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Defining a role for sphingosine kinase 1 in p53-dependent tumors

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Abstract

p53 is a crucial tumor suppressor that is mutated or deleted in a majority of cancers. Exactly how p53 prevents tumor progression has proved elusive for many years; however, this information is crucial to define targets for chemotherapeutic development that can effectively restore p53 function. Bioactive sphingolipids have recently emerged as important regulators of proliferative, apoptotic and senescent cellular processes. In this study, we demonstrate that the enzyme sphingosine kinase 1 (SK1), a critical enzyme in the regulation of the key bioactive sphingolipids ceramide, sphingosine and sphingosine-1-phosphate (S1P), serves as a key downstream target for p53 action. Our results show that SK1 is proteolysed in response to genotoxic stress in a p53-dependent manner. p53 null mice display elevation of SK1 levels and a tumor-promoting dysregulation of bioactive sphingolipids in which the anti-growth sphingolipid ceramide is decreased and the pro-growth sphingolipid S1P is increased. Importantly, deletion of SK1 in p53 null mice completely abrogated thymic lymphomas in these mice and prolonged their life span by ~30%. Deletion of SK1 also significantly attenuated the formation of other cancers in p53 heterozygote mice. The mechanism of p53 tumor suppression by loss of SK1 is mediated by

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elevations of sphingosine and ceramide, which in turn were accompanied by increased expression of cell cycle inhibitors and tumor cell senescence. Thus, targeting SK1 may restore sphingolipid homeostasis in p53-dependent tumors and provide insights into novel therapeutic approaches to cancer.

Keywords

Ceramide; sphingosine-1-phosphate; p53; sphingosine kinase; cancer; senescence

Introduction

Cancer is a complex process in which aberrant growth signals lead to the expansion of damaged cells that would normally not proliferate. Moreover, discerning the intricate molecular mechanisms that lead to tumor growth should enable the development of more effective cancer therapies that target them (Kang *et al.*, 2005; Lees and Weinberg, 1999). One class of molecules that have emerged as critical regulators of several cellular processes of direct relevance to cancer, including growth, differentiation, apoptosis, and senescence are the bioactive sphingolipids (Hannun and Obeid, 2008; Pruett *et al.*, 2008; Pyne and Pyne, 2010). Dysregulation of sphingolipid metabolic pathways has been demonstrated in several cancers, suggesting that alterations in sphingolipid metabolism may represent an important step in tumorigenesis (Cuvillier and Levade, 2003; Long *et al.*, 2010; Ryland *et al.*, 2011; Watson *et al.*, 2010).

SK1 holds a crucial position in sphingolipid metabolism with its function required for homeostatic processing of the pro-apoptotic sphingolipids, ceramide and sphingosine. Moreover, the product of SK1, sphingosine-1-phosphate (S1P), is not only a metabolic intermediate required for exit from the sphingolipid metabolic pathway, but has been shown to act as a pro-growth signal in stark contrast to the functions of its upstream sphingolipid family members (Pitson, 2010). Thus, it appears that SK1 acts as a balance between pro-death and pro-growth bioactive signaling lipids and, intuitively, alterations in this enzyme's activity could be involved in carcinogenesis. Indeed, previous work from our lab showed that SK1 message levels are universally increased in various types of human cancers (Johnson *et al.*, 2005). Furthermore, multiple independent studies have shown that the sphingosine kinase 1 (SK1)/sphingosine-1-phosphate (S1P) pathway plays a role in cancer cell growth and viability and in the resistance of tumors to chemotherapy (Akao *et al.*, 2006; Bayerl *et al.*, 2008; Li *et al.*, 2009; Long *et al.*, 2010; Pchejetski *et al.*, 2008; Watson *et al.*, 2010).

Similar oncogenic properties have long been attributed to the loss of the tumor suppressor p53 (also known as TP53), which has been the most studied tumor suppressor protein due to its involvement in more than 50% of human cancers (Soussi, 2007; Vogelstein B, 2000; Weisz *et al.*, 2007). Since alteration of p53 signaling has been recognized as a central component of human carcinogenesis, nearly 20 different types of genetically modified mice targeting p53 in various ways have been made over the past twenty years to aid in the study of this medically relevant tumor suppressor (Soussi, 2007; Zilfou and Lowe, 2009). The

most common phenotype observed in mice which lack both p53 alleles is decreased survival due to their development of thymic lymphoma, whereas in mice which lack only one p53 allele, osteosarcomas and soft tissue sarcomas predominate, and animal survival is not as dramatically decreased as in complete knockout (KO) animals (Donehower *et al.*, 1992; Jacks *et al.*, 1994; Lozano). Thus, p53 deficient mice can serve as an appropriate *in vivo* model for the study of carcinogenesis in multiple tissues.

Interestingly, some studies have suggested a link between the p53 tumor suppressor protein and bioactive sphingolipids, especially ceramide and sphingosine 1-phosphate (S1P) (Lopez-Marure *et al.*, 2000; Oskouian *et al.*, 2006; Pruschy *et al.*, 1999) as reviewed in (Heffernan-Stroud and Obeid, In press). Genotoxic stress has been shown to induce the pro-apoptotic sphingolipid, ceramide in a p53-dependent manner (Dbaibo *et al.*, 1998), and, indeed, several inducers of apoptosis have been shown to increase ceramide in cells concomitant with p53 activation (El-Assaad *et al.*, 2003; Kim *et al.*, 2002; Sawada *et al.*, 2004). Moreover, we found that treatment of Molt-4 T-cell leukemia cells with the DNA damaging agent Actinomycin D results in degradation of SK1, the key enzyme that regulates the balance between ceramides, sphingosine, and S1P, and these effects were not observed in cells overexpressing the papilloma virus E6 protein which targets p53 to degradation, suggesting a role for p53 in this process (Taha *et al.*, 2004). Furthermore, Oskouian *et al.* showed that overexpression of the enzyme that breaks down S1P, S1P lyase, can potentiate an apoptotic response to DNA damage in a p53-dependent manner (Oskouian *et al.*, 2006). These studies suggest that some of the enzymes controlling ceramide and S1P levels in cells may act as effectors/modulators of the p53 tumor suppressor pathway; however, the mechanisms and significance of these relations to sphingolipids are not known.

