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Binding of the sphingolipid S1P to hTERT stabilizes telomerase at the nuclear periphery by allosterically mimicking protein phosphorylation[†]

Shanmugam P. Selvam^{1,2}, Ryan M. De Palma^{1,2}, Joshua J. Oaks^{1,2}, Natalia Oleinik^{1,2}, Yuri K. Peterson^{2,3}, Robert V. Stahelin^{4,5}, Emmanuel Skordalakes⁶, Suriyan Ponnusamy^{1,2}, Elizabeth Garrett-Mayer², Charles D. Smith^{2,3}, and Besim Ogretmen^{1,2,*}

¹Department of Biochemistry and Molecular Biology, Medical University of South Carolina, 86 Jonathan Lucas Street, Charleston, SC 29425

²Hollings Cancer Center, Medical University of South Carolina, 86 Jonathan Lucas Street, Charleston, SC 29425

³Department of Pharmaceutical Sciences, Medical University of South Carolina, 86 Jonathan Lucas Street, Charleston, SC 29425

⁴Department of Biochemistry and Molecular Biology, Indiana University School of Medicine-South Bend, South Bend, IN 46617

⁵Department of Chemistry and Biochemistry and the Mike and Josie Harper Cancer Research Institute, University of Notre Dame, South Bend, IN 46556, USA

⁶Gene Expression and Regulation Program, The Wistar Institute, Philadelphia, Pennsylvania, USA

Abstract

During DNA replication, the enzyme telomerase maintains the ends of chromosomes, called telomeres. Shortened telomeres trigger cell senescence, and cancer cells often have increased telomerase activity to promote their ability to proliferate indefinitely. The catalytic subunit, human telomerase reverse transcriptase (hTERT), is stabilized by phosphorylation. Here, we found that the lysophospholipid sphingosine 1-phosphate (S1P), generated by sphingosine kinase 2 (SK2), bound hTERT at the nuclear periphery in human and mouse fibroblasts. Docking predictions and mutational analyses revealed that binding occurred between a hydroxyl group (C'3-OH) in S1P

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*Corresponding author. ogretmen@musc.edu.

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and Asp⁶⁸⁴ in hTERT. Inhibiting or depleting SK2 or mutating the S1P binding site decreased the stability of hTERT in cultured cells and promoted senescence and loss of telomere integrity. S1P binding inhibited the interaction of hTERT with MKRN1, an E3 ubiquitin ligase that tags hTERT for degradation. Murine Lewis lung carcinoma (LLC) cells formed smaller tumors in mice lacking SK2 than in wild-type mice, and knocking down SK2 in LLC cells before implantation into mice suppressed their growth. Pharmacologically inhibiting SK2 decreased the growth of subcutaneous A549 lung cancer cell-derived xenografts in mice, and expression of wild-type hTERT, but not an S1P-binding mutant, restored tumor growth. Thus, our data suggest that S1P binding to hTERT allosterically mimicks phosphorylation, promoting telomerase stability and hence telomere maintenance, cell proliferation, and tumor growth

INTRODUCTION

Human telomerase is an RNA-dependent DNA polymerase that contains a catalytic component, hTERT (human telomerase reverse transcriptase), and an internal RNA template, TR (1, 2). Telomerase extends the ends of chromosomes and protects telomeres from replication-dependent attrition, enabling cancer cells to proliferate indefinitely by overcoming the end replication problem (3–5). Telomerase is over-expressed in >80% of all cancer types (6, 7). Inhibition of telomerase leads to telomere damage, subsequent senescence, and tumor suppression (8–11). Lamins are key structural components of the nuclear lamina, an intermediate filament meshwork that lies beneath the inner nuclear membrane, attaching chromatin domains to the nuclear periphery and localizing some nuclear envelope proteins. Fibroblasts obtained from lamin B1 mutant mouse embryos displayed premature senescence (12). In fact, in budding yeast, telomeres are reversibly bound to the nuclear envelope, and small ubiquitin-like modifier protein (SUMO)-dependent association with the nuclear periphery was proposed to restrain bound telomerase (13). Phosphorylation of hTERT increases its stability, and protein phosphatase 2 (PP2A)-dependent dephosphorylation of hTERT inhibits telomerase function (14).

The bioactive sphingolipids, ceramide and sphingosine 1 phosphate (S1P), exert opposing functions: ceramide is emerging as a tumor suppressor molecule, whereas S1P promotes tumor growth (15–19). Ceramide inhibits hTERT expression by inducing histone deacetylase 1 (HDAC1)-dependent deacetylation of Sp3 (a Sp1 family transcription factor), which represses hTERT promoter function (20). S1P is generated by cytoplasmic sphingosine kinase 1 (SK1) or nuclear SK2 (21, 22). S1P generated by SK1 promotes tumor growth and metastasis (23–25). SK1-generated intracellular S1P binds and promotes TRAF2 (TNF receptor-associated factor 2) dependent NFκB (nuclear factor κB) signaling (21). SK2-generated nuclear S1P directly binds and inhibits HDAC1 and HDAC2 (22). SK2-generated S1P binding also induces prohibitin-2 activity, leading to cytochrome-c oxidase assembly and mitochondrial respiration (26).

Considering S1P in the context of telomerase, we investigated how the binding of SK2-generated S1P alters hTERT abundance and the function of telomerase.

RESULTS

SK2-generated S1P promotes hTERT stability

To examine the possible roles of S1P in the regulation of hTERT, we determined whether down-regulation of SK1 or SK2 affected hTERT abundance or stability in human lung cancer cells. Small interfering RNA (siRNA)-mediated knockdown of SK2 but not SK1 decreased hTERT protein abundance without affecting that of its mRNA in various human lung cancer cell lines (Fig. 1A and fig. S1, A and B). Compared with controls, stable knockdown of SK2 using one of two shRNAs targeting distinct sequences decreased the abundance of hTERT in H1299 and H1650 cells (fig. S1, C and D) and hTERT stability in A549 cells treated with cycloheximide (fig. S1, E and F). These data suggested that SK2 promotes hTERT abundance and protein stability.

Like the effects of SK2 knockdown, genetic loss of SK2 promoted the degradation of hTERT protein. In the presence of CHX, ectopically expressed Flag-tagged hTERT showed decreased protein stability in MEFs from mice lacking SK2 compared to those that were wild-type or those lacking SK1 (Fig. 1B). Ectopic expression of V5-tagged wild-type SK2 (V5-SK2^{WT}), but not the catalytically inactive mutant (V5-SK2^{G212E}) (fig. S1G), prevented the degradation of hTERT-FLAG in SK2-deficient MEFs (Fig. 1C). Pharmacologic inhibition of SK2 using ABC294640 (27) reduced nuclear but not cytoplasmic abundance of 17C-sphingosine-1-phosphate (17C-S1P), an analogue of S1P that contains 17 carbons (28), after labeling cells with 17C-sphingosine, which was used as a substrate in cells (Fig. 1D and fig. S1I). Calnexin and lamin B abundance were measured as cytoplasmic and nuclear markers, respectively (Fig. 1E). Likewise, ABC294640 decreased hTERT abundance in A549, H157 and H1650 cells (Fig. 1, F and G; and fig. S1H). Together, these data so far suggest that knockdown or inhibition of nuclear SK2-generated S1P, but not SK1-generated S1P, results in decreased hTERT stability.

