Antitelomerase Therapy Provokes ALT and Mitochondrial Adaptive Mechanisms in Cancer

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**SUMMARY**

To assess telomerase as a cancer therapeutic target and determine adaptive mechanisms to telomerase inhibition, we modeled telomerase reactivation and subsequent extinction in T cell lymphomas arising in Atm−/− mice engineered with an inducible telomerase reverse transcriptase allele. Telomerase reactivation in the setting of telomere dysfunction enabled full malignant progression with alleviation of telomere dysfunction-induced checkpoints. These cancers possessed copy number alterations targeting key loci in human T cell lymphomagenesis. Upon telomerase extinction, tumor growth eventually slowed with reinstatement of telomere dysfunction-induced checkpoints, yet growth subsequently resumed as tumors acquired alternative lengthening of telomeres (ALT) and aberrant transcriptional networks centering on mitochondrial biology and oxidative defense. ALT+ tumors acquired amplification/overexpression of PGC-1β, a master regulator of mitochondrial biogenesis and function, and they showed marked sensitivity to PGC-1β or SOD2 knockdown. Genetic modeling of telomerase extinction reveals vulnerabilities that motivate coincidental inhibition of mitochondrial maintenance and oxidative defense mechanisms to enhance antitelomerase cancer therapy.

**INTRODUCTION**

Telomeres are nucleoprotein complexes at chromosome ends that function to maintain chromosomal integrity. Genetic models have defined their critical roles in cancer (Artandi and DePinho, 2010), aging, and degenerative diseases (Sahin and DePinho, 2010). Telomeres are synthesized by telomerase consisting of a reverse transcriptase catalytic subunit (TERT) and an RNA template subunit (TERC) (Feng et al., 1995; Nakamura et al., 1997). As normal or premalignant cells divide, the end-replication problem of conventional DNA polymerases, coupled with low or absent telomerase activity, results in loss of telomere sequences and eventual telomere uncapping, which activates cellular checkpoints similar to those provoked by DNA double-stranded-breaks (DSBs) (Harley and Sherwood, 1997). Like classical DSBs, telomere dysfunction has been shown to induce p53 and associated cellular responses, such as senescence and/or apoptosis (Chin et al., 1999; d’Adda di Fagagna et al., 2003; Karl-seder et al., 1999; Takai et al., 2003; van Steensel et al., 1998). Upon mutational inactivation of p53, continued cell cycling and survival of cells with telomere dysfunction provide a procarcinogenic mutator mechanism characterized by translocations and regional amplifications and deletions (Artandi et al., 2000; Chang et al., 2003; Gonza´ lez-Sua´ rez et al., 2000; Rudolph et al., 2001).
The occurrence of telomere erosion and importance of telomerase-mediated telomere maintenance in fully established cancers are evidenced by typically short telomeres relative to normal tissues and robust telomerase activity in most human cancers (Shay and Wright, 2006). This profile of shorter telomeres and telomerase activity in cancer has motivated the clinical development of telomerase inhibitors including a 13-mer antisense oligonucleotide in a number of cancer types (Agrawal et al., 2011; Shay and Wright, 2006). At present, uncertainty surrounds whether antitelomerase therapy will be hampered by the potential lag time needed for telomere erosion-associated tumor cell killing, and/or whether re-entry into telomere-based crisis will engender genomic instability that may allow for emergence of adaptive responses and resistance mechanisms, such as alternative lengthening of telomeres (ALT) mechanism which enables telomere maintenance via homologous recombination (Cesare and Reddel, 2010). Notably, in mTERC+/− mice, p53 deficiency alleviates tumor suppression imparted by telomere dysfunction (Chin et al., 1999) and, in transformed cells with intact p53-dependent DNA damage checkpoint, there is activation of ALT (Chang et al., 2003).

The study of cancer pathogenesis and specific role of telomeres therein have been enabled by the use of genetically engineered mouse models of cancer, which provide an in vivo assessment of the complex adaptive responses to targeted cancer treatments. Here, we exploited the experimental merits of mice to model and study more precisely telomere crisis, telomerase reactivation and telomerase extinction in cancer development, progression and treatment in the in vivo setting. To that end, we engineered an inducible TERT knockin allele and studied telomere dynamics in mice mutant for Atm, which develop T cell lymphomas with high penetrance (Xu et al., 1996). The Atm mutant model was selected to study telomerase activation and its extinction as this model retains a robust p53-mediated checkpoint response upon telomere uncapping which strongly suppresses emergent tumors (Qi et al., 2003; Wong et al., 2003). Next, to test the impact of telomerase reactivation on lymphoma kinetics in this model, continuous-release 4-OHT tablets were implanted subcutaneously at 18 weeks, an age when lymphomas in Atm+/− mice with intact telomeres start to emerge. 4-OHT-treated G3-4 mice developed overt lymphomas more rapidly and with higher penetrance than, not only vehicle-treated G3-4 mice, but also G1 mice (Figure 1A). In contrast, there were no differences in tumor latency and penetrance in the 4-OHT and vehicle-treated G1 mice (Figure 1A). On the histopathological level, vehicle-treated G3-4 tumors were smaller in size and displayed less aggressive malignant features relative to 4-OHT-treated G3-4 tumors as well as telomere intact G0 and G1 tumors (Figures 1B–1D; data not shown). Finally, 4-OHT treatment itself does not impact on lymphomagenesis as reflected by similar survival curves of G3-4 Atm+/− TERT+/+/TERT+/− mTERC+/−/C0 mice treated with either vehicle or 4-OHT (Figure 1A), supporting the view that acceleration of 4-OHT on G3-4 Atm+/− TERT+/−/TERT+/−/C0 tumors stems from telomerase reactivation and not from nontelomere activities of 4-OHT.