Because SK1 message levels are universally increased in various types of cancers (Johnson *et al.*, 2005) and p53 may regulate SK1 in response to genotoxic stress in leukemia cells, we hypothesized that dysregulation of the SK1/S1P pathway could be involved in p53-altered cancers. To test this hypothesis, we evaluated the p53-dependent regulation of SK1 in mouse non-cancer cells as well as SK1 expression in p53 null tumors. Next we evaluated the effect of genetic ablation of SK1 in p53 knockout (KO) mice on spontaneous tumor development and survival to determine if restoration of the sphingolipid profile could offer protective tumor cell senescence. Finally, we expanded these studies to include other tissue/tumor types by crossing SK1 knockout (KO) mice with p53 heterozygote mice and assessing the effects of SK1 gene dosage on tumorigenesis and survival.

Results

p53 negatively regulates SK1

First, we set out to investigate a mechanistic connection among genotoxic stress, p53, and SK1. To this end, we utilized mouse embryonic fibroblasts (MEFs) from WT and p53 KO mice and evaluated the effects of UV irradiation on SK1. We found that induction of p53 was required for a reduction in SK1 protein to occur following genotoxic stress (Figure 1a), thus, establishing p53 as an upstream regulator of sphingolipid metabolism. These studies also revealed that p53-dependent downregulation of SK1 was occurring at the protein, rather than message level (Figure 1a,b). Additionally, we were able to inhibit the effects of p53-

mediated genotoxic stress on SK1 with a caspase-2 inhibitor, suggesting proteolytic degradation as the mechanism of p53-dependent SK1 regulation (Figure 1c) as is consistent with previous studies suggesting regulation of SK1 through proteolysis (Loveridge *et al.*, 2010; Taha *et al.*, 2005). This finding is also in keeping with several reports showing p53-dependent activation of caspase-2 (Baptiste-Okoh *et al.*, 2008; Cuenin *et al.*, 2008; Ho *et al.*, 2009; Vakifahmetoglu *et al.*, 2008). We found that genotoxic stress led to p53-dependent downregulation of SK1, which, in turn, decreased the pro-growth signaling product of SK1, S1P (Figure 1d). Importantly, this downregulation of SK1 also led to the accumulation of the upstream, anti-growth signaling sphingolipid, ceramide (Figure 1d). This regulation of SK1 and these potentially tumor-suppressive changes in sphingolipid signaling molecules required p53 (Figure 1d), and could constitute an important component of the p53 tumor suppressor pathway.

In light of these results, we hypothesized that cells lacking p53 would lack proper regulation of SK1. Indeed, treating WT, p53 KO, SK1 KO, and DKO MEFs with labeled substrate for SK1, C17-sphingosine, showed increased production of C17-S1P in the p53 KO cells (Figure 2a). These studies also confirmed that the main enzyme responsible for S1P production in these cells is SK1 rather than SK2, since very little S1P was produced in cells lacking SK1 (Figure 2a). Next, we investigated whether p53 was required for the regulation of SK1 *in vivo*. Because p53 KO mice develop thymic lymphomas (Donehower *et al.*, 1992; Jacks *et al.*, 1994), we compared SK1 expression and activity in the thymus glands of WT and p53 KO mice at 60 days of age, a time during which there is negative selection and significant cell turnover in the developing murine thymus. Consistent with our cellular studies, we observed no differences in SK1 message levels between WT and p53 KO thymus glands (Supplementary Figure 1). In contrast, using immunohistochemistry, we observed more intense staining for SK1 protein in tissues lacking regulatory p53 when compared to those with WT p53 (Figure 2b). In addition, SK1 activity was significantly increased in tissues lacking p53 (Figure 2c), demonstrating that SK1 is deregulated which can lead to changes in sphingolipid metabolism that signal for tumor-promotion in the thymic tissue of p53 KO mice.

Spontaneous tumor formation in p53 KO mice requires SK1

Next, we investigated the functional consequences of p53 effects on SK1. Ceramide exerts apoptotic and growth-suppressive effects (Jayadev *et al.*, 1995; Kolesnick and Fuks, 2003; Obeid *et al.*, 1993), whereas S1P exerts opposing anti-apoptotic and pro-growth effects (Lee *et al.*, 1998; Olivera *et al.*, 1999; Olivera and Spiegel, 1993); indeed, ceramide has been proposed as a tumor suppressor lipid and S1P as a tumor-promoting lipid (Cuvillier *et al.*, 1996; Hannun and Obeid, 2008). Therefore, to determine if SK1 deregulation in p53 KO mice contributes to tumor development, we crossed p53 KO mice (Jacks *et al.*, 1994) with SK1 KO mice (Allende *et al.*, 2004) to ascertain whether enforced SK1 downregulation was protective against carcinogenesis. We initially focused on thymic lymphoma as the major and most consistent tumor in p53 KO mice (Donehower *et al.*, 1992; Jacks *et al.*, 1994). The results revealed a significant and gene-dosage-dependent effect in which tumor burden was decreased in p53 KO mice lacking a single SK1 allele. Remarkably, p53 KO mice lacking both SK1 alleles were almost totally protected from thymic lymphoma development,

compared to p53 KO mice expressing WT SK1 (Figure 3a). In necropsy studies, thymic mass decreased with loss of SK1 expression in p53 KO mice (Figure 3b), and histological evaluation confirmed protection from thymic lymphoma in the DKO mice (Figure 3c). Such protection from carcinogenesis in the DKO mice led to increases in median survival by more than 36%, compared to p53 KO mice expressing both SK1 alleles (Figure 3d). These results suggest that deregulation of SK1 in p53 KO mice may contribute to early tumor development and that SK1 knockout could protect these mice from thymic lymphoma development thereby increasing survival.