S1P selectively binds hTERT involving the C'3-OH group of S1P

Lipid-protein associations regulate the function and stability of various proteins (22, 29, 30). Thus, we examined whether S1P directly binds and stabilizes hTERT. To determine whether hTERT associates with endogenous S1P, we ectopically expressed wild-type, FLAG-tagged hTERT (hTERT^{WT}-FLAG) in A549 cells and measured 17C-S1P binding by immunoprecipitation for FLAG followed by lipid extraction and liquid chromatography and mass spectrometry (fig. S1J). We observed that hTERT^{WT}-FLAG was significantly associated with endogenously generated 17C-S1P compared to the vector control (Fig. 2A, and fig. S1J).

To uncover the structural details of the association between hTERT and S1P, we performed homology modeling and molecular docking studies using the X-ray structure of *Tribolium castaneum*-TERT (tcTERT) for S1P binding (31). The model was refined using amino acid alignment between tcTERT and hTERT (isoform 2, referred herein as hTERT) containing possible putative S1P-binding domains with at least two hydrophobic pockets that do not interfere with the DNA-binding and telomerase activity (fig. S2A). We then analyzed our results from the hTERT protein docking simulations using S1P as a ligand. The data

suggested that the Asp⁶⁸⁴ residue of hTERT, which is present in hTERT2 but not hTERT1, might be involved in S1P binding (Fig. 2B and fig. S2B), possibly interacting with C'3-OH of S1P. To test this model, we incubated lysates from A549 cells overexpressing hTERT with increasing concentrations of biotinylated-S1P in the presence or absence of non-labeled stereoisomers of S1P (*D-erythro*-S1P, *D-erythro*-3-O-CH₃-S1P, and *L-erythro*-S1P) (fig. S2C). Biotinylated-S1P-bound proteins were captured by avidin-conjugated agarose beads, and examined by SDS/PAGE and Western blotting using an antibody against hTERT. Biotinylated-S1P bound hTERT effectively compared to biotin control, and that presence of non-labeled *D-e*-S1P, but not *L-e*-S1P or *D-e*-3-O-CH₃-S1P, prevented the interaction between hTERT and biotinylated-S1P (fig. S2C). Incubation of A549 cell extracts with biotinylated lysophosphatidic acid, which is structurally similar to S1P but lacks the C'3-OH (fig. S2D) did not result in any detectable hTERT binding compared to biotinylated-S1P (fig. S2D). Binding between biotinylated-S1P and hTERT was also detected in H157 cells (fig. S2E). Thus, these data suggest that hTERT associates with S1P in vitro and in cells with high selectivity for the C'3-OH of S1P.

A hydrophobic region of hTERT that includes Asp⁶⁸⁴ is key for S1P binding

To examine whether the Asp⁶⁸⁴ residue is involved in S1P binding, we used a mutant hTERT with D684A conversion (hTERT^{D684A}-FLAG), which retains telomerase activity (32), comparable to hTERT^{WT}-FLAG, as measured by the polymerase chain reaction (PCR) based telomeric repeat amplification protocol (TRAP) assay (fig. S2F). Then, we examined the binding of biotinylated-S1P to hTERT^{WT}-FLAG or hTERT^{D684A}-FLAG expressed in A549 cells. The D684A mutation prevented the binding of hTERT to biotinylated-S1P compared to either hTERT^{WT}-FLAG or another mutant of hTERT (hTERT^{R669A}-FLAG) in which the mutation is in close proximity to Asp⁶⁸⁴ but was not predicted to interfere with S1P binding (Fig. 2C). To control for endogenous hTERT, we expressed hTERT^{WT}-FLAG or hTERT^{D684A}-FLAG in GM00847 fibroblasts which lack endogenous hTERT. hTERT^{WT}-FLAG but not hTERT^{D684A}-FLAG associated with biotinylated-S1P (Fig. 2D). Overall, these data suggest— as predicted by our molecular modeling— that a hydrophobic pocket, localized between the thumb and fingers domains and involving the Asp⁶⁸⁴ residue of hTERT, plays a key role in S1P binding to the C'3-OH of S1P.

S1P directly binds tcTERT and hTERT in vitro and in transfected cells

To quantify the interaction between S1P and TERT, we performed surface plasmon resonance (SPR) using S1P- or lysophosphatidic acid (LPA)-containing lipid vesicles and purified recombinant tcTERT protein. Although tcTERT does not contain the Asp⁶⁸⁴ residue, our modeling studies suggested that both tcTERT and hTERT have similar hydrophobic pockets that might be involved in S1P binding (Fig. 2B). Purified tcTERT was injected at increasing concentrations to detect binding to S1P or LPA vesicles [containing membrane phospholipids PC (phosphatidylcholine), PE (phosphatidylethanolamine) and either S1P or LPA at a ratio of 70:20:10] or control vesicles [containing POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) and POPE (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine) at a ratio of 80:20], as previously described (29, 33). Purified recombinant tcTERT bound the S1P vesicles in a concentration-dependent manner, whereas tcTERT did not bind the LPA vesicles (Fig. 2E). The dissociation constant (K_d) for

tcTERT was $\sim 4.8 \mu\text{M}$ for the S1P vesicles (Fig. 2F). Thus, these data show the direct binding between S1P and tcTERT in vitro.

To quantify the interaction between S1P and hTERT (which, unlike tcTERT, contains the integral Asp⁶⁸⁴ residue), we expressed hTERT^{WT}-FLAG and hTERT^{D684A}-FLAG in A549 cells, and partially purified hTERT using agarose beads recognizing FLAG. Partially purified hTERT was incubated with biotinylated-S1P with or without increasing concentrations of unlabeled S1P. Bound biotinylated-S1P was measured using a biotin-sensitive ELISA assay. The K_d for hTERT^{WT}-FLAG was $\sim 430 \text{ nM}$, whereas binding between biotinylated-S1P and the hTERT^{D684A}-FLAG was undetectable in the presence of 0.1–10 μM S1P (Fig. 2, G and H). The stoichiometry was calculated as 1:1 for S1P and hTERT. Collectively, these data suggest that S1P-TERT binding involves the hydrophobic domain between the thumb and finger domains of tcTERT and hTERT, and that the Asp⁶⁸⁴ residue of hTERT is essential for S1P binding. Using SPR, we also predicted that S1P may bind tcTERT in vitro at the conserved hydrophobic domain. However, this binding required much higher concentrations of purified tcTERT, because the Asp⁶⁸⁴ residue is not conserved in tcTERT.

SK2-generated S1P binds hTERT in cells

To determine whether the association between hTERT and S1P is physiologically relevant, we measured their interaction by proximity ligation assay (PLA) using antibodies that recognize S1P (34) and hTERT, respectively. DAPI staining was used to visualize nuclei. The association between S1P and hTERT was detected in the nuclei of A549 cells transfected with Scrambled control siRNA but not in those transfected with siRNA against SK2 (Fig. 3A). Inhibition of SK2 with ABC294640 attenuated the S1P-hTERT association in A549 cells (Fig. 3A). Ectopic expression of hTERT^{WT}-FLAG, but not hTERT^{D684A}-FLAG, resulted in 4.5 fold increase in S1P binding compared to vector-transfected GM00847 cells (Fig. 3B). Interactions between S1P and other nuclear proteins, such as histone deacetylase 3 (HDAC3) or SET [Su(var), Enhancer-of-zeste, Trithorax domain containing oncoprotein], used as negative controls, were undetectable in A549 cells by PLA (Fig. 3C).