The capacity to regulate endogenous telomerase activity in vivo prompted serial analyses of malignant properties and cellular checkpoint responses as functional readouts of telomere status during lymphomagenesis and progression in G3-4 mice. Quantitation of DNA damage foci by anti-γH2AX and -53BP1 staining showed markedly increased foci in vehicle-treated G3-4 tumor cells and a dramatic decrease in such foci in 4-OHT-treated G3-4 tumor cells (Figures 2A and 2B; Figure S2). Correspondingly, vehicle-treated G3-4 tumor cells exhibited decreased proliferation (Ki67) and increased apoptosis (cleaved caspase 3), senescence (SA-β-Gal), and p53 signaling (p53 phosphorylation and p21 expression), which are all alleviated by telomerase reactivation (Figures 2C–2F).

**Telomerase Reactivation Promotes Tumorigenesis by Stabilizing Telomeres and Alleviating Telomere Dysfunction-Induced Checkpoints**

We generated mice harboring an Atm null allele (Xu et al., 1996) and a 4-Hydroxynortamoxifen (4-OHT)-inducible Telomerase Reverse Transcriptase-Estrogen Receptor (TERT-ER(T2)) fusion knockin allele (Jaskelioff et al., 2011). In the absence of 4-OHT, mice homozygous for TERT(T2) (designated TERT+/+/TERT+/−) or TERT+/−/TERT+/−/C0 are telomerase activity deficient and sustain same cytogenetic and cellular phenotypes of conventional TERT or TERC knockout model (Jaskelioff et al., 2011). Upon 4-OHT treatment, TERT-ER protein activity can be restored to levels comparable to the native TERT protein (Jaskelioff et al., 2011). To generate lymphoma-prone mice with telomere dysfunction, Atm+/−/TERT+/−/TERT+/−/TERT+/−/C0 mice were intercrossed to produce first generation (G1) Atm+/−/TERT+/−/TERT+/−/C0 and Atm+/−/TERT+/−/TERT+/−/C0 mice. G1 Atm+/−/TERT+/−/TERT+/−/C0 mice were then serially intercrossed to successive generations in the absence of 4-OHT, culminating in G3 and G4 Atm+/−/TERT+/−/TERT+/−/C0 experimental cohorts (Figure S1A available online). For brevity, the Atm+/−/TERT+/−/TERT+/−/C0, G3 and G4 Atm+/−/TERT+/−/C0 mice are designated G0, G1, and G3-4, respectively.

G0 and G1 mice developed T cell lymphomas at similar latencies and penetrance (Figure S1B), which is consistent with adequate telomere reserves to maintain capping in G1 mice and avoid activated DNA damage checkpoints. In contrast, G3-4 lymphomas emerge with a longer latency and lower penetrance relative to G1 controls (Figure 1A), consistent with previous work (Maser et al., 2007; Qi et al., 2003; Wong et al., 2003). Next, to test the impact of telomerase reactivation on lymphoma kinetics in this model, continuous-release 4-OHT tablets were implanted subcutaneously at 18 weeks, an age when lymphomas in Atm+/−/C0 mice with intact telomeres start to emerge. 4-OHT-treated G3-4 mice developed overt lymphomas more rapidly and with higher penetrance than, not only vehicle-treated G3-4 mice, but also G1 mice (Figure 1A). In contrast, there were no differences in tumor latency and penetrance in the 4-OHT and vehicle-treated G1 mice (Figure 1A). On the histopathological level, vehicle-treated G3-4 tumors were smaller in size and displayed less aggressive malignant features relative to 4-OHT-treated G3-4 tumors as well as telomere intact G0 and G1 tumors (Figures 1B–1D; data not shown). Finally, 4-OHT treatment itself does not impact on lymphomagenesis as reflected by similar survival curves of G3-4 Atm+/−/TERT+/−/TERT+/−/TERT+/−/C0 mice treated with either vehicle or 4-OHT (Figure 1A), supporting the view that acceleration of 4-OHT on G3-4 Atm+/−/TERT+/−/TERT+/−/C0 tumors stems from telomerase reactivation and not from nontelomere activities of 4-OHT.