Decreased tumor burden in p53-SK1 double KO mice via induction of cellular senescence

To investigate how knockout of SK1 protects p53 KO mice from the development of thymic lymphoma, we first measured sphingolipids in thymus glands from mice with varied p53 and SK1 expression including p53 WT SK1 WT (WT), p53KO SK1 WT (p53 KO), p53 WT SK1 KO (SK1 KO), and p53 KO SK1 KO (DKO). Consistent with deregulated SK1 activity (Figure 2c), thymic tissue from p53 KO mice had decreased upstream sphingolipid ceramide and increased S1P, the product of SK1 (Figure 4a). The p53 KO thymus glands also had increased sphingosine (Figure 4a), which could indicate decreased activity of a ceramide synthase, but most likely indicates increased ceramide breakdown by a ceramidase (Supplementary Figure 1). As expected, thymus glands from SK1 KO mice contained significantly less S1P and more sphingosine than those from WT mice (Figure 4a). Of note, knockout of SK1 suppressed the pro-growth signaling sphingolipid, S1P, in the p53 KO mice to that of WT levels (Figure 4a). Likewise, knockout of SK1 also restored the levels of the anti-growth ceramide to WT levels in the thymic tissue of DKO mice (Figure 4a), demonstrating a role for SK1 in maintaining ceramide homeostasis. Taken together, these results demonstrate significant effects of p53 (and its loss) on the levels of bioactive sphingolipids. The results also implicate SK1 in mediating these changes.

Similar changes were also observed for sphingolipids in the blood of these mice (Supplementary Figure 2). Thus, knockout of SK1 seemed to function to re-balance these two bioactive sphingolipids to WT levels in p53 KO tissues. However, sphingosine was significantly increased in DKO tissue compared to either KO mouse alone (Figure 4a middle panel). Again these changes are consistent with decreased phosphorylation of sphingosine with knockout of SK1, but also suggest an increase in ceramide catabolism with knockout of p53. Overall, our data suggest that knockout of SK1 protects p53 KO mice from tumor development by restoring dysregulated ceramide and S1P in these tissues to those of WT levels.

To determine the pathophysiologic significance of bioactive sphingolipid dysregulation, we analyzed thymic tissue from mice of the four genotypes for apoptosis and senescence, functions known to be reciprocally regulated by ceramide and S1P (Hannun and Obeid, 2008; Taha *et al.*, 2006). First, we suspected that a decrease in ceramide and the increase in S1P in p53 KO thymus glands may predispose these mice to carcinogenesis as a result of an inadequate apoptotic response. However, we did not find a significant difference between genotypes in the percentage of apoptotic cells present in the thymus glands (Figure 4b). When split into T-cell subtypes, p53 KO thymi had decreased apoptosis of double-positive T

cells, but this was offset by increased apoptosis of double-negative and single-positive $CD8^+$ cells (Supplementary Figure 3). However, such differences in apoptosis among the cell subtypes appeared as minor differences in overall cell population between genotypes rather than differential susceptibility to apoptosis (Supplementary Figure 3). Therefore, we concluded that a decrease in apoptosis was not responsible for the increased thymus size observed in p53 KO mice. This conclusion is further supported by an elegant study in which an apoptosis-deficient p53 mutant mouse that retained p53-dependent cell cycle arrest and senescence was still protected from thymic lymphoma development, thus ruling out a role for apoptosis in the tumor suppressive action of p53 (Liu *et al.*, 2004). In contrast, evaluation of expression of p16 and p21, regulators of cell cycle arrest and senescence (Alesse *et al.*, 1998; Bringold and Serrano, 2000; el-Deiry *et al.*, 1994; Lukas *et al.*, 1995) revealed differential expression of p21 and p16 in the thymi of the various genotypes (Figure 4c). These proteins are known to be regulated by sphingolipids such that ceramide induces their expression (Lee *et al.*, 2000; Venable and Obeid, 1999). Quantitatively, levels of p21, and to a slightly lesser extent p16, closely correlated with the corresponding changes in both ceramide and S1P observed in these tissues (Supplementary Figure 4), thus, expression of p16 and p21 was decreased in the p53 KO, increased in the SK1 KO, and reverted to near WT levels in the DKO model. These results suggest that the predominant effect of SK1 loss is to restore ceramide and S1P levels and consequently the expression of p16 and p21, which are suppressed in tissues lacking p53.

Because both p16 and p21 are important mediators of cell senescence (Jung *et al.*), we next evaluated thymus senescence. The results showed that staining for senescence-associated β-galactosidase was profoundly more intense in the DKO tissues than what was observed in the p53 KO tissues (Figure 4d). These results clearly suggest that induction of senescence is a major response of the DKO cells. Interestingly, the β-galactosidase staining correlated best with the levels of sphingosine (Figure 4a), which has previously been associated with senescence pathways (Chao *et al.*, 1992; Debacq-Chainiaux *et al.*, 2010; Yoon *et al.*, 2009). Collectively, these results suggest that by correcting ceramide and S1P levels, and possibly increasing sphingosine, knockout of SK1 rectifies the expression of cell cycle inhibitors and induces a strong cellular senescence response to prevent tumor development in p53 KO tissues.