S1P or hTERT colocalizes with a nuclear periphery protein Lamin B

To demonstrate the nuclear selectivity of hTERT-S1P interaction, we examined the colocalization of S1P with the nuclear membrane marker lamin B in wild-type or SK2-deficient MEFs or A549 cells using immunofluorescence microscopy. S1P was co-localized with lamin B in wild-type or control-transfected cells, respectively, but S1P was too diminished in SK2-deficient cells to detect abundance at the nuclear periphery (Fig. 3, D and E). Likewise, in transfected A549 cells, hTERT^{WT}-FLAG but not hTERT^{D684A}-FLAG co-localized with lamin B in the nucleus (fig. S3). Thus, these data suggest that, in the presence of SK2 and its product S1P, wild-type but not mutant hTERT localizes to the nuclear periphery.

S1P binding protects hTERT from ubiquitination and proteasomal degradation

To define whether binding by SK2-generated S1P affects hTERT stability by inhibiting ubiquitin-mediated proteasomal degradation, we treated A549 cells with the SK2 inhibitor ABC294640 alone or combined with the proteasome inhibitor lactacystin. Indeed, lactacystin prevented ABC294640-induced suppression of hTERT abundance (Fig. 4A). Likewise, exogenous S1P also prevented the ABC294640-induced loss of hTERT (Figure 4A). Moreover, in A549 cells, ABC294640 induced the ubiquitination of endogenous hTERT (Fig. 4B, fig. S4A) and increased the amount of HA-ubiquitin that had conjugated to exogenous hTERT^{WT}-FLAG regardless of lactacystin treatment (Fig. 4C and fig. S4B). Likewise, in wild-type MEFs, the stability of ectopically expressed mutant hTERT (hTERT^{D684A}-FLAG) was less than that of the wild-type construct in the presence of cycloheximide, an inhibitor of de novo translation (Fig. 4D). However, inhibition of proteasome activity by MG132 stabilized the abundance of hTERT^{D684A}-FLAG (Fig. 4D). Accordingly, although hTERT^{WT}-FLAG was stable in wild-type MEFs (Fig. 4E), its abundance was decreased in SK2-deficient MEFs when treated with cycloheximide, unless simultaneously treated with MG132 (Fig. 4F). Equal transfection efficiency for ectopic expression of wild-type and mutant hTERT without cycloheximide and MG132 exposure in wild-type and SK2-deficient MEFs was measured by Western blotting (fig. S4, C and D). Together, these data suggest that hTERT is degraded through the proteasomal degradation pathway, particularly in the absence of SK2.

S1P-hTERT binding prevents MKRN1-mediated degradation of hTERT

The E3 ubiquitin ligase MKRN1 interacts with the C-terminus (residues 946-1132) of hTERT (35), a region that also contains Gly⁹³², Cys⁹³¹, Phe⁹⁸⁶, His⁹⁸³, and Ser⁹⁸⁴ residues and is located within the putative S1P binding domain of hTERT (Fig. 2B and fig. S2B), suggesting that S1P-hTERT binding may disrupt MKRN1 binding to hTERT. As observed above, knocking down SK2 or inhibiting it with ABC294640 decreased hTERT stability in A549 cells, but simultaneously knocking down MKRN1 (Fig. 5A) almost completely prevented the degradation of hTERT in the presence of cycloheximide (Fig. 5, B and C). Expressing a wild-type (MKRN1^{WT}) but not a catalytically inactive RING domain mutant construct of MKRN1 (MKRN1^{H307E}) increased the ubiquitination of hTERT in the presence of MG132 (Fig. 5D), suggesting that MKRN1 ubiquitylates hTERT. In wild-type MEFs, FLAG-tagged hTERT coimmunoprecipitated with V5-tagged MKRN1, but was decreased in the presence of exogenous S1P (Fig. 5E, fig. S4E). In contrast, the addition of C3-O-CH3-S1P (D-erythro-3-O-methyl S1P, an analogue of S1P which lacks C'3-OH), which does not bind hTERT, did not inhibit the interaction between hTERT and MKRN1 (Fig. 5F and fig. S4E). The association between MKRN1 and the S1P-binding mutant hTERT^{D684A}-FLAG was also detectable, and as expected neither the presence of S1P nor C3-O-CH3-S1P had any effect (Fig. 5F, fig. S4E). Furthermore, siRNA-mediated reduction of MKRN1 abundance blunted the suppression of A549 cell growth in soft agar that was induced by SK2 knockdown (Fig. 5G). Together, these data indicate that SK2-generated S1P promotes hTERT stability and subsequent cell proliferation by inhibiting hTERT's interaction with and ubiquitination by MKRN1 and, hence, preventing its subsequent degradation.

S1P-hTERT binding regulates the endogenous MKRN1-hTERT interaction

We then determined the effects of S1P produced by SK2 on the endogenous hTERT-MKRN1 association. We expressed wild-type or a catalytically inactive mutant of SK2 (SK2^{G212E}) in A549 and H1650 cells and assessed the interaction between hTERT and MKRN1 by immunoprecipitation in the presence or absence of geldanamycin, which induces MKRN1-dependent hTERT degradation (35). Ectopic expression of wild-type but not mutant SK2 prevented the geldanamycin-induced interaction between endogenous hTERT and MKRN1 (Fig. 6A and fig. S5, A and B). In primary human lung fibroblasts (NHLF cells), we stably expressed vector or SK2^{WT}, and measured hTERT-MKRN1 association using PLA. In vector-transfected controls, there was a high degree of hTERT-MKRN1 association, which was primarily detected in the cytoplasm, and over expression of SK2^{WT} (Fig. 5, C and D) almost completely abrogated this interaction (fig. S5C). In addition, expression of wild-type but not mutant SK2 prevented the geldanamycin-induced inhibition of the growth of A549 and H1650 cells on soft agar (Fig. 6B and fig. S5E). Thus, these data demonstrate that SK2-generated S1P inhibits the association between endogenous hTERT and MKRN1.