Telomerase Reactivation in Spontaneous Tumors with Unstable Genomes Promotes Aggressive Malignant Properties

A higher percentage of G3-4/4-OHT mice exhibited widespread tumor cell infiltration in spleen, kidney, liver, lung, and bone marrow relative to vehicle-treated G3-4 mice as well as 4-OHT- and vehicle-treated G1 mice (Figures 3A and 3B). The more aggressive nature of 4-OHT-treated G3-4 lymphomas was particularly evident from infiltration into brain (4/16) which was not.
observed in G1 necropsies (n = 16) (Figures 3A and 3B), demonstrating that the malignant progression of initiated tumors is enabled by somatic reactivation of telomerase. This acquisition of more aggressive tendencies may stem in part from increased genome instability during the formative stages of tumor development prior to telomerase activation. Indeed, telomere dysfunction-induced bridge-fusion-breakage events can generate non-reciprocal translocations as well as regional amplifications and deletions at sites of breakage that, under biological selection, can result in cancer-promoting copy number changes (Ar-tandi et al., 2000; Maser et al., 2007; O’Hagan et al., 2002). To examine this possibility, we compared cytogenetic and genomic profiles of 4-OHT-treated G3-4 tumors and 4-OHT-treated G1 tumors, the latter without a period of telomere-driven crisis. Consistent with previous work (Maser et al., 2007), spectral karyotype (SKY) analysis showed a 3-fold increase in chromosomal rearrangements including nonreciprocal translocations in 4-OHT-treated G3-4 tumors (n = 3) relative to 4-OHT-treated G1 tumors (n = 3) (Figures 3C and 3D). These cross-species shared copy number alterations (CNAs) in G3-4-4-OHT tumors identified 4,928 genes resident within regions of genomic gain and 2,297 genes resident within regions of genomic loss with the use of Segment Gain or Loss (SGOL) algorithm (Wiedemeyer et al., 2010; Extended Experimental Procedures). Comparison of G3-4-4-OHT tumors to human T-ALLs (Maser et al., 2007) showed that 565 of 4,928 amplified genes (11.5%) and 300 of 2,297 deleted genes (13%) are targeted does not discernibly alter the scope of clonal genomic events in these cancers (Figure 3E; Figure S3B). Along these lines, we assessed whether the observed CNAs in G3-4-4-OHT mice might contribute to the lymphomagenesis by examining whether murine and human lymphomas sustain orthologous CNA events than might be expected by chance as conducted previously (Maser et al., 2007). Array-CGH profiles of G3-4-4-OHT tumors identified 4,928 genes resident within regions of genomic gain and 2,297 genes resident within regions of genomic loss with the use of Segment Gain or Loss (SGOL) algorithm (Wiedemeyer et al., 2010; Extended Experimental Procedures). Comparison of G3-4-4-OHT tumors to human T-ALLs (Maser et al., 2007) showed that 565 of 4,928 amplified genes (11.5%) and 300 of 2,297 deleted genes (13%) are targeted for copy number alteration in both species (Table S1), which are significantly higher than those expected by chance (n = 10,000; p = 3e-04 and 2e-04 for amplification and deletion, respectively) (Figure S3C). These cross-species shared copy number altered genes include several known tumor suppressors and oncogenes implicated in T-ALL biology such as Crebbp, Ikaros, Abl, Notch1, Myc, and PTEN (Figure 3E). These data and previous work (Maser et al., 2007; O’Hagan et al., 2002) suggest that telomere dysfunction provides a mechanism that promotes structural genome alterations of cancer-relevant loci, which not only drive primary tumorigenesis but also confer additional malignant properties such as enhanced invasiveness.

Genetic Extinction of Telomerase Inhibits Cancer Growth and Leads to Eventual Tumor Re-Emergence