Multiple p53-dependent tumor types are reliant on SK1

Next, we wanted to investigate whether the protective effect of SK1 KO extended to other p53-dependent tumor types in addition to thymic lymphoma. Unlike the aggressive thymic lymphomas observed in p53 KO mice, p53 heterozygote mice tend to develop sarcomas and splenic lymphomas and survive longer than p53 KO mice (Jacks *et al.*, 1994). Therefore, we crossed p53 heterozygote mice with SK1 KO mice to create p53 +/− SK1 +/+, p53 +/− SK1 +/−, and p53 +/− SK1 −/− mice and monitored them for tumor development. The results showed that decreased expression of SK1 protected against tumor development in p53 heterozygote mice (Figure 5a), with knockout of SK1 increasing the percent of p53 heterozygote mice surviving tumor-free to 42.9% (compared to 7.7% for those expressing both SK1 alleles). Knockout of SK1 offered substantial protection from the development of lymphoma, osteosarcoma, lung adenocarcinoma, and rhabdomyosarcoma in the p53

heterozygote mice (Figure 5a). Importantly, this decreased tumor burden observed in p53 heterozygote mice lacking SK1 resulted in a 27% increase in their median survival when compared to those with WT SK1 (Figure 5b). These results indicate a more generalized role for SK1 in p53-induced carcinogenesis that applies to several tissues and types of tumors.

Interestingly, analysis of spleens from WT mice and p53 +/− SK1 WT mice with and without tumors, and spleens from p53 +/− SK1 KO mice revealed that ceramide levels best matched the expression of cell cycle inhibitors. Whereas normal spleens from p53 heterozygous mice contained less ceramide than WT spleens, this decrease was more profound in tumor tissues from afflicted spleens in the p53 heterozygous mice (Figure 5c). Importantly, knockout of SK1 in p53 heterozygote mice restored ceramide to near WT levels (Figure 5c). These differences in ceramide, in turn, closely matched the expression of the cell cycle inhibitors p21 and p16 (Figure 5d), suggesting that knockout of SK1 protects p53 heterozygote mice from tumors through a mechanism similar to that within p53 KO mice. These data suggest that restoring the downregulation of SK1, as seen with WT p53, can, in turn, restore ceramide and cell cycle inhibitor expression to protect p53-deficient mice from the development of various types of tumors, including osteosarcoma and splenic lymphoma as well as thymic lymphoma.

Discussion

This work has identified a novel connection between the p53 pathway and its regulation of sphingolipid metabolism. The results show that p53, through its activation of caspase-2, negatively regulates SK1 in response to genotoxic stress, and that loss of p53 results in significant changes in bioactive sphingolipids through SK1 leading to increased S1P levels and decreased ceramide. Functionally, knockout of SK1 significantly increased survival and decreased tumor burden in both p53 KO and p53 heterozygote mice, demonstrating that p53-dependent regulation of SK1 is essential for its tumor suppressive effects. This improved survival in p53-deficient mice with knockout of SK1 was associated with restoration of sphingolipid homeostasis (decreased S1P, increased ceramide) and induction of cellular senescence in the thymuses of these mice.

There is an evolving body of literature supporting a role for SK1 in cancer pathogenesis (Bergelin *et al.*, 2009; Pitson *et al.*, 2005; Vadas *et al.*, 2008; Xia *et al.*, 2000). SK1 message level is increased in several tumor types (Akao *et al.*, 2006; Bayerl *et al.*, 2008; Johnson *et al.*, 2005; Kawamori *et al.*, 2006), and knock down of SK1 was shown to be protective from the development of colon cancer in an experimental model of azoxymethane-induced colon carcinogenesis (Kawamori *et al.*, 2009). Moreover, it has also been suggested that knockdown of SK2 can slow the growth of breast cancer xenografts through an immune-mediated process (Weigert *et al.*, 2009). Therefore several groups are currently working on the production and characterization of selective SK1 and SK2 inhibitors so that their specific roles in anti-cancer therapies may be better understood (Hengst *et al.*, 2010; Mathews *et al.*, 2010). Recent studies investigating the application of SK1 inhibitors as anti-cancer agents have proved promising (Paugh *et al.*, 2008). Although not yet commercially available, the same group found that treatment with their SK1 inhibitor could slow the growth of glioblastoma xenografts through the inhibition of Akt (Kapitonov *et al.*, 2009). Interestingly,

some recently developed SK1 inhibitors work through proteasomal degradation of SK1 (Loveridge *et al.*, 2010), further supporting that proteolysis may be an important mechanism of SK1 regulation as suggested by our study. As selective inhibitors are developed and made commercially available, their *in vivo* optimization will allow for investigation as to whether the tumor-protective effect of SK1 knockout found in this study can in fact be mimicked pharmacologically.

Finally, in this report, we uncover tumor cell senescence as a novel mechanism by which loss of SK1 is protective from tumor development. Moreover, the associated changes in sphingolipid profile and protein expression could mechanistically explain the increased proliferation in p53 KO tumors and the protective senescence response in DKO mice. The results clearly demonstrate significant derangement in ceramide, sphingosine, and S1P levels in p53 knock out cells and tissues and in p53 KO-induced tumors. These changes occur in directions known to promote carcinogenesis (decreased ceramide and increased S1P) (Hannun and Obeid, 2008; Ogretmen and Hannun, 2004; Taha *et al.*, 2006). Together with previous studies demonstrating the p53-dependent effects on ceramide in human leukemia and neuroblastoma cell lines as well as in mouse fibroblasts (Dbaibo *et al.*, 1998; Kim *et al.*, 2002), our data suggest that the major effect of p53 on sphingolipid metabolism is to induce proteolysis of SK1 (Taha *et al.*, 2004), which subsequently leads to ceramide accumulation and loss of S1P. Because SK1 knockout could potentially be mimicked by treatment with a small molecule inhibitor, this method of inducing tumor cell senescence may offer a promising therapeutic avenue for patients with altered p53 status, which has proved to be an elusive aspect of cancer for many years.