S1P binding at Asp⁶⁸⁴ mimics phosphorylation of hTERT at Ser⁹²¹

To determine how S1P binding protects hTERT from MKRN1-mediated proteasomal degradation, we compared the stabilizing effect of S1P binding to that of the known phosphorylation modification (14). Molecular modeling and simulation data suggested that binding of S1P to hTERT at the Asp⁶⁸⁴ residue might mimic the phosphorylation of hTERT at Ser⁹²¹ (fig. S6, A and B). This was tested in experiments in which S921A (a non-phosphorylatable mutation) or S921D (a phosphomimetic mutation) conversions were introduced into the D684A mutant of hTERT to generate hTERT^{D684A-S921A}-FLAG and hTERT^{D684A-S921D}-FLAG double mutants. The colocalization of the double mutants with lamin B at the nuclear periphery, their association with MKRN1, and their stability were assessed in comparison to that of the wild-type construct (hTERT^{WT}-FLAG) in GM00847 cells. hTERT^{WT}-FLAG, but not hTERT^{D684A}-FLAG, was localized to the nuclear periphery, as visualized by confocal microscopy and immunofluorescence. Whereas hTERT^{D684A-S921A}-FLAG was mainly cytoplasmic, localization of hTERT in the lamin B-containing nuclear periphery was restored in cells expressing the phosphomimetic and S1P binding-defective hTERT^{D684A-S921D}-FLAG (Fig. 6C). Moreover, hTERT^{S921A}-FLAG, which contains intact S1P binding at Asp⁶⁸⁴, was also localized mainly in the nucleus (Fig. 6C). The MKRN1-hTERT association [measured by a proximity ligation assay (PLA)] was enhanced in cells expressing hTERT^{D684A}-FLAG but was prevented by expression of hTERT^{D684A-S921D}-FLAG (Fig. 6D). These data are consistent with the restoration of hTERT stability we observed after expression of the phosphomimetic mutant, hTERT^{S921D}-FLAG, compared to either hTERT^{D684A-S921A}-FLAG or hTERT^{D684A}-FLAG (Fig. 6E). Collectively, these data suggest that Ser⁹²¹ phosphorylation mimetic restores hTERT stability in the absence of S1P binding in cells expressing hTERT^{D684A}-FLAG. These studies also suggest that lipid binding of hTERT by S1P allosterically mimics hTERT phosphorylation at its C-termini including Ser⁹²¹, preventing hTERT-MKRN1 association, and stabilizing hTERT at the nuclear periphery.

Nuclear S1P-hTERT binding prevents telomere damage and delays senescence

We then determined whether S1P-hTERT binding has any impact on telomere damage, dysfunction, or senescence – key biological processes counteracted by telomerase. Control NHLF cells became positive for β -galactosidase (β -gal), a cell senescence marker, after ~10 passages in culture. Expressing SK2^{WT} delayed the emergence of senescence up to passage 14, but concomitantly knocking down hTERT restored senescence on the basis of β -gal positivity at passage 10 (Fig. 7, A and B; and fig. S7, A to C). We then assessed telomere integrity in these cells by detecting the complex between phosphorylated histone H2AX (γ -H2AX) and telomeric repeat-binding factor 2 (TRF2), which is recruited to the damaged or dysfunctional telomeres (36, 37), using a telomere-dysfunction induced foci (TIF) assay. Ectopic SK2 expression prevented telomere dysfunction compared to vector-transfected controls, and TERT knockdown reestablished telomere damage (Fig. 7B and fig. S7D).

Accordingly, primary SK2-deficient MEFs became senescent at passage 5, whereas senescence in their age-matched wild-type or SK1-deficient MEFs was not observed until passage 7 (Fig. 7C). Reconstitution of hTERT^{WT}, but not hTERT^{D684A} prevented telomere damage (Fig. 7D) and delayed senescence (Fig. 7E and fig. S8A) in SK2-deficient MEFs. Telomere restriction fragment (TRF) length measurement by Southern blotting showed no detectable differences in telomere lengths in wild-type or SK2-deficient MEFs at passages 3 and 6 (fig. S8B). As controls, we used genomic DNA isolated from subcutaneous A549 xenografts expressing either vector or hTERT^{WT} isolated from immunocompromised mice after 28 days of growth. Ectopic expression of hTERT lengthened telomeres in the isolated tumor cells (fig. S8C). Collectively, these data suggest that SK2 delays senescence and promotes telomere maintenance through its activity on hTERT.

SK2-generated S1P promotes hTERT stability and tumor growth

Because cellular senescence plays key roles in tumor suppression (38–40), we determined the effects of systemic versus cellular S1P produced by SK2 on the regulation of tumor growth. We measured the enlargement of Lewis lung carcinoma (LLC)-allograft-derived tumors in wild-type and SK2-deficient mice. Knocking down SK2 in LLC cells prior to implantation almost completely inhibited tumor growth compared to controls (Fig. 7F). Systemic loss of SK2 through whole-body deletion decreased the growth of control LLC allografts by ~50% compared to wild-type mice (Fig. 7F) without affecting murine TERT abundance. These data indicate that molecular targeting of tumors rather than systemic SK2 inhibits TERT expression and suppresses tumor growth in vivo, but that systemic SK2 inhibition seems to play a role in the regulation of tumor growth by an independent, unknown mechanism.

Finally, we measured the effects of stable expression of hTERT^{WT} or hTERT^{D684A} on ABC294640-mediated inhibition of A549 cell-derived xenograft-tumor growth in immunocompromised mice. hTERT^{WT} conferred resistance to ABC294640-mediated tumor suppression when compared to vector-transfected controls (Fig. 7G). However, expression of hTERT^{D684A} had no effect on ABC294640-mediated tumor suppression compared to controls (Fig. 7G). Overall, these results suggest that pharmacologically targeting SK2 in the tumor or inhibiting S1P-hTERT binding in cancer cells may suppress tumor growth.

DISCUSSION

Here, our data revealed that SK2 generated nuclear S1P directly binds TERT, and regulates hTERT stability in the lamin B positive nuclear periphery by inhibiting MKRN1-dependent hTERT ubiquitination and degradation. Binding of hTERT by S1P appears to function as an allosteric-phospho mimetic of hTERT^{S921} at its C-termini including Ser⁹²¹, which prevents MKRN1-hTERT association, stabilizing hTERT at the nuclear periphery. Nuclear S1P-hTERT binding plays important biological functions regulated by telomerase, such as protection of cells from telomere damage, delaying senescence and preventing tumor suppression. Accordingly, SK2 inhibition or prevention of S1P-hTERT binding accelerated senescence and telomere damage, and suppressed tumor growth or proliferation (Figure 7H).

Protein stability of hTERT is regulated by a direct and selective interaction between the C'3-OH of S1P and the Asp⁶⁸⁴ residue of hTERT, which is predicted to localize within a hydrophobic region between the thumb and fingers domains of hTERT. The binding of S1P to purified recombinant tcTERT (31) revealed that S1P directly binds TERT in vitro. In cells, the binding of nuclear S1P to hTERT prevented MKRN1-hTERT interaction, modulating hTERT ubiquitination and degradation. Many signaling proteins such as oncogenic RAS, hedgehog (HH) and autophagy-related Atg8 family proteins, are covalently modified with sphingolipids (30, 41) or phospholipids, which is key for their correct protein localization and function in cells (42–44). Recently, a nuclear phospholipid, phosphatidylinositol 5-phosphate (PI5P), was shown to bind and activate UHRF1 (ubiquitin-like with PHD and RING finger domains 1), a nuclear factor that maintains DNA methylation patterns during replication (45). Our data demonstrated that S1P binding prevents the association between MKRN1 and hTERT, thereby promoting the stability of hTERT. Because MKRN1 interacts with the C-terminus (residues 946–1132) of hTERT (35), lipid binding of hTERT by S1P, mimicking phosphorylation status at its C-terminus, including Ser⁹²¹, seemed to interfere physically with MKRN1-hTERT association. We believe that this is the initial discovery for a function of protein-lipid binding (hTERT binding by S1P) as an allosteric phosphomimetic to regulate a protein-protein (hTERT-MKRN1) interaction, to control hTERT stability in the nuclear periphery, leading to delayed senescence.