The TERT-ER system affords exploration of the impact of genetic extinction of telomerase activity in established cancers that, similar to human cancers, had evolved to first experience telomere dysfunction, and then subsequently acquire telomerase activity. In particular, this genetic model system enables assessment of tumor biological impact and potential adaptive mechanisms of antitelomerase therapy following reacquisition of telomerase dysfunction. Freshly harvested 4-OHT-treated G3.4 tumor cells mice were passaged directly through SCID mice.
preimplanted with vehicle or 4-OHT tablets (Figure S4A). Of 15 primary tumors lines (ten mice per each passage of treatment; 10^7 cells per intraperitoneal injection), 11 lines generated xenograft tumors. In first and second passages (P1 and P2), these lines showed no differences in the two treatment arms in terms of penetrance and latency (Figure S4A; P1 and P2). However, at third passage (P3), 9 of 11 lines showed lower penetrance and longer latency in the vehicle-treated arm relative to the 4-OHT-treated arm, a profile consistent with the view that multiple rounds of cell division are needed to achieve sufficient telomere erosion and re-entry into crisis. Six of these nine lines showed complete loss of tumor formation in the vehicle-treated arm, whereas three lines yielded escaping tumors in several vehicle-treated recipient mice (Figure S4A; P3). When these escaping P3 tumors were passaged to P4, they reacquired aggressive malignant properties approaching those of matched 4-OHT controls (Figure 4A; P4), suggesting acquisition of resistant mechanisms to counteract telomerase deficiency. Moreover, 2 of the original 11 tumor lines did not show any attenuation of tumor growth in the vehicle-treated arm compared with the 4-OHT-treated arm (data not shown), suggesting early acquisition of such resistant mechanisms (see below).

To assess how G3-4-4-OHT tumors respond to telomerase extinction on molecular level, we audited p53 signaling, DNA damage foci and apoptosis in serial passages of the two treatment arms. In 4-OHT-treated P1 to P4 and vehicle-treated P1 tumor cells, p53 signaling, DNA damage foci and apoptosis levels were similar to those of parental tumor cells.
Figure 3. Telomerase Reactivation Promotes Invasiveness of Late-Generation TERT\textsuperscript{ER/ER\textsuperscript{+}} Atm\textsuperscript{−/−} Lymphomas

(A) Representative images of infiltrated organs (lung, kidney, liver, and brain) in G\textsubscript{3,4} TERT\textsuperscript{ER/ER\textsuperscript{+}} Atm\textsuperscript{−/−} mice treated with 4-OHT. Scale bars represent 100 mm.

(B) Quantification of infiltrating incidence of G\textsubscript{1} and G\textsubscript{3,4} TERT\textsuperscript{ER/ER\textsuperscript{+}} Atm\textsuperscript{−/−} mice treated with 4-OHT or vehicle.

(C) Representative spectral karyotype (SKY) images from metaphases of G\textsubscript{1} and G\textsubscript{3,4} TERT\textsuperscript{ER/ER\textsuperscript{+}} Atm\textsuperscript{−/−} lymphomas treated with 4-OHT.

(D) Quantification of nonreciprocal translocations (NRTs) detected by SKY in G\textsubscript{1} (n = 3) and G\textsubscript{3,4} (n = 3) TERT\textsuperscript{ER/ER\textsuperscript{+}} Atm\textsuperscript{−/−} lymphomas treated with 4-OHT (p < 0.0001, t test).

(E) Recurrence plot of CNAs defined by array-CGH for 18 G\textsubscript{3,4}-4-OHT lymphomas. The x axis shows the physical location of each chromosome. The percentage of tumors harboring gains (dark red, log\textsubscript{2} ≥ 0.3), amplifications (bright red, log\textsubscript{2} ≥ 0.6), losses (green, log\textsubscript{2} ≤ −0.3), and deletions (dark green, log\textsubscript{2} ≤ −0.6) for each locus is depicted. Locations of physiologically relevant CNAs of TCR loci and some known cancer genes are indicated with asterisks.

See also Figure S3 and Table S1.
In contrast, vehicle-treated P2 tumor cells showed significantly higher levels of p53 signaling, DNA damage foci and apoptosis, and all of these processes were reduced in the resistant vehicle-treated P3-4 tumor cells (Figures 4B–4E), raising the possibility that these resistant tumors may have stabilized their telomeres to alleviate these checkpoints. Intriguingly, while DNA damage foci were reduced in the resistant tumors compared with the vehicle-treated P2 tumors, these foci remain higher than telomerase+ (4-OHT-treated) tumors (Figures 4D and 4E), consistent with residual genotoxic stress in the resistant tumors. However, despite ongoing DNA damage signaling, apoptosis was not observed, which is consistent with compromised p53 signaling in the resistant tumors (Figure S4B).