Materials and Methods

Reagents

Actinomycin D and Etoposide were purchased from Sigma. For western blots (specific procedures detailed in SI Materials and Methods) we used complete-mini protease inhibitor tablets (Roche, Indianapolis, IN, USA), ripa buffer (Boston Bioproducts, Ashland, MA, USA), BCA protein assay (Pierce, Rockford, IL, USA), and precast 10% Tris-HCl gels (BioRad, Hercules, CA, USA). SA-β-galactosidase staining kits were purchased from Cell Signaling (Danvers, MA, USA). SK1 primary antibodies were custom made for our laboratory either at MUSC (human) or by BioSource/Fisher (mouse). Other primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-conjugated secondary antibodies were obtained from Jackson Labs (Bar Harbor, ME, USA), except for rabbit secondary which was purchased from Santa Cruz Biotechnology.

Cell Culture

Following harvest, mouse embryonic fibroblasts (MEFs) were maintained in Dulbecco's Modified Eagles Medium (DMEM) containing high glucose (Gibco), supplemented with L-glutamine, 10% (v/v) fetal bovine serum (Gibco or Hyclone) and 1% (v/v) antimycotic-antibiotic solution (Gibco). Cells were kept in a humidified incubator at 37 °C with 5% CO_2 Where indicated, UV irradiation of cells was performed using a Biorad GS Gene Linker cabinet that emits UVC light (λ = 253.7 nm).

Animals

Under our protocol, ARC# 2690, which was approved by the MUSC IACUC, we created a breeding colony of C57BL/6 mice with varying p53 and SK1 status. The SK1 KO mice were originally obtained from Dr. Rick Proia (Allende *et al.*, 2004) crossbred with WT C57BL/6 mice in the DLAR at MUSC and maintained as an SK1 KO colony. Two male p53 heterozygote mice were obtained from the laboratory of Dr. C. Schweinfest and bred as previously described (Jacks *et al.*, 1994) and additional p53 heterozygote breeders were purchased from Jackson Labs (stock number 002101). Genotyping of mice was performed using a Qiagen (Valencia, CA, USA) DNAeasy Blood and Tissue kit and specific primers from IDT (Coralville, IA, USA. Mating pairs of p53 +/− SK1+/− or WT or SK1 KO mice were set up and MEFs were harvested and genotyped between embryonic day 12.5–14.5 of gestation. Further breeding and genotyping information and MEF collection details are provided in the SI

Assessing Survival

Mice were monitored daily for tumor development and general health by an unbiased caretaker. Mice that appeared to be suffering or in pain, unable to eat, or immobile were sacrificed and necropsied. Survival was measured by the number of days between each mouse's date of birth and date of death, or date of sacrifice due to humane reasons. Runts and mice with genetic issues common to the C57BL/6 background, namely blindness and malocclusion, were sacrificed and not included in the survival study.

Assessing Tumor Burden

At 4 months-of-age, p53 KO mice harboring each possible SK1 genotype: WT, HZ, or KO, were sacrificed and necropsied with the assistance of a veterinary pathologist (KLH). Each organ was inspected and weighed, and then representative sections were fixed for histological evaluation. Thymic mass was the primary indicator of tumor burden in the p53 KO mice, whereas, p53 HZ mice suffered a more diverse range of tumors with more varied latency. Thus, p53 HZ mice were sacrificed and necropsied at different ages due to individual unthriftiness, or they were necropsied after they were found dead to determine tumor burden. Each organ was inspected and any abnormal tissues or masses were fixed for histological evaluation by KLH.

Animal Imaging

Micro-magnetic resonance imaging of mice was performed by Dr. P. Chou under the guidance of Dr. B. Memet in the MUSC Animal Imaging Core Facility with a 7 T MR Bruker Biospec USR (Bruker Inc., Billerica, MA, USA) actively shielded scanner with a 30-cm horizontal bore and Paravision software.

Tissue Homogenization

Following tissue collection as described in SI Materials and Methods, tissues were minced with dissecting scissors in 350 μL of SK1 lysis buffer or RLT buffer (Qiagen) in a 2-mL microcentrifuge tube before being homogenized on ice with a handheld Tissue Tearor (Biospec, Bartlesville, OK, USA) set at level 25 for 1 min. Samples were then transferred to

a clean microcentrifuge tube and sonicated for 10 sec to ensure homogeneity before being aliquoted for protein or other analysis.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were hydrated through histoclear and an ethanol gradient, slides were boiled in citrate buffer for antigen retrieval, and they were immersed in 10% H_2O_2 in methanol to quench endogenous peroxide activity. Sections were then blocked with normal serum and incubated overnight at 4 °C with rabbit anti-SK1 primary antibody followed by a 30-min incubation with biotinylated anti-rabbit IgG, and then ABC-HRP (Vector Laboratories, Burlingame, CA, USA). The slides were then developed with DAB/H2O2 (Thermo Scientific, Hudson, NH, USA), counterstained with hematoxylin, and dehydrated with an ethanol gradient and Histoclear.

Senescence-associated β-galactosidase (SA-β-gal) Staining

Frozen 10-mm sections of thymi were fixed in 0.2% glutaraldehyde/PBS for 10 min at 4 °C, washed with PBS (pH 6.0), stained with the SA-β-gal staining kit (Cell Signaling) overnight at 37 °C, washed with H_2O, counterstained with eosin, and dehydrated in an ethanol gradient with histoclear as previously described (Sun *et al.*, 2007).

SK1 Activity Assay

After tissue homogenization in SK1 lysis buffer, either an aliquot of protein (30 μg) from each homogenized tissue sample, or 30 μL of sample was normalized to its protein concentration and assayed for sphingosine kinase activity in the presence of ^{32}P-ATP and sphingosine for 30 min at 37 ºC. At the end of the reaction, lipids were extracted and subjected to TLC. ^{32}P-sphingosine-1-phosphate was measured using autoradiography. Quantification of S1P-specific radioactivity was performed by scraping the S1P-specific bands into scintillation vials that had their contents quantified via scintillation counting.