S1P binding-dependent hTERT stabilization altered senescence by protecting against telomere damage in primary human lung fibroblasts and wild-type MEFs, whereas SK2 loss in MEFs increased telomere damage and accelerated senescence. Furthermore, hTERT^{WT}, and not hTERT^{D684A}, protected SK2-deficient MEFs from telomere damage and delayed senescence, which seemed to be independent of telomere length control. These data are intriguing, as senescence in MEFs might not be regulated solely by telomere shortening; rather, senescence in MEFs might be controlled at least in part by induction of telomere damage. However, our inability to detect any changes of telomere length in wild-type versus SK2-deficient MEFs might be due to their extra-long telomeres (>21 kb), which are difficult to measure accurately using Southern blotting. It is also possible that non-canonical roles for S1P-bound hTERT (isomer 2) might play a role in regulating telomere damage or senescence without affecting telomere length. This, however, is unclear and needs further investigation. Interestingly, SK2-deficient mice do not exhibit any detectable obvious

phenotype due to telomerase instability or accelerated senescence. This is consistent with TERT-deficient mice, which do not show any aging phenotype for the first three generations (G1-G3), and have phenotypic changes starting G4 through G6, due to the particularly long telomeres in these animals (49). It is known that even partially reduced expression of telomerase has profound effects on telomere maintenance, as mice heterozygous for mTERT or mTERC show haplo-insufficiency in telomere maintenance (50). These data are consistent with our studies, in which mTERT/hTERT instability in response to genetic loss or molecular knockdown of SK2 resulted in accelerated telomere dysfunction, senescence and tumor suppression.

Overall, these data suggest that lipid binding of hTERT by S1P allosterically mimics hTERT phosphorylation at Ser⁹²¹, which then prevents hTERT-MKRN1-E3 ubiquitin ligase association, enhancing hTERT stability at the nuclear periphery. Increased hTERT stability at the nuclear periphery by S1P binding has important biological implications for the regulation of telomerase-dependent control of telomere damage and cellular senescence, key processes involved in aging and cancer biology. It is possible that this novel nuclear signaling mechanism mediated by interaction with S1P might regulate not only telomerase but also other proteins with diverse biological functions via protein phosphorylation mimicry.

MATERIALS and METHODS

Cell lines and culture conditions

A549, H157, H1650 cells were cultured in DMEM medium (Cellgro) with 10% FBS (Atlanta Biologics) and 1% penicillin and streptomycin (Cellgro). H1341 small cell lung cancer cells (SCLC) were cultured in RPMI-1640 (ATCC) with 10% FBS and 1% penicillin and streptomycin. GM00847 cells were purchased from Coriell repositories (New Jersey, USA). Wild-type, SK1-deficient and SK2-deficient MEFs were obtained from Dr. Kelley Argraves (Medical University of South Carolina). GM00847 cells and MEFs were cultured in DMEM media and incubated at 37°C with 5% CO₂. Primary human lung fibroblasts (NHLF) were purchased from Lonza, Inc. and cultured in fibroblast growth media as described by the manufacturer.

Plasmids

Plasmids containing hTERT^{WT} and hTERT^{D684A} in pcDNA vectors were obtained from Dr. Julian Chen (Arizona State University). MKRN1^{WT} and MKRN1^{H307E} plasmids were obtained from Dr. Mark T. Muller (University of Central Florida). SK2^{WT} and SK2^{G212E} plasmids were obtained from Dr. Sarah Spiegel (Virginia Commonwealth University). pBABE-puro (plasmid #1764), and pBABE-puro-hTERT (plasmid #1771), pMD2.G (plasmid #12259) and psPAX2 (plasmid #12260) were purchased from Addgene.

qPCR

Total RNA isolation was performed using RNeasy (Qiagen) and one µg of total RNA was used for cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad). Taqman probes

(hTERT, Hs00162669; SK1, Hs00184211; SK2, Hs00219999; MKRN1, Hs00831972), were used for qPCR (Life Technologies)

Antibodies

The antibodies used for Western blotting in this study were: hTERT (1531-1, clone Y182, Epitomics), SK2 (ab37977, Abcam), MKRN1 (ab72054, Abcam), rabbit V5 (ab9116, Abcam), Ubiquitin (3933S, Cell Signaling Technology), HA (3724, Cell Signaling Technology), Calnexin (Sc6465, Santa Cruz Biotechnology), Lamin B (Sc6216, Santa Cruz Biotechnology), mouse V5 (R96025, Invitrogen, USA).

Exogenous S1P treatment

Exogenous S1P (Avanti Polar Lipids) was suspended in 4 mg/ml fat-free BSA in 1X PBS, pH 7.4 at 125 μ M. S1P was incubated in a sonicator water bath for 5 min followed by incubation at 37°C–55°C for 20 min. D-erythro-C3-O-CH₃-S1P and L-erythro-S1P were synthesized at the Lipidomics Shared Resource Facility, Medical University of South Carolina.

Stable shRNA knockdown of SK2

SK2 shRNA (TRCN00000036973) and non-targeting shRNA containing plasmids were purchased from Open Biosystems, Inc. Cells were co-transfected with pCMV-psPAX2 and pMD2 plasmids in 293T cells using the viral transduction protocol as described by the RNAi consortium. The viral supernatants were added to A549 cells, and selection was performed using puromycin at 1 μ g/mL for 14 days. The following shRNAs were purchased from Thermo Scientific, Inc: sh-SK2#1(TRCN00000036973): CCGGCTACTTCTGCATCTACACCTACTCGAGTAGGTGTAGATGCAGAAGTAGT TTTTG; sh-SK2#2(TRCN00000036969): CCGGGCTTCGTGTCAGATGTGGATACTCGAGTATCCACATCTGACACGAAGC TTTTGTG; shSK2#3(TRCN00000036970): CCGGGTTGCTCAACTGCTCACTGTTCTCGAGAACAGTGAGCAGTTGAGCAAC TTTTGTG.

Immunoprecipitation and Western blotting

FLAG-hTERT was expressed in cells followed by treatment with CHX (Sigma) at 50 μ g/ml for up to 4 hours. Cells were lysed with the FLAG lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1mM EDTA, 1% Triton X-100) with protease inhibitor cocktail. Immunoprecipitation was performed using beads conjugated with antibody against FLAG (Anti-FLAG M2 affinity gel, A2220, Sigma). Immunoprecipitation and elution of bound proteins with FLAG beads were carried out according to manufacturer's instructions. Immunoprecipitation with hTERT antibody (4 μ g/ml) was carried out using 250 μ g total protein lysate made up to 500 μ l volume using FLAG lysis buffer. Lysates were precleared with 30 μ l of protein A/G beads and incubated in a rotary shaker for 1 hour at 4°C. Samples were spun at 1,000 rpm for 1 min and the supernatant was transferred to a new micro centrifuge tube and incubated with hTERT antibody (4 μ g/ml) overnight followed by the addition of protein A/G beads and incubation at 4°C for 1 hour. Samples were centrifuged at

1,000 rpm for 1 min and washed twice with lysis buffer and 1X PBS followed by the addition of gel loading dye. Samples were boiled using heating block and centrifuged to collect eluted proteins followed by western blotting using an antibody against hTERT. A rabbit IgG control was used alongside hTERT pulldown.