Emergence of ALT in Tumors following Extinction of Telomerase

To elucidate adaptive mechanisms following telomerase extinction, we cataloged telomere lengths in serially passaged tumors in the two treatment arms. In passaged 4-OHT-treated P1 to P4 tumor cells, telomere lengths remained unchanged from those in parental tumor cells, while vehicle-treated P1 and P2 tumor cells showed progressive telomere shortening (Figure 5A). However, vehicle-treated P3-4 tumor cells showed a sharp increase in average and maximal telomere lengths that exceeded those in matched 4-OHT controls (Figure 5A); however, these vehicle-treated P3-4 tumor cells tumor cells showed more telomere signal-free ends by telomere-FISH (Figures 5B and 5C), a picture consistent with higher heterogeneity in the distribution of telomere lengths in resistant tumors. In addition, costaining of promyelocytic leukemia (PML) bodies and telomeres showed an increase in ALT-associated PML bodies (APBs) (Figures 5D and 5E), and telomere-FISH showed more extrachromosomal telomere fragments in the vehicle-treated P3-4 tumors relative to the matched 4-OHT controls (Figures 5F and 5G). Finally, vehicle-treated P3-4 tumor cells showed an increased telomere Sister Chromatid Exchange (tSCE) rate than matched 4-OHT controls (Figures 5H and 5I). These collective data provide clear evidence of ALT in vehicle-treated P3-4 tumor cells that have resumed robust tumor growth in vivo. In addition, the two tumor lines, which did not show any attenuation of tumor growth in the vehicle-treated arm, also showed ALT features including APBs and extra-chromosomal telomere fragments (Figures S5A and S5B), consistent with the early acquisition of ALT.

ALT Tumors Show Upregulation of a Master Regulator of Mitochondrial Biogenesis/Function and Oxidative Defense

Serial loss of telomere function and genome destabilization in this genetically defined system provided an unprecedented opportunity to discern on a genome scale the associated adaptive molecular events in these ALT+ tumor cells via integrated transcriptomic and aCGH profile analyses. Cluster analysis of transcriptomes of 4-OHT-treated P3-4 tumors (n = 3), and parental 4-OHT-treated tumor revealed significant differences relative to the matched 4-OHT-treated P2 tumors, 891 upregulated and 1,345 downregulated genes in ALT+ tumors with 1.5-fold change and p < 0.01 (t test; Figure 6A; Table S2). Pathway (IPA) analysis of the ALT-specific transcriptome showed strong representation of networks centering on
mitochondrial biology and oxidative stress regulation (Figure S6A). Consistent with the known role of DNA recombination in ALT (Fan et al., 2009; Zhong et al., 2007), MRE11 and FANCA were also upregulated in these ALT+ tumors (Figures S6B and S6C). With regard to mitochondria and oxidative pathways, Q-PCR verified aberrant expression of several key genes in these networks including upregulation of a master regulator of mitochondrial biology and oxidative defense, PGC-1β, and its targets such as NRF2, SOD2, and Catalase, among others (Figure 6B).

As noted, telomere dysfunction-induced DNA double-strand breakage process and biological selection can lead to functionally relevant CNAs, prompting us to identify potential genetic events that may inform how ALT+ cells cope with telomere-based crisis. To that end, we performed array-based comparative genomic hybridization (aCGH) analysis of ALT+ versus telomerase+ P2 tumors from the two treatment arms. While serially transplanted telomerase+ tumors showed minimal CNA differences relative to the parental tumor, ALT+ tumors acquired numerous CNAs (see below). Strikingly, integrated transcriptomic and copy number analysis revealed PGC-1β as the only gene in the mitochondrial function and oxidative stress regulation pathways showing both upregulated expression and copy number gain in the ALT+ tumors relative to telomerase+ tumors (Table S3); these genomic data raised the possibility that PGC-1β might be a major driver of the adaptive response to telomere dysfunction.

To ask whether PGC-1β amplification/overexpression is a common event in our ALT+ tumors, we profiled eight independent telomerase+ tumors and five independent ALT+ tumors, and found that three of five ALT+ tumors (60%) show amplification of a large region of chromosome 18, which includes PGC-1β gene, whereas none of the telomerase+ tumors (n = 8) sustain such genomic alterations (Figure 6C). To reinforce the potential role of PGC-1β upregulation as a possible adaptive response to ALT, we examined PGC-1β expression levels in ALT+ tumors without PGC-1β amplification, and established that PGC-1β expression is significantly higher in ALT+ tumors than their corresponding telomerase+ tumors (Figure S6D). ALT+ Tumors Have Higher Levels of Mitochondrial Dysfunction and Reactive Oxygen Species Relative to Telomerase+ Tumors

PGC-1α and PGC-1β are master regulators of mitochondrial biogenesis and function, play integral roles in the regulation of genes governing reactive oxygen species (ROS) defense, and are downregulated in normal tissues experiencing telomere dysfunction as a result of activated p53-mediated repression (Sahin et al., 2011). This framework, coupled with above genomic observations, prompted us to hypothesize that telomerase extinction and ensuing telomere dysfunction repress the mitochondrial biogenesis and function in cancer cells, and the adaptive response to such telomere-based crises targets the p53-PGC axis in an effort to restore the mitochondrial function. To assess this possibility, we assessed PGC network expression, mitochondrial mass and mitochondrial function in passaged 4-OHT-treated and vehicle-treated P1-P4 tumor cells. Mitochondria DNA content were maintained in the telomerase+ arm but sharply reduced in the vehicle-treated P2 tumor cells yet significantly restored in the ALT+ P3-4 tumor cells (Figure 6D). Correspondingly, in contrast to the telomerase+ arm or early passage of vehicle-treated tumor cells, vehicle-treated P2 tumor cells showed reduced expression of PGC-1β and its major targets NRF-1, ERRα, PPARα, and TFAM which are critical for mitochondrial biogenesis and function (Figure 6E; note: PGC-1α is not expressed in these murine T cell lymphomas). These expression patterns were significantly normalized in the ALT+ P3-4 tumor cells (Figure 6E).

