L}$*; p53*$^{L/L}$ mouse. DNA index (DI) represents ratio of analyzed DNA content to reference nuclei in GO/G1 phase. (**d**) Summary of interphase ploidy analyses. Average DNA indices are shown for each genotype. (**e**) Metaphase spread from cultured cells derived from an *Sprr2f-Cre; Pot1a*$^{L/L}$*; p53*$^{L/L}$ tumor. Arrows show fused bi-armed chromosomes. (**f**) Spectral karyotype of this cell line shows aneuploidy (near-tetraploidy) and reciprocal translocations.

manipulations of telomere length strongly influence tumor spectra in genetically engineered mouse models. p53 inactivation in mice with unaltered (that is, long) telomeres promotes lymphomas and sarcomas, whereas p53 inactivation in mice with artificially short telomeres (that is, mTERC knockout) promotes epithelial carcinogenesis characterized by genomic and chromosomal instability.[17] These important studies provided an important intellectual framework for several well-known features of epithelial carcinogenesis, including its striking age dependence, and provided a rationale for the rampant genomic instability and chromosomal aberrations that characterize most epithelial cancers. It has been suggested that mice with short telomeres represent a more faithful model of human epithelial carcinogenesis. However, the long telomeres of laboratory mouse strains have confounded efforts to replicate telonomic instability, since it is telomeric status, as opposed to telomerase activity per se that cooperates with p53 deficiency to accelerate tumorigenesis. Thus, mTERC knockout mice must be bred for several generations before critical telomere shortening ensues. Consequently, previous efforts have relied upon complex, multigenerational breeding schemes that are logistically challenging, time consuming and labor intensive. The utility of such models is also greatly complicated by the fact that global telomere shortening occurs in all tissues, resulting in premature aging phenotypes, multi-organ carcinogenesis and premature death from a variety of causes. Our study demonstrates that simultaneous conditional inactivation of p53 and components of the shelterin complex such as Pot1a is an alternative and experimentally more viable strategy to model the telonomic instability that characterizes human epithelial malignancies.

Our ploidy analyses of tumors *in vivo* and SKY of cultured tumor cells were in concordance with one another, revealing that the majority of Pot1a p53 tumors not only had abnormal karyotypes, but were tetraploid or near-tetraploid. Many epithelial cancers have near-tetraploid karyotypes, and tetraploidization is believed to be an important step during tumorigenesis. Tetraploid-derived tumors often display massive chromosomal instability, a phenomenon that is not entirely understood but may be related to the presence of supernumerary centrosomes. Polyploidization may also further promote aneuploidy and enhance tumorigenesis through several mechanisms, such as masking the effects of otherwise deleterious recessive mutations. In this manner, polyploidization may confer a survival advantage by providing a genetic 'buffer' for cells undergoing genomic instability.[50] Our findings that most Pot1a p53 tumors appear to have undergone tetraploidization during their evolution is particularly interesting in light of recent studies showing that persistent telomere damage mediated by Pot1 deficiency induces tetraploidy. Specifically, mouse embryonic fibroblasts deficient for both Pot1a/b and p53 become tetraploid. The endoreduplication event leading to tetraploidy in these cells with dysfunctional telomeres is triggered by an ATM/ATR signaling cascade that blocks mitotic entry. Despite failure of mitotic entry, cells that persist in this state ultimately switch to a state resembling G1, re-enter S phase, and become tetraploid.[19] Our studies represent an intriguing *in-vivo* correlate of these findings, lending further support that telomere-driven tetraploidization is a relevant biological phenomenon during telomere damage-driven tumorigenesis. Along these lines, it is also notable that a tetraploid DNA index is much more common in Type II vs Type I endometrial carcinomas.[51] As a side note, it is also noteworthy that both Pot1a p53 tumors subjected to SKY exhibited t(3:16) reciprocal translocations. The functional significance of this specific chromosomal aberration remains unclear, although chromosomal translocations involving both chromosome 3 and 16 have been documented in tumors deficient for both ATM and mTERC.[52]

Finally, another important finding of this study is that Pot1a proved a potent tumor suppressor in the mouse, at least in the context of p53 deficiency. Pot1 or other components of the shelterin complex might represent functional tumor suppressors in human cancers. Although few studies have specifically evaluated this possibility, whole-genome studies have not identified frequent mutations in Pot1 or other shelterin components in human tumors. For example, the COSMIC database[53,54] lists only three human tumors with somatically acquired mutations in the *POT1* gene, and the functional significance of these mutations is unclear. It is also possible that, since Pot1 is encoded by a single gene in humans, its inactivation may have other more severe deleterious effects on the cell, or that other unknown variables mitigate against its potential action as a classic tumor suppressor. Although *POT1* mutations have not been identified as the basis of any hereditary disease, some patients with dyskeratosis congenita have mutations in the shelterin protein TIN2.[54]

In conclusion, the idea that Pot1 and other components of the shelterin complex might function as 'caretaker' tumor suppressor genes that protect against genomic instability in Type II EMCAs and other cancers is an idea worthy of future investigation, both

Table 1. *In-vivo* ploidy determination of interphase nuclei by image analysis/Feulgen method