For assays in A549 cells, cells were plated at 200,000 cells per well in a 6-well plate for 18 hours prior treatment with either DMSO (vehicle control) or ABC294640 (Apogee, Inc.) at 80 μ M for 8 hours. After treatment, cells were centrifuged at 1,300 rpm for 3.5 min, and washed with 1X PBS, pH 7.4 (Gibco). Cells were lysed with 1X CHAPS lysis buffer (10 mM Tris-HCl, pH7.5, 1 mM $MgCl_2$, 1 mM EDTA, 0.1 mM Benzamidine, 5 mM β -mercaptoethanol, 0.5% CHAPS, 10% Glycerol) including a protease inhibitor cocktail (Sigma) for 20 min on ice. Cell lysates were centrifuged at 12,000 g for 15 min at 4°C, and supernatants were used for Western blotting. 4–20% SDS-PAGE was carried out using Criterion Bio-Rad apparatus followed by semi-dry transfer using PVDF membranes. The membrane was blocked with 5% milk+ 0.1% Tween-20 in 1X PBS, pH 7.4. Primary antibodies were used at 1:1000 dilution overnight at 4°C followed by rabbit or mouse secondary antibodies (Jackson Research Laboratories) conjugated with HRP (Horse radish peroxidase) at room temperature for 1 hour. The rabbit secondary antibody was used at 1:2500 dilution for detecting hTERT and 1:5000 dilution for the rest of the antibodies. Actin was used as an internal control for western blotting. The membranes were either washed with 1X PBS-T or 1X TBS-T and developed using ECL plus chemiluminescence detection kit (GE Healthcare).

17C-Sphingosine labeling and measurement of S1P

A549 cells were treated with C17-Sphingosine (5 μ M) for 30 min, and cytoplasmic and nuclear fractions were analyzed for C17-S1P formation using liquid chromatography and mass spectrometry, as previously described (28).

Homology modeling of hTERT

Currently, there is no experimental structural information available for hTERT. A model of a complete hTERT-RNA-DNA complex has been published (PMID: 21606328), and was optimized for mechanistic interactions with the RNA template and DNA. The goal of our modeling and simulation studies was to better understand the topology and chemistry of potential TERT lipid interaction sites, not to predict the overall structure or catalytic mechanism of the full-length enzyme complex. BLAST searching of hTERT (accession O14746) amino acid sequence against the PDB database indicated a highly similar sequence from *Tribolium castaneum* TERT (tcTERT) containing the RBD and RT domain. The hTERT amino acid sequence has 41% coverage and 27% identity to the tcTERT structure. To begin homology modeling, the amino acid sequence from hTERT was aligned with the amino acid sequence of the X-ray crystal structure of tcTERT. Pairwise sequence alignment was performed with BioEdit v7.0 using standard Clustal alignment parameters and the BLOSUM62 matrix. Alignment of tcTERT and hTERT indicated 127 identities and 104 similarities, giving an overall similarity of 31% (231/744). Before homology modeling the tcTERT structure was protonated at pH 7.5 and the structure was energy-minimized with heavy atoms constrained.

Alignment homology modeling was performed using MOE Homology Model. Ten models were generated and the best model selected using fine grain intermediates, GB/VI scoring and OPLS-AA Forcefield. OPLS was chosen since it tolerates small molecules like S1P better than most other energy fields (AMBER, CHARMM, MMFF). There are a series of short inserts in the hTERT that are not present in tcTERT. A large insertion is located right before the RT domain. The insert is a region of low confidence in the predicted model. Using Moe, this region was refined using a loop library. Comparison of the homology models of hTERT with tcTERT revealed that the overall RMSD divergence of the crystal structures to the homology model was 1.27 Angstroms. As expected, the highest structural divergence was centered on the largest insert of hTERT between the RBD and RT domains.

Molecular docking of hTERT with S1P

To further understand the potential regulatory mechanism of TERT, S1P was docked to hTERT. Before the simulation, S1P chirality was formalized and the molecule protonated at pH 7.5. Docking was set to probe the entire protein surface. Initial placement calculated 500 poses for S1P using Triangle Matching with London dG scoring. The top 250 poses were then refined using the Forcefield based refinement and ASE scoring. Four of the top five poses showed Asp⁶⁸⁴ within 20 Angstroms of S1P. The large number of poses and the two stage docking was done to fully explore the entire surface and place scoring emphasis on shape and hydrophobicity of the interaction. The best pose was then subjected to bimolecular energy minimization of the S1P-hTERT complex.

Detection of S1P-hTERT binding

A549 cells were lysed in 1X CHAPS lysis buffer. One mg of total protein was incubated with biotinylated-S1P (5 μ M). For cold competition assays, non-biotinylated sphingolipids were pre-incubated with cell lysates before binding reactions were carried out using biotinylated-S1P. Streptavidin beads were added to the reaction mixture, and the assay was performed as described by the manufacturer (Miltenyi Biotec) (29,30).

A549 cells transfected with the hTERT^{WT}-FLAG or hTERT^{D684A}-FLAG (or empty vector) were lysed by freezing–thawing in a lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% NP-40 with a protease inhibitor cocktail (1:500). Cell lysates (400 μ g of protein) were incubated with 150 μ l beads conjugated with an antibody against FLAG (Sigma-Aldrich) for 18 hours at 4 °C with agitation. Then, the beads were washed, and incubated with or without unlabeled S1P in the presence of increasing concentrations of biotinylated-S1P in 150 μ l binding buffer containing 50 mM Tris (pH 7.5), 137 mM NaCl, 1 mM MgCl₂, 2.7 mM KCl, 15 mM NaF and 0.5 mM NaV₃O₄ for 30 min at 30 °C. biotinylated-S1P-bound hTERT proteins were eluted with 40 μ l FLAG peptide (250 μ g/ml) and quantified using biotin-ELISA (Eagle Biosciences). The results were analyzed using the GraphPad Prism 4 software.

Binding of S1P with tcTERT using surface plasmon resonance

Surface plasmon resonance (SPR) was used to measure the kinetics of S1P-tcTERT binding in vitro. Lipid vesicles containing S1P (PC:PE:S1P, 70:20:10), LPA (PC:PE:LPA, 70:20:10) or control vesicles (POPC:POPE, 80:20) were injected with purified tcTERT (250 nM – 6

μM) at a flow rate of 30 μl/min in 10 mM HEPES, pH 7.4 containing 0.16 M KCl. All SPR measurements were performed at 25°C as previously described (29). For each experiment, POPC:POPE (80:20), used as a control surface, and S1P or LPA (10 mol% S1P or LPA) vesicles, used as active surfaces, were injected with tcTERT. Each data set was repeated three times to verify the binding of tcTERT to control or active surfaces.

Quantitative detection of S1P-hTERT binding

A549 cells transfected with Flag-tagged hTERT^{WT} and hTERT^{D684A} vectors, or empty vector were lysed by freeze–thawing in buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% NP-40 and 1:500 protease inhibitor cocktail. Lysates (400 μg of protein) were incubated with 150 μl conjugated agarose-conjugated to antibodies specific for FLAG (Sigma-Aldrich) at 4°C for 18 hours with agitation. Then, the beads were extensively washed and incubated without or with unlabeled S1P in the presence of increasing concentrations of biotinylated-S1P (starting at 250 nM) in 150 μl binding buffer. Bound hTERT proteins were eluted with 40 μl Flag peptide (250 μg/ml). Biotinylated-S1P bound to the eluted proteins was quantified using biotin-ELISA (Eagle biosciences). The results were analyzed using GraphPad Prism 4 software. The stoichiometry was determined using the following equation: $n = NKd/(L + N)$, in which $N=L/M$ (L = Free Ligand concentration, M = protein concentration, and Kd = dissociation constant).

