Type II endometrial cancer (EMCA) represents only 10% of all EMCA, 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 EMC. 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 tetruploid or near-tetruploid. 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.

Oncogene (2013) 32, 2211–2219; doi:10.1038/onc.2012.232; published online 11 June 2012

Keywords: endometrial cancer; shelterin; p53; telomeres; pot1a; mouse models

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. 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. 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. They are characterized by greater nuclear atypia and are thus categorized as high nuclear grade, likely a reflection of their aneuploidy and abnormal karyotypes. Type II cancers carry a much worse prognosis: they represent only 10% of all EMCA but have a much greater propensity to metastasize and account for 40% of EMCA-related deaths. Type I and II EMCAs have largely distinct mutational spectra and this knowledge has been exploited in the development of murine EMCA models. Pot is the most frequently mutated gene in Type I EMCA and Pten, have been conditionally inactivated through the use of PR-Cre develop well-differentiated (Type I like) invasive endometrial adenocarcinomas. Bi-allelic inactivation of the tumor suppressor Lkb1 in endometrial epithelium with Spr2fCre results in highly invasive adenocarcinomas that despite their aggressive behavior are well-differentiated endometrioid adenocarcinomas that thus also resemble Type I EMCA. TP53 is the most commonly mutated gene in Type II EMCA. Over 90% of uterine serous carcinomas and >70% of endometrioid intraepithelial carcinomas (EICs), the precursor of invasive Type II EMCA, harbor TP53 mutations. TP53 mutations are also detected in advanced, high-grade Type I cancers. Interestingly, mice or mice with conditional p53 inactivation in the uterus do not develop endometrial carcinomas by 5 months of age, suggesting that inactivation of p53 alone is not sufficient for development of Type II EMCA 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. Telomere shortening and ensuing dysfunction are believed to have an especially important role in the initiation and
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,18 and exhibit rampant genomic instability and an increased potential for progression.13 Telomere shortening and p53 inactivation together drive a vicious cycle of genomic instability through breakage-fusion-bridge cycles.20 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 machinery20 and regulates telomere length.21 In functional human telomeres, the shelterin component POT1 suppresses ATM 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 other genetic alterations through homologous DNA recombination.18 More recently, telomere-dependent tetraploidization has been uncovered as an additional mechanism by which telomere dysfunction leads to genomic instability.19

Our objective was to study the contribution of dysfunctional telomeres to endometrial carcinogenesis. To perform conditional genetic analyses, we employed the endometrial epithelial-specific deleter line Spr2f-Cre. In Spr2f-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 Spr2f-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) Spr2f-Cre; Pot1aL/L, (2) Spr2f-Cre; p53L/L, (3) Spr2f-Cre; Pot1aL/L; p53L/L and (4) sibling control Pot1aL/L; mice (n = 20 per genotype). Spr2f-Cre; Pot1aL/L mice did not develop any tumors up to 15 months of age (Figure 1d), while Spr2f-Cre; p53L/L mice developed uterine cancer only with very long latency and incomplete penetrance. In contrast, Spr2f-Cre; Pot1aL/L; p53L/L mice developed endometrial carcinomas with obvious myometrial invasion by 9 months of age. Spr2f-Cre; Pot1aL/L; p53L/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 Spr2f-Cre; Pot1aL/L; p53L/L uterine tumors histologically. For comparison, Spr2f-Cre; Pot1aL/L endometrium was histologically normal (Figure 2c). Spr2f-Cre; p53L/L tumors were surprisingly well differentiated, exhibited typical endome-trioid histology and modest nuclear atypia (Figure 2d), and thus closely resembled human Type I endometrioid adenocarcinomas (Figure 2a).11 In contrast, Spr2f-Cre; Pot1aL/L; p53L/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 ELCs in 100% of Spr2f-Cre; Pot1aL/L; p53L/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.10 Spr2f-Cre; Pot1aL/L; p53L/L tumors also displayed tripolar mitoses (Figure 2k) and anaphase bridges (Figure 2l), evidence of genomic instability and telomere dysfunction.

We also note that neoplastic foci in Spr2f-Cre; Pot1aL/L; p53L/L tumors were somewhat heterogeneous 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 EMCA is associated with advanced tumors and extremely aggressive behavior. Concordantly, Spr2f-Cre; Pot1aL/L; p53L/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,
Although a slight decrease in the Ki67 index in the endometrium of Sprr2f-Cre; Pot1a<sup>L/L</sup> mice was observed (Figure 3j), this difference was not statistically significant (P = 0.43). Interestingly, fewer cells from Pot1a<sup>L/L</sup> 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 EMCA

Interaction of POT1 with TRF2 and single-stranded telomeres inhibits telomere elongation by telomerase. 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. 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<sup>L/L</sup>; p53<sup>L/L</sup> 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. To further explore this point, quantitative telomere determinations (quantitative fluorescence in-situ hybridization—Q-FISH) were conducted with three independently derived Pot1a<sup>L/L</sup>; p53<sup>L/L</sup> EMCA cell lines and a control Lkb1 EMCA cell line derived from the extremely well-differentiated Sprr2f-Cre; Lkb<sup>L/L</sup> EMCA model. Q-FISH confirmed similar telomere lengths in Pot1a<sup>L/L</sup>; p53<sup>L/L</sup> and Lkb<sup>L/L</sup> EMCA cell lines (Figures 4b and c). We also did not