With regard to mitochondrial function, mitochondrial respiration assays (normalized for mtDNA content) showed that vehicle-treated P2 tumor cells have dramatically impaired mitochondrial respiration relative to telomerase+ cells (Figure 6F). ALT+ P3-4 tumor cells show substantial improvement in mitochondrial respiration, although function remains somewhat compromised relative to those of telomerase+ cells (Figure 6F; Figure S6E). Finally, the capacity of PGC-1β to regulate oxidative defense genes (St-Pierre et al., 2006) and the integral role of mitochondria in ROS control (Murphy, 2009) prompted examination of intracellular ROS levels in ALT+ and telomerase+ tumor cells. Using the fluorescent dye DCF-DA protocol, FACS analysis showed significantly increased ROS levels in vehicle-treated P2 tumor cells relative to 4-OHT-treated P0 to P4, and vehicle-treated P1 tumor cells (Figure 6G; Figure S6F). In ALT+ tumor cells, ROS levels are lower than vehicle-treated P2 tumor cells, albeit higher than telomerase+ tumor cells (Figure 6G; Figure S6F). Thus, telomere dysfunction in cancer cells is commonly associated with repression of PGC1β and its network resulting in mitochondrial mass decline and the adaptive responses to telomere dysfunction appear to involve not only activation of ALT telomere maintenance mechanism but also upregulation of genetic pathways promoting partial restoration of mitochondrial mass and function. Importantly, however, relative to telomerase+ cells, ALT+ tumor cells still have modestly elevated mitochondrial dysfunction and ROS which may reflect ongoing genotoxic stress relating to inefficiencies of telomere maintenance via ALT as reflected by moderately increased DNA damage foci above baseline in these tumors (see Figures 4D and 4E above). ALT+ Tumors Show Increased Sensitivity to Inhibition of PGC-1β or SOD2

Because relatively functional mitochondria and moderate levels of ROS are required for cancer cell survival (Weinberg et al., 2010), we asked whether shRNA-mediated knockdown of PGC-1β would have a negative or differential impact on telomerase+ and ALT+ tumor cells. Multiple independent shRNAs achieved PGC-1β knockdown in both telomerase+ and ALT+ tumor cells (Figure 7A). Consistent with the pivotal role of PGC coactivators in mitochondrial function and ROS regulation, both telomerase+ and ALT+ tumor cells showed significantly reduced mitochondrial numbers, compromised mitochondrial function, and increased ROS after PGC-1β knockdown (Figures 7B–7D). Notably, ALT+ tumor cells showed greater compromise in all three categories relative to telomerase+ tumor cells (Figures 7B–7D), raising the possibility that ALT+ tumor cells may show greater sensitivity to PGC-1β inhibition with increased mitochondrial dysfunction and ROS toxicity. Correspondingly,
Figure 5. TERT<sup>E16K</sup> Atm<sup>−/−</sup> Lymphomas Developed ALT in the Absence of Telomerase

(A) Telomere lengths of serially transplanted lymphomas treated with 4OHT or vehicle measured by telomere-FISH coupled laser scanning cytometry (n = 3–5).

(B) Telomere signal-free ends were measured by telomere-FISH in serially transplanted lymphomas treated with 4OHT or vehicle.
shRNA-mediated knockdown of PGC-1β showed a minimal impact on telomerase+ tumor growth and a profound impact on ALT+ cells (Figure 7E; median survival from 15 weeks to 19 weeks versus 17 weeks to over 30 weeks; \( p = 0.0065 \), Cox model). These findings are consistent with increased apoptosis in ALT+ tumors with PGC-1β knockdown (Figure S7A). In addition, since SOD2 is a major ROS antioxidant in mitochondria, regulated by PGC-1β, and highly expressed in ALT+ cells (Figure 6B), we tested the impact of SOD2 knockdown in ALT+ and telomerase+ cells. As expected, SOD2 knockdown increased ROS levels in both telomerase+ and ALT+ cells, although ROS levels in ALT+ cells (both basal or after SOD2 knockdown) were substantially higher than those in telomerase+ cells (Figures 7F and 7G). On the biological level, while non-targeting shRNA-transduced telomerase+ and ALT+ cells showed similar cell survival rates (MTT assay), SOD2 knockdown in ALT+ cell decreased survival greater than in telomerase+ cells (Figure 7H; \( p = 0.02 \)), suggesting that ALT+ cells are more sensitive to increase of ROS than telomerase+ cells. The sensitivity of ALT+ cells to ROS was further confirmed by NAC rescue experiments on ALT+ cells with PGC-1β and SOD2 knockdown (Figures S7B and S7C). Together, these data suggest that targeting PGC or ROS detoxifying genes may enhance the therapeutic impact of antitelomerase therapy, particularly in the context of ALT+-resistant cells.