Sphingolipidomic Analysis

Cell samples were prepared as detailed in SI Materials and Methods. Each sample was vortexexed and then 250 uL was aliquoted into a labeled 15-mL conical flask. This and the remaining sample in a 1.5-mL microcentrifuge tube were then snap frozen in a dry ice/methanol bath and frozen at −80 °C prior to sphingolipidomic analysis or BCA protein assay, respectively. Tissue samples were homogenized as described above and, after protein analysis, an aliquot of homogenized sample (1 mg protein) was transferred to a 15-mL conical flask and frozen at −80 °C until sphingolipidomic analysis. Alternatively, 100 μL of homogenized sample was submitted and lipids were subsequently corrected to the sample protein concentration. Sphingolipid masses were determined by ESI/MS/MS. Analysis of ceramides, sphingosine, and sphinosine-1-phosphate was performed on a Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer operating in a multiple reaction-monitoring positive ionization mode, as described (Bielawski *et al.*, 2009; Bielawski *et al.*, 2006).

Statistical Analysis

Data are represented as mean ± SEM, unless otherwise indicated. Unpaired student's t-test, One-way ANOVA with Dunnett's post-test, or logrank test statistical analyses were performed using Prism/GraphPad software (La Jolla, CA, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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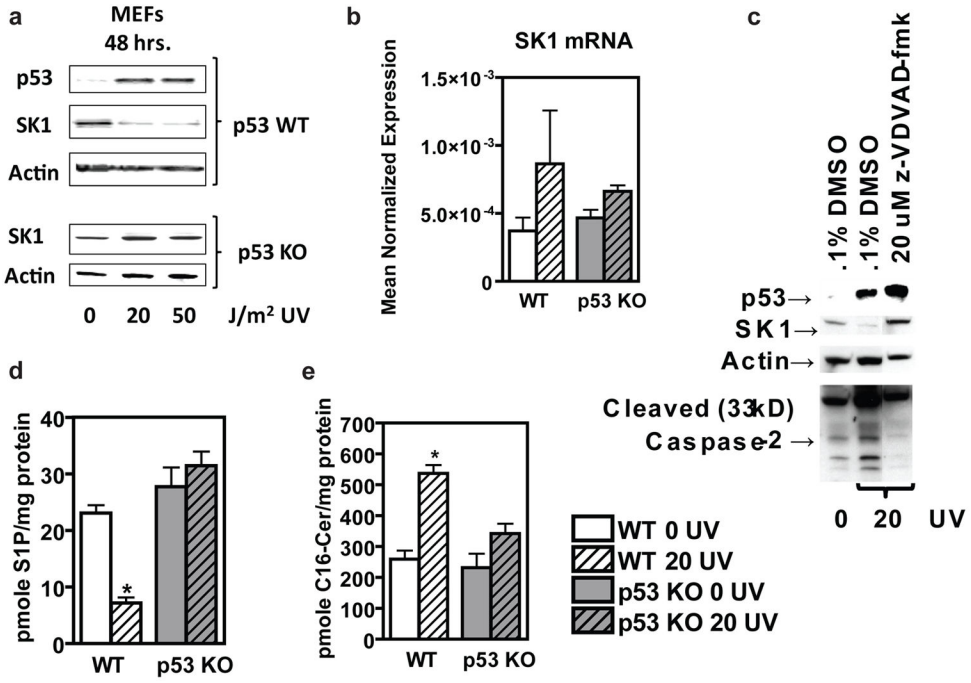

Figure 1.

Primary mouse cell lines respond to genotoxic stress through p53-dependent, caspase-2 mediated proteolysis of SK1 to alter the balance of pro- and anti-growth sphingolipids. (a) Mouse embryonic fibroblasts (MEFs) expressing WT p53 or with p53 knocked out, were treated with indicated doses of UV radiation (J/m^2), cultured for 48 hours, and then whole cell lysate was examined by (a) Western blotting with antibodies to the indicated proteins, or, (b) harvested in RLT buffer, prepared for qRT-PCR, with enzyme expression normalized to β-actin expression for each reaction performed in triplicate, or, (c) WT MEFs were pre-treated with 20 uM z-VDVAD-fmk (caspase-2 inhibitor) dissolved in DMSO or DMSO control in fresh media and incubated for one hour prior to treatment with 0 or 20 J/m^2 UV radiation, incubated for 48 hours before being harvested by scraping on ice using Tris-Triton lysis buffer supplemented with protease and phosphatase inhibitors from Sigma for western blotting with antibodies to the indicated proteins (n=3), or (d) harvested for sphingolipidomic analysis to measure S1P and C_{16}-ceramide by LC/MS, expressed as pmol lipid per mg protein ± SEM (n=3, and *p < 0.05 by Student's *t* test relative to WT 0 UV).

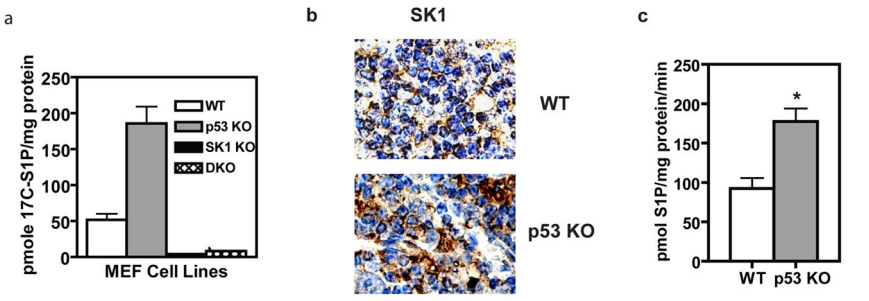

Figure 2.