Double-strand breaks characterize Pot1a p53 EMCA

Shelterin protects telomeres from being recognized as DNA double-stranded breaks and thereby maintains telomere length homeostasis. When dysfunctional telomeres are recognized as DNA damage, 3BP1, γ-H2AX, the Mre11 complex, Rif1 and the phosphorylated form of ATM, ATM S1981-P, accumulate at the site of DNA double-stranded breaks and thereby maintains telomere length. However, telomerase activity is required for telomere elongation by telomerase. We thus assessed 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<sup>L/L</sup>; p53<sup>L/L</sup> 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. To further explore this point, quantitative telomere determinations (quantitative fluorescence in-situ hybridization—Q-FISH) were conducted with three independently derived Pot1a<sup>L/L</sup>; p53<sup>L/L</sup> EMCA cell lines and a control Lkb1 EMCA cell line derived from the extremely well-differentiated Sprr2f-Cre; Lkb<sup>L/L</sup> EMCA model. Q-FISH confirmed similar telomere lengths in Pot1a<sup>L/L</sup>; p53<sup>L/L</sup> and Lkb<sup>L/L</sup> EMCA cell lines (Figures 4b and c). We also did not...
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). 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.

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; Lkb1L/L murine EMCA model. All three of these tumors had GO/G1 DNA indices of $(2n)$, similarly to Pot1aL/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; Pot1aL/L; p53L/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 $(4n)$ ($P = 0.002$ 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
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 chromosome 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 Spr2f-Cre; Lkb1L/L and representative Spr2f-Cre; Pot1aL/L; p53L/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 Pot1aL/L control mouse. (b) Uterine tumor from 20-week-old Spr2f-Cre; Lkb1L/L mouse. (c) Uterine tumor from Spr2f-Cre; Pot1aL/L; p53L/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 Spr2f-Cre; Pot1aL/L; p53L/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. 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, multi-generational 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. 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. 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. 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.

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 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.

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 Pot1aL/L #1 | 0.92 Diploid |
| Control Pot1aL/L #2 | 0.94 Diploid |
| Control Pot1aL/L #3 | 0.94 Diploid |
| Sprn2f-Cre; Lkb1L/L; p53L/L | 1.01 Diploid |
| Sprn2f-Cre; Lkb1L/L; p53L/L | 1.02 Diploid |
| Sprn2f-Cre; Lkb1L/L; p53L/L | 0.95 Diploid |
| Sprn2f-Cre; Pot1aL/L; p53L/L | 1.86 Aneuploid |
| Sprn2f-Cre; Pot1aL/L; p53L/L | 2.01 Aneuploid |
| Sprn2f-Cre; Pot1aL/L; p53L/L | 1.37 Aneuploid |
| Sprn2f-Cre; Pot1aL/L; p53L/L | 1.52 Aneuploid |
| Sprn2f-Cre; Pot1aL/L; p53L/L | 1.66 Aneuploid |
| Sprn2f-Cre; Pot1aL/L; p53L/L | 1.36 Aneuploid |
| Sprn2f-Cre; Pot1aL/L; p53L/L | 1.54 Aneuploid |
| Sprn2f-Cre; Pot1aL/L; p53L/L | 1.90 Aneuploid |
| Sprn2f-Cre; Pot1aL/L; p53L/L | 1.41 Aneuploid |
| Sprn2f-Cre; Pot1aL/L; p53L/L | 2.21 Aneuploid |

DNA indices were determined on histologically normal endometrial epithelial cells (Pot1aL/L controls) or malignant epithelial cells in Sprn2f-Cre; Lkb1L/L and Sprn2f-Cre; Pot1aL/L; p53L/L mice.

### Table 2. Quantitation of chromosomal abnormalities in metaphase spreads from three independently derived cancer cell lines from Sprn2f-Cre; Pot1aL/L; p53L/L mice

| # Metaphase anoma | % Normal metaphase | % Aberration metaphases | % Cells w/ breaks | % Cells w/ fusions | Fusion/ metaphase | Poly/ tetraploid | c-anaphase |
|-------------------|--------------------|-------------------------|-------------------|-------------------|-----------------|----------------|-----------|
| #1                | 15.5               | 20                      | 4.4               | 13.3              | 0.18            | 66.7           | 15.5      |
| #2                | 13.9               | 22.2                    | 5.5               | 19.4              | 0.25            | 77.8           | 2.8       |
| #3                | 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 UT Southwestern Medical Center and the Institutional 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; Biologend, 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 previously12 and the slides were counterstained with hematoxylin. Q-FISH was performed using a Cy-3-labeled T2Aq3 PNA probe as described. 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 values 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 plus 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 fibroblasts. Epithelial origin and genotypes of the cultured tumor cells was confirmed by PCR for the fibroblasts. Epithelial origin and genotypes of the cultured tumor cells was confirmed by PCR for the fibroblasts. Epithelial origin and genotypes of the cultured tumor cells was confirmed by PCR for the fibroblasts. Epithelial origin and genotypes of the cultured tumor cells was confirmed by PCR for the fibroblasts.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGEMENTS
We thank Florence Sibetya for help with the ploidy analyses, Teresa Gallardo for technical assistance, and the MD Anderson Molecular Cytogenetics Core for cytogenetic services (under Cancer Center Support Grant NOI CA016672). SC acknowledges support from the NCI (RO1CA129037) and the Michal and Betty Kadoorie Cancer Genetic Research Program. This work was supported by grants to DHC from the NIH (R01CA137181, R01CA141576) and the Cancer Prevention Research Institute of Texas (RP100550).

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