	DNA index (GO/G1)	*Ploidy*
Control *Pot1a*$^{L/L}$ #1	0.92	Diploid
Control *Pot1a*$^{L/L}$ #2	0.94	Diploid
Control *Pot1a*$^{L/L}$ #3	0.94	Diploid
Sprr2f-Cre; Lkb1$^{L/L}$#1	1.03	Diploid
Sprr2f-Cre; Lkb1$^{L/L}$#2	1.02	Diploid
Sprr2f-Cre; Lkb1$^{L/L}$#3	0.95	Diploid
Sprr2f-Cre; Pot1a$^{L/L}$*; p53*$^{L/L}$ #1	1.86	Aneuploid
Sprr2f-Cre; Pot1a$^{L/L}$*; p53*$^{L/L}$ #2	2.01	Aneuploid
Sprr2f-Cre; Pot1a$^{L/L}$*; p53*$^{L/L}$ #3	1.37	Aneuploid
Sprr2f-Cre; Pot1a$^{L/L}$*; p53*$^{L/L}$ #4	1.52	Aneuploid
Sprr2f-Cre; Pot1a$^{L/L}$*; p53*$^{L/L}$ #5	1.66	Aneuploid
Sprr2f-Cre; Pot1a$^{L/L}$*; p53*$^{L/L}$ #6	1.36	Aneuploid
Sprr2f-Cre; Pot1a$^{L/L}$*; p53*$^{L/L}$ #7	1.54	Aneuploid
Sprr2f-Cre; Pot1a$^{L/L}$*; p53*$^{L/L}$ #8	1.90	Aneuploid
Sprr2f-Cre; Pot1a$^{L/L}$*; p53*$^{L/L}$ #9	1.41	Aneuploid
Sprr2f-Cre; Pot1a$^{L/L}$*; p53*$^{L/L}$ #10	2.21	Aneuploid

DNA indices were determined on histologically normal endometrial epithelial cells (*Pot1a*$^{L/L}$, controls) or malignant epithelial cells in *Sprr2f-Cre; Lkb1*$^{L/L}$ and *Sprr2f-Cre; Pot1a*$^{L/L}$*; p53*$^{L/L}$ mice.

Table 2. Quantitation of chromosomal abnormalities in metaphase spreads from three independently derived cancer cell lines from *Sprr2f-Cre; Pot1a*$^{L/L}$*; p53*$^{L/L}$ mice

	# Metaphase anomalies	*% Normal metaphase*	*% Aberration metaphases*	*% Cells w/ breaks*	*% Cells w/ fusions*	*Fusion/ metaphase*	*% Poly/ tetraploid*	*c-anaphase*
#1	45	15.5	20	4.4	13.3	0.18	66.7	15.5
#2	36	13.9	22.2	5.5	19.4	0.25	77.8	2.8
#3	32	0	100	12.5	100	3.9	84.4	0

The data reveal that most cells harbor significant abnormalities.

through direct studies of human tumors and the use of mouse models.

MATERIALS AND METHODS

Mouse husbandry and breeding

Mice were housed in a pathogen-free animal facility and all experiments were performed with the approval of the UTSW Animal Care and Use Committee.

Tissue processing, X-gal staining and immunohistochemistry

Tissues were fixed in 10% formalin overnight and then processed for embedding in paraffin. Five-micrometer sections were deparaffinized in xylene and hydrated in an ethanol series. Slides were subjected to antigen retrieval by boiling in 10 mM sodium citrate and then cooling at room temperature for 20 min. The antibodies used were pH2AX (phosphorylated at serine 139) also known as γ-H2AX (1:2000, catalog no. 613401; Biolegend, San Diego, CA, USA) and Ki67 (1:250, catalog no. RM9106; Lab Vision, Fremont, CA, USA). The detection system was Immpress (Vector, Burlingame, CA, USA). Whole-mount X-gal staining and sectioning was performed as described.[11]

Telo-CISH and Q-FISH

Telo-CISH was performed as described previously[12] and the slides were counterstained with hematoxylin. Q-FISH was performed using a Cy-3-labeled T2AG3 PNA probe as described.[55] A total of 39, 37, 38 and 38 nuclei were counted for the Lkb1, Pot1a p53 #1, Pot1a p53 #2 and Pot1a p53 #3 cell lines, respectively.

Ploidy determinations of interphase nuclei in tissue sections by image analysis

Formalin-fixed paraffin-embedded tissue sections were Feulgen-stained and analyzed with AutoCyte Quic-DNA software (AutoCyte, Burlington, NC, USA). Stromal cells from each sample were used as reference and assigned DNA index = 1. For the epithelial cells and tumors, ~300 randomly selected nuclei were counted. DNA contents for the highest peak (mean) were assigned GO/G1 and used to determine ploidy. A mean between 0.9 and 1.1 was scored as diploid; higher values were scored as aneuploid.

Establishment of primary cultures, chromosome analyses, and SKY

Primary tumors were minced and trypsinized for 20 min at 37 °C. After pelleting, cells were resuspended and grown in low-glucose DMEM + 10% fetal bovine serum media and passaged multiple times to select against fibroblasts. Epithelial origin and genotypes of the cultured tumor cells was confirmed by PCR for the *Pot1a* and *p53* null alleles. Exponentially growing cells were exposed to colcemid (0.04 μg/ml; Gibco BRL, Carlsbad, CA, USA) for 35–40 min at 37 °C and to hypotonic treatment (0.075 M KCl) for 20 min at room temperature. Cells were fixed in a methanol and acetic acid (3:1 by volume) mixture for 15 min, and washed three times in the fixative. Slides were prepared following the standard air-drying procedure as described.[56]

SKY was performed on the cytological preparations described above using the mouse chromosome SKY probe from Applied Spectral Imaging (Vista, CA, USA) according to manufacturer's instructions to determine chromosomal rearrangements. The slides were analyzed using a Nikon Eclipse 80i (Japan) fluorescent microscope. Images were captured and karyotyped using the Applied Spectral Imaging system.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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