Immunofluorescence

A549 cells (50,000 per well) were plated on glass cover slips in a 6 well plate for 18 hours. Cells were fixed and permeabilized using 4% paraformaldehyde (20 min) and 0.1% triton X-100 in 1X PBS, pH7.4 for 10 min. The cells were then blocked with 1% BSA, dissolved in 1X PBS, pH 7.4 for 1 hour. Cells were incubated for 18 hours at 4°C with antibodies specific for S1P (Sphingomab, LT1002, 20 μg/ml) mouse IgG antibody lamin B or hTERT (1:1000) in blocking solution followed by Alexa Fluor 488, Alexa Fluor 594- or Cy5-conjugated secondary antibodies (1:500) for one hour.. Immunofluorescence was performed using a Leica TSC SP2 AOBS TCS confocal or Olympus FV10i microscope with 543 nm and 488 nm channels for visualizing red and green fluorescence. Images were taken at 63X magnification. At least three random fields were selected for images.

Visualization of S1P in nuclear membranes using PLA

A549 cells, or wild-type and SK2-deficient MEFs were grown in DMEM growth media. Cells were then fixed with formalin. Fixed and permeabilized cells were incubated with an antibody specific for S1P (Sphingomab, LT1002, 20 μg/ml) and an antibody which recognizes hTERT at 4°C for 18 hours. PLA was then performed and visualized by IF-CM using the Duolink in situ hybridization kit as described by the manufacturer (Olink Biosciences).

Stable expression of vector, hTERT^{WT} and hTERT^{D684A} in A549 cells

pBabe-puro, pBabe-WT and pBabe-hTERT^{D684A} plasmids were obtained from Addgene. Plat-A amphotropic cells were plated at 70% confluence and transfected with the plasmids using the Effectene transfection reagent for 48 hours. After centrifugation of viral

supernatant at 1,250 rpm for 5 min, it was filtered through 0.45 µm filter, and viral transduction of A549 cells were performed using polybrene at 8 µg/ml. The selection of transfected cells was performed using puromycin at 1 µg/ml for 14 days. Western blotting was performed to assess the expression of hTERT.

Detection of MKRN1-hTERT interaction

Co-localization of MKRN1 and hTERT in primary human lung fibroblasts was carried out by PLA using the Duolink in situ hybridization kit (Olink Biosciences). Antibodies against MKRN1 (ab119096) (1:50) and against hTERT (ab32020) (1:50) were used for 18 hours at 4°C, and PLA signals were detected using IF-CM and the Duolink ImageTool (Olink Biosciences). Association of MKRN1 and hTERT was also detected by IP and Western blotting in A549 and H1650 cells expressing vector, SK2^{WT} and SK2^{G212E} in the absence/presence of 5 µM Geldanamycin (GA) for 4 hours.

Telomere dysfunction induced foci (TIF) assay

A549 cells and primary human lung fibroblasts were fixed using 4% formaldehyde for 20 min, and blocked with 1% goat serum in 0.3M Glycine, 1% BSA, and 0.1% Tween in 1X PBS, pH 7.4 for 2 hours. The fixed cells were then incubated with primary antibodies that recognize γ-H2AX (5 µg per ml, ab2893, Abcam) and TRF-2 (5 µg per ml, IMG-124A, Imgenex) for 18 hours. They were then incubated with secondary antibodies containing red (Alexa 568) and blue (Cy5) fluorophores against γ-H2AX and TRF-2 respectively (colocalization resulted in purple images). Images were captured using IF-CM, and co-localization was quantified using ImageJ Fiji software (51,52).

TRAP assay

Cells were lysed in 1X CHAPS buffer and total protein was quantified using Bradford's assay. One µg of total protein was used per assay reaction. Assay conditions were followed as per manufacturer's instructions (Millipore) and the reaction products were separated on 12.5% acrylamide gel under non-reducing conditions. The TRAP products on gels were then stained using SyBr green (Invitrogen).

Telomere restriction fragment (TRF) length measurement

TRF length analysis using genomic DNAs was performed using the TRF assay kit (Roche) and the Telo TAGGG probe by Southern blotting as described by the manufacturer.

Detection of senescence by SA-β-gal staining

Primary human lung fibroblasts and MEFs were used up to 17 and 6 passages respectively for the detection of senescence using the SA-beta galactosidase assay kit as described by the manufacturer (Cell Signaling Technology).

Anchorage independent growth on soft agar

Cells were grown in 2X DMEM and 5% agar at a ratio of 9:1. Around 20,000 cells per well were grown in agar media mix and incubated at 37°C. DMEM was changed for cells every 3 days for 14–21 days.

Animal xenograft studies

SCID mice were purchased from Harlan Laboratories. Age and sex matched mice were used. All animal protocols were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina. A549 cells (2×10^6) that stably transfected with control shRNA or shRNA targeting SK2 were implanted into the flanks of SCID mice (n=4 mice, each containing 2 tumors on both flanks). When the tumors were palpable, the mice were treated daily with either vehicle control or ABC294640 by oral gavage at 100 mg per kg body weight for 21 days. Tumor volume was measured using digital calipers. At the end of the 21 days of treatment, these mice were euthanized and tumor tissues were collected. Similarly, Lewis lung carcinoma (LLC) cells stably transfected with shRNA against SK2 or control (scrambled) shRNA were injected subcutaneously into either wild-type or SK2-deficient C57/BL6 mice (n=4 mice, each containing 2 tumors on both flanks). Allografted tumor volumes were measured using digital calipers every three days for 14 days.

Statistical analysis

All data are presented as means $\pm$ SD, and group comparisons were performed with a two-tailed Student's *t* test. $P < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. SK2-generated S1P regulates hTERT protein abundance and stability

(A) Endogenous hTERT protein abundance in A549 cells transfected with SK1 or SK2 siRNA. (B) Immunoprecipitation for FLAG then Western blotting for hTERT in lysates from wild-type, SK1-deficient or SK2-deficient MEFs expressing FLAG-hTERT or vector in the absence/presence of CHX (50µg/ml, 0 to 4 hours). Densitometry from 3 blots shown below. (C) Quantification of FLAG pulldown then Western blotting for hTERT in lysates from SK2-deficient MEFs co-transfected with hTERT^{WT}-FLAG and either SK2^{WT} or SK2^{G212E} with or without CHX for 4 hours. (D) Effects of ABC294640 on 17C-S1P formation in the nuclear (Nuc) and cytoplasmic (Cyto) fractions of A549 measured by liquid chromatography/mass spectrometry. (E) Cytoplasmic and nuclear fractionation of A549 cells were performed after treatment with ABC294640, and analyzed by Western blotting using antibodies against lamin B and calnexin, nuclear and cytoplasmic markers, respectively. (F and G) Effects of ABC294640 treatment (80 µM) on hTERT abundance in A549 (F) or H157 and H1650 cells (G) at 0 to 8 hours (h) measured by Western blotting. In all panels, blots are representative of 3 independent experiments, and data are means ± SD from 3 independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001 by a Student's *t*-test.