**DISCUSSION**

One of the hallmarks of cancer cells is the shift from mitochondrial to glycolytic metabolism as the prime source of energy...
and anabolic support (Vander Heiden et al., 2009; Warburg, 1956). While this bioenergetic shift conveys decreased reliance on mitochondria as a major energy source for cancer cells, our genetic studies indicate a continued vital role in the maintenance of mitochondrial function in cancer. This view aligns with multiple lines of evidence suggesting that mitochondrial competence is indeed important for cancer cell viability (Jose et al., 2011; Koppenol et al., 2011) and efficient oncogene-mediated transformation (Gao et al., 2009; Weinberg et al., 2010; Wise et al., 2008; Yuneva et al., 2007). More specifically, our study uncovers a critical role of robust mitochondrial function in cancer cells, particularly for those cancer cells with ALT-maintained telomeres. Why might ALT+ cancer cells exhibit exquisite sensitivity to inhibition of PGC expression or antioxidant defense? We speculate that continued DNA damage signaling at ALT-maintained telomeres perhaps due to altered chromatin (Heaphy et al., 2011) or inefficient capping via this mechanism could lead to mitochondrial damage. Mitochondria are the major intracellular source of ROS in mammalian cells, and dysfunctional mitochondria produce more ROS, which at excessively high levels is...
known to be detrimental to cell survival (Lin and Beal, 2006; Murphy, 2009; St-Pierre et al., 2006). In this context, we propose that the pressure to maintain mitochondrial function reflects the need to maintain tolerable intracellular ROS levels. This oxidative defense mechanism may be particularly important for telomeres as ROS are particularly injurious toward the G-rich sequences of telomeres (Hall et al., 1996; Oexle and Zwiner, 1997; Retéli et al., 1993), thereby causing increased telomere dysfunction in the absence of efficient telomerase-mediated repair. Correspondingly, our combined genomic and functional studies establish that ALT+ cells strive to maintain adequate mitochondrial and oxidative defense functions and show exquisite sensitivity to loss of such function. On the molecular level, this work also establishes that the intimate PGC-directed link between telomeres and mitochondria identified recently in normal tissues (Sahin et al., 2011) is operative in cancer cells (Figure 7I).

About 15% of human cancers maintain their telomeres in the absence of telomerase activity by the ALT mechanism. The existence of a telomerase-independent telomere maintenance mechanism was first identified in yeast with telomerase deficiency, and this mechanism was demonstrated to be dependent on RAD52, a gene involved in homologous recombination (Lundblad and Blackburn, 1993). Subsequent studies established that the ALT mechanism in human cancer cells requires certain DNA recombination proteins, including the MRN complex (MRE11, RAD50, and NBS1) (Jiang et al., 2005; Zhong et al., 2007), SMC5-SMC6 complex (Potts and Yu, 2007), flap endonuclease 1 (FEN1) (Saharia and Stewart, 2009), MUS81 (Zeng et al., 2009), Fanconi anemia group D2 (FANCD2), and Fanconi anemia group A (FANCA) (Fan et al., 2009). In our model, MRE11 and FANCA genes are indeed upregulated in ALT+ cells relative to telomerase+ cells (Figures S4B and S4C). These observations provide genetic evidence in mammalian cells of the importance of these factors for DNA recombination-dependent ALT mechanism in this cancer model.

Our studies also highlight that, while ALT+ cells suppress apoptosis and senescence as efficiently as telomerase+ cells, these cells do not fully restore mitochondrial function which we speculate may relate to ongoing genotoxic stress partly associated with less efficient telomere capping by ALT-mediated telomere maintenance (Cesare et al., 2009) as well as p53-independent repression of PGC (Sahin et al., 2011). In ALT+ cells, the importance of this mitochondrial maintenance program is underscored by the upregulated PGC network signaling on both the genomic (copy number) and transcriptional levels (Figure 7I). That the PGC network is a vital regulator in our ALT+-resistant modeling of telomerase extinction in cancer increases our understanding of how tumor cells might respond and adapt to telomerase inhibition and illuminates a clinical path hypothesis utilizing combination regimens targeting telomerase and PGC-mediated adaptive mechanisms in cancer.