SK1 activity is increased in the absence of its regulator, p53. (a) MEFs (passage 4–7) of the indicated genotype were treated with 1 nM 17C-labeled sphingosine and following a 30 minute incubation, cells were harvested for sphingolipidomic analysis and 17C-containing S1P was normalized to the amount of protein submitted for each sample (n=4 and $p < 0.01$ by One-way ANOVA with Dunnett's post test). (b) Immunohistochemistry for SK1 on formalin-fixed thymic sections from WT or p53 KO mice (pictures representative of n=3). (c) Homogenates of WT thymus and p53 KO thymic lymphoma from 4 month-old mice were incubated with sphingosine and ^{32}P labeled ATP to determine SK1 activity (n=3, and $*p < 0.05$ by Student's *t* test).

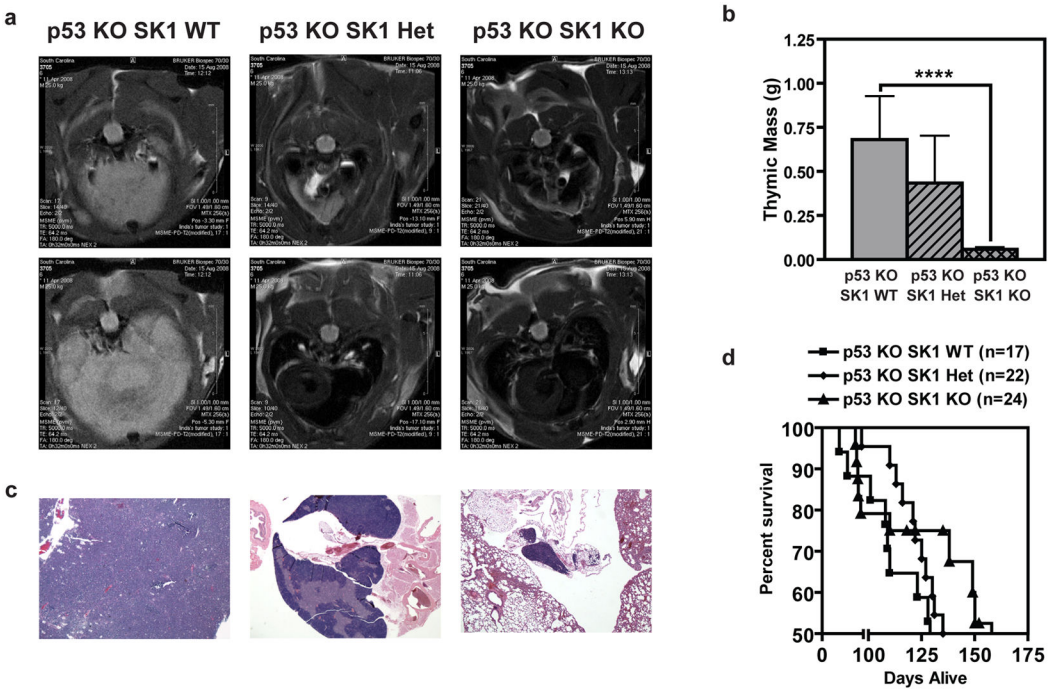

Figure 3.

KO of SK1 protects p53 KO mice from thymic lymphoma. (a) Representative MRI of chest cavity from a mouse of each genotype. Post-imaging, necropsy, and histology confirmed that the large white mass observed was thymic lymphoma, the characteristic lesion observed in p53 KO mice, with a smaller mass observed in SK1 Het mice and a normal thymus and clear chest cavity in the DKO mouse. (b) Weight of thymi dissected from male mice immediately after euthanization at 120 days-of-age. Data are averages of thymic weight from 5–9 mice of each genotype ± SEM. Asterisks indicate statistically significant differences ($P < 0.02$ by Student's *t* test). (c) H & E stain of thymi with histology representative of what was found for each genotype. (d) Kaplan-Meier survival analysis of p53 KO mice with different SK1 genotypes. Each point represents a mouse that died naturally or was sacrificed due to tumor burden or unthriftiness, whereas the n listed includes mice that are still living, all of which contribute to the survival estimate curve. Differences between the survival curves are statistically significant (by log-rank comparison $P < 0.04$).

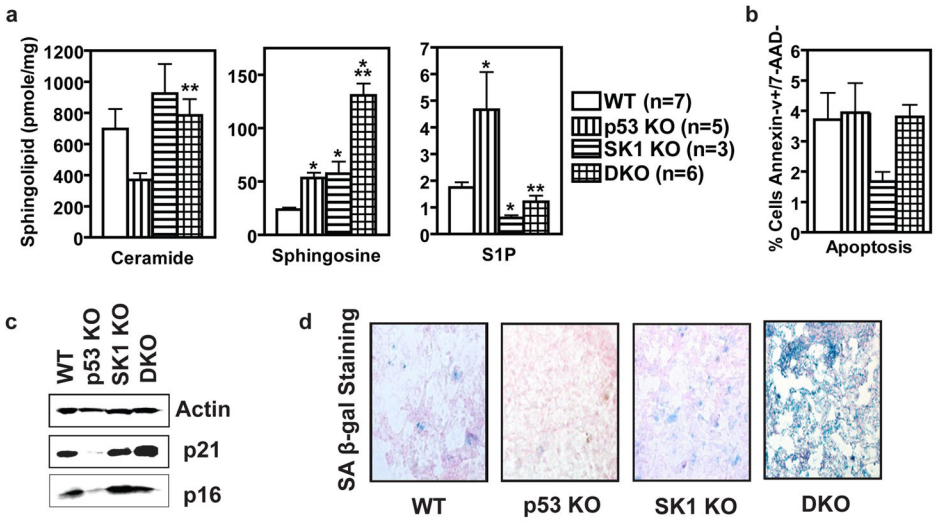

Figure 4.