Fig. 2. SK2-generated S1P interacts with hTERT by lipid-protein binding

(A) Ectopic expression of hTERT^{WT}-FLAG in A549 cells (right) treated with 17C-sphingosine was measured by immunoprecipitation for the FLAG tag and assessed for bound 17C-S1P (data in fig. S1J). (B) Overlay of the hTERT model with crystal structure of tcTERT. Distinct domains of TERT are shown in color and the S1P interacting residue Asp⁶⁸⁴ located at the interface of the palm and thumb domains is in red stick. (C and D) Binding of biotinylated-S1P (B-S1P) to FLAG-tagged wild-type or mutant hTERT in A549 cells (C) or GM00847 cells (D) assessed by avidin bead pulldown then Western blotting for hTERT. (E and F) In vitro binding of tcTERT to either a S1P [POPC:POPE:S1P (70:20:10)] or LPA [POPC:POPE:LPA (70:20:10)] vesicles relative to the control [POPC:POPE (80:20)] (E). K_d analysis of tcTERT interaction with S1P vesicles (F). (G and H) In vitro binding of biotin-tagged S1P to FLAG-tagged wild-type or D684A mutant

hTERT (0.4 mg/ml) partially purified from A549 cells. In all panels, data are means $\pm$ SD from 3 independent experiments. * P <0.05, ** P <0.01, *** P <0.001 by a Student's t-test.

Fig. 3. Interaction of hTERT with SK2-generated S1P colocalizes with lamin B at the nuclear periphery

(A) PLA detection of the subcellular localization of the S1P-hTERT interaction in A549 cells either transfected with control (Scr) or SK2 siRNA (upper) or treated with vehicle or ABC294640 (lower). Nuclei were counterstained with DAPI. Scale bar, 20 μ m. (B) PLA detection of S1P-hTERT binding in GM00847 cells transfected with vector, hTERT^{WT}-FLAG or hTERT^{D684A}-FLAG. (C) PLA for S1P binding to hTERT compared to HDAC3 or SET (nuclear proteins) in A549 cells. (D and E) Colocalization of S1P (red) and lamin B (green) in the nucleus assessed by immunofluorescence confocal microscopy in wild-type or

SK2-deficient MEFs (D) or A549 cells transfected with control or SK2 shRNA. Scale bar, 100 μm . In all panels, images are representative of 3 independent experiments, and data are means $\pm$ SD from 3 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by a Student's t-test.

Fig. 4. S1P-hTERT binding prevents the ubiquitination and proteasomal degradation of hTERT (A) Western blotting for hTERT to assess protein stability in A549 cells pretreated with S1P, lactacystin or vehicle followed by the SK2 inhibitor ABC294640. (B and C) Pulldown for hTERT then Western blotting for ubiquitin showing the effects of ABC294640 on the ubiquitination of endogenous hTERT in A549 cells (B) or of FLAG-hTERT in the presence of protease inhibitor lactacystin in A549 cells expressing HA-ubiquitin (C). (D) Pulldown for FLAG then Western blotting for hTERT showing the stability of FLAG-tagged wild-type or mutant (D684A) hTERT in the presence or absence of cycloheximide (CHX) or MG132 in wild-type MEFs. (E and F) Pulldown for FLAG then Western blotting for hTERT showing the stability of FLAG-hTERT^{WT} expressed in wild-type (E) or SK2-deficient MEFs (F) in the presence of CHX alone or with MG132. In all panels, blots are

representative of 3 independent experiments, and data are means $\pm$ SD from 3 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by a Student's t-test.

Fig. 5. Binding of SK2-generated S1P protects hTERT from MKRN1-mediated degradation (A) MKRN1 knockdown in A549 cells stably transfected with control (Scr) or SK2 shRNA. (B and C) Western blotting for endogenous hTERT stability after MKRN1 knockdown in stable shScr and shSK2 A549 cells treated with CHX (B) or ABC294640 (C) for 4 hours (h). In (C), bottom blot is actin loading control. (D) Pulldown for FLAG then Western blotting for ubiquitin in wild-type MEFs coexpressing FLAG-hTERT and either wild-type (Wt) or RING mutant (H307E) MKRN1 and pretreated with MG132 for 2 hours. (E) Coimmunoprecipitation of FLAG-hTERT with V5-MKRN1 in A549 cell extracts in the presence of S1P or LPA (5 μ M). Samples shown are from the same representative blot but not in contiguous lanes. (F) Coimmunoprecipitation of FLAG-tagged wild-type (Wt) or mutant (D684A) hTERT with MKRN1 in A549 cell extracts in the presence of S1P or C3-

O-CH3-S1P. **(G)** Effect of siRNA-mediated MKRN1 knockdown on the anchorage-independent growth on soft agar exhibited by A549 cells stably transfected with SK2 or control (Scr) shRNA. In all panels, blots are representative of 3 independent experiments, and data are means $\pm$ SD from 3 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, by a Student's t-test.

Fig. 6. SK2-generated S1P prevents hTERT-MKRN1 interaction by mimicking hTERT phosphorylation at Ser⁹²¹

(A and B) Effects of wild-type or catalytically inactive (G212E) SK2 on endogenous hTERT-MKRN1 interaction (A) and proliferation on soft agar (B) in A549 cells, either untreated or treated with GA. (C) Colocalization of FLAG-tagged wild-type or mutant hTERT (red) with lamin B (green) detected by immunofluorescence confocal microscopy in GM00847 cells. Scale bar, 20 μ m. (D) PLA detection of the interaction between MKRN1 and FLAG-tagged wild-type or mutant hTERT in GM00847 cells. (E) Western blotting to assess the stability of wild-type or mutant hTERT in the presence or absence of cycloheximide (CHX). In all panels, images are representative of 3 independent experiments, and data are means $\pm$ SD from 3 independent experiments. * P <0.05, ** P <0.01, *** P <0.001, by a Student's t -test.

Fig. 7. SK2-generated S1P-hTERT plays key roles in the control of senescence, telomere damage and tumor growth

(A) β-Gal staining in PLFs (at passages 7–17) that stably coexpress pCDH and control shRNA (shScr), or SK2 and either shScr or shTERT. (B) Telomere damage assessed by the TIF assay (γ-H2AX and TRF2 colocalization) in PLFs at passage 12, cotransfected as in (A). (C) β-Gal staining in SK2-deficient or wild-type MEFs at passage 5. Positive cells were counted from 3 to 4 fields. (D and E) Telomere damage assessed by the TIF assay (D) and senescence by β-Gal detection (E) in SK2-deficient MEFs transfected with wild-type (Wt) or mutant (D684A) hTERT. (F) Volumes of allograft tumors derived from LLC cells stably transfected with shScr or shSK2 in the flanks of aged-matched wild-type or SK2-deficient mice. (G) Volumes of xenograft tumors derived from A549 cells stably transfected with vector, hTERT^{WT} or hTERT^{D684A}, in mice treated with vehicle or ABC294640 for 21 days. In panels (A to E), data are means ± SD from 3 independent experiments; in (F and G), data

are means $\pm$ SD from 4 mice each, each containing two tumors. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by a Student's t-test. **(H)** A model of our findings, revealing a nuclear lyso-phospholipid-mediated mechanism through which S1P binding to a pin-pointed region in hTERT functions as an allosteric phosphomimetic and stabilizes hTERT. Various ways to reduce S1P abundance or binding may induce the degradation of hTERT and, in turn, the acceleration of telomere damage and senescence in tumors.