EXPERIMENTAL PROCEDURES

Mice

Abl, Terc, and TERT-ER mice described previously (Jaskelioff et al., 2011; Maser et al., 2007; Wong et al., 2003) were interbred and backcrossed to high grade of C57BL/6 (over 95%). The mating strategy to obtain experimental cohorts G0, G1, and G2 (shown is Figure S1A). 4-OHT time release pellets (5 mg; Innovative Research of America) were inserted subcutaneously at age of 18 weeks to reach steady-state blood levels of 1 ng ml−1 4-OHT for 60 consecutive days.

Histology, Tumor Characterization, and Sample Preparation

Antibodies used for IHC include anti-Ki67 (Dako), anti-S3BP1 (Bethyl Labs), anti–γH2AX (Bethyl Labs), and anti-Cleaved Caspase 3 (Cell Signaling). For FACS analysis, cells were immunostained with CD4, CD8, and CD3 antibodies (eBioscience) and analyzed on a BD FACSCanto II (BD Biosciences).

Cytogenetic Analysis

For metaphase preparation, metaphases were obtained from colcemid-treated cells incubated in 105 mM KCl hypotonic buffer for 15 min before fixation in 3:1 methanol-acetic acid. Spectral karyotyping (SKY) was done using the SkyPaint Kit and SkyView analytical software (Applied Spectral Imaging) according to manufacturer’s protocols. For telomere-FISH (fluorescent in situ hybridization), metaphase spreads were applied with telomere-specific TTAGGG–FITC PNA (peptide nucleic acid) probes and centromere-specific Cent-Cy3 or Cent-pacific blue PNA probes, and counter-stained with DAPI (for microscopy) or TO-TO3 (for laser scanning). Laser scanning cytometry quantification was performed with an iCys Research Imaging Cytometer (Compucyte) as described earlier (Jaskelioff et al., 2011; Sahin et al., 2011).

Telomere Sister Chromatid Exchange Assay

Telomere Co-FISH staining was performed as previously described (Potts and Yu, 2007). Details are described in Extended Experimental Procedures.

RT-qPCR, Mitochondria DNA Content Measurement, and Western Blot

RT-qPCR primers are described in Table S4. Mitochondria DNA content was measured by the relative levels of Cox1 versus beta globin by qPCR. Cox1 and beta globin primers are described in Table S4. Antibodies used for Western blotting are anti-phospho p53 (ser15, Cell Signaling), anti-p21 (Santa Cruz Biotechnology), anti-H2AX (Bethyl Labs), and anti-Cleaved Caspase 3 (Cell Signaling). For Western blotting, proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk in TBS-T and probed overnight with primary antibodies. Blots were visualized with enhanced chemiluminescence reagents (Amersham Biosciences) according to manufacturer’s instructions. Probes for immunofluorescence are described in Table S4.

shRNA Knockdown

Lentiviral based shRNA constructs targeting mouse PGC-1α and SOD2 were ordered from The Dana-Farber/Harvard Cancer Center DNA Resource Core. Verified shRNA sequences were cloned into inducible lentiviral constructs PLKO-TRC-901 (IPTG inducible; Broad Institute) and PLKO-Tet (Doxycycline inducible; Novartis).

Pathway Analysis

Eight hundred ninety-one upregulated and 1,345 downregulated genes with 1.5-fold change and p < 0.01 in ALT+ tumors relative to telomerase+ tumors were applied with Knowledge-based Pathway (IPA) analysis. The significantly represented pathways were compared with the pathways obtained from the similar analysis on ten ALT+ and eight telomerase+ osteosarcomas (Lafferty-Whyte et al., 2009). Overlapped pathways are listed.

Array-CGH Profiling and Analyses

Array-CGH, SGGOL score analysis, homologous mapping, and permutation analysis are described in Extended Experimental Procedures.
Mitochondrial Oxygen Consumption Measurements in Live Cells

Oxygen consumption rates (OCRs) were measured using the Seahorse XF24 instrument (Seahorse Biosciences). Basal mitochondrial respiration were measured at four time points, and respiration nonlinked to mitochondrial ATP synthesis were measured at four time points after adding 1 μM oligomycin. Nonmitochondrial OCRs were obtained by adding 5 μM antimycin A, and uncoupled respiration was obtained by adding 1 μM FCCP.

ROS Measurement

For determination of ROS levels, tumor cells were stained with 5 μM CM-H2DCFDA (Invitrogen) at 37°C for 30 min, followed by FACS analysis on a BD FACSCanto II (BD Biosciences).

COX Model Test

Effects of shPGC-1α knockdown on Tel+ and ALT+ cells were tested by Cox analysis. Details are described in Extended Experimental Procedures.

ACCESSION NUMBERS

Microarray (GSE39044) and aCGH (GSE35045) data were deposited in GEO (http://www.ncbi.nlm.nih.gov/geo/) under the accession numbers indicated.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and four tables and can be found with this article online at doi:10.1016/j.cell.2011.12.028.

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