Loss of SK1 leads to tumor cell senescence in p53 KO thymus. (a) Thymi were harvested from mice of the indicated genotypes, homogenized, and submitted to lipid analysis. C_{16}-ceramide, sphingosine, and S1P were measured by LC/MS. Data are averages of lipid levels determined for thymi from three or more mice and are expressed as pmol lipid per mg protein ± SEM. Asterisks indicate statistically significant differences ($P < 0.05$ by Student's *t* test relative to WT, *, or DKO relative to p53 KO, **). (b) Thymi were harvested from mice of the indicated genotypes, processed into a single cell suspension, stained and analyzed via flow cytometry. Data are averages ± SEM of the percentages of Annexin-V positive, 7-AAD negative-staining cells in thymuses from three or more mice. (c) Protein markers of cell cycle arrest and senescence. Thymi from mice of the indicated genotypes were homogenized, and equal amounts were examined by Western blotting. (d) Senescence associated β-gal staining of thymuses from age-matched mice of the indicated genotypes.

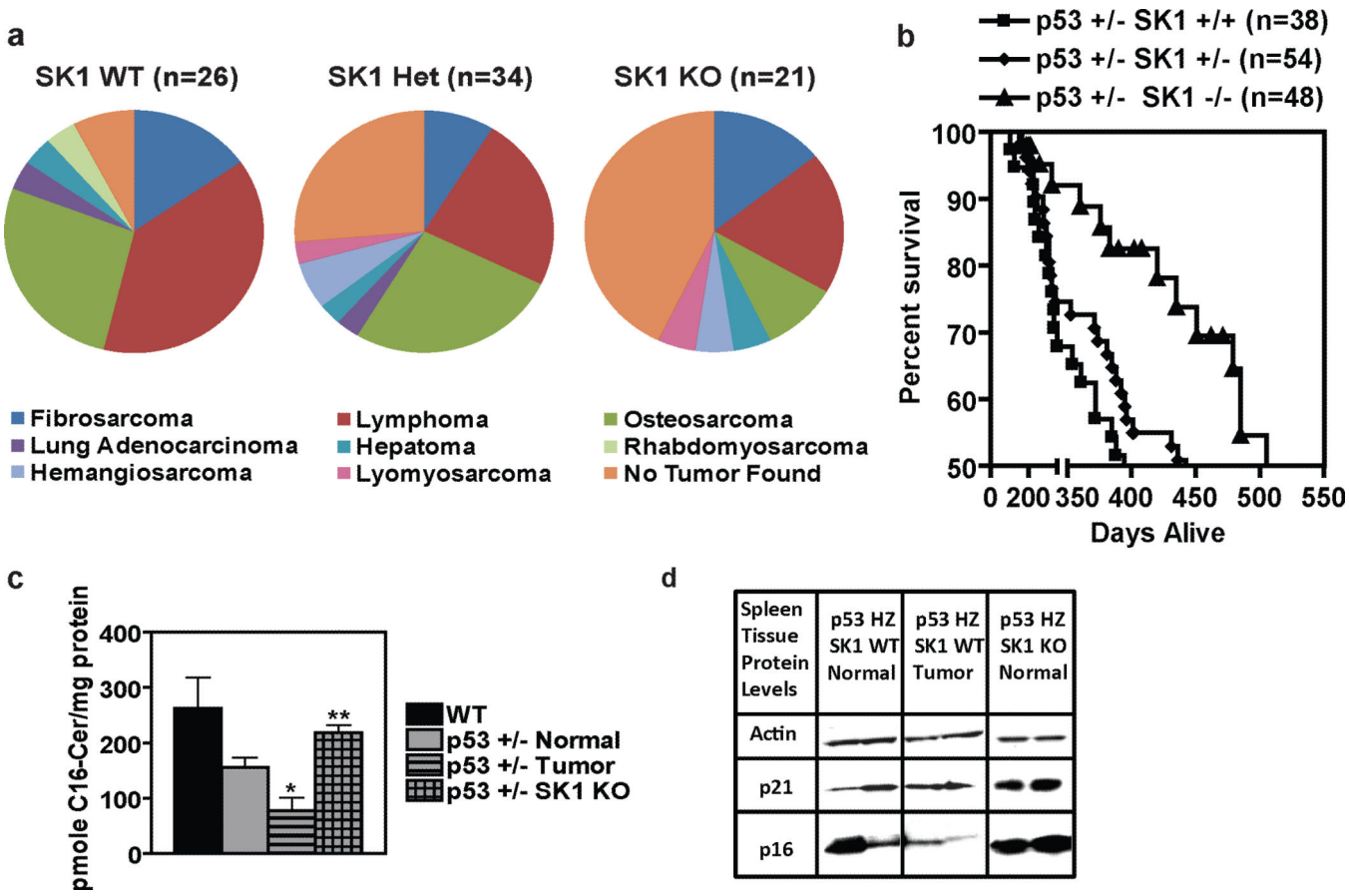

Figure 5.

KO of SK1 protects p53 heterozygote mice from tumor development. (a) Pie chart representation of tumor burden found during necropsy and confirmed via histology of p53 heterozygote mice with the indicated SK1 genotype. (b) Kaplan-Meier survival analysis of p53 heterozygote mice with different SK1 genotypes. Each point represents a mouse that died naturally or was sacrificed due to tumor burden or unthriftiness, whereas the n listed includes mice that are still living, all of which contribute to the survival estimate curve. Differences between the survival curves are statistically significant (by log-rank comparison $P < 0.02$). (c) Spleens were harvested from mice of the indicated genotypes and phenotypes, homogenized, and submitted to lipid analysis. C_{16}-ceramide was measured by LC/MS. Data are averages of lipid levels determined for thymuses from three mice and are expressed as pmole lipid per mg protein ± SEM. Asterisk indicates a statistically significant difference ($P < 0.05$ by Student's *t* test relative to the other groups). (d) Protein markers of cell cycle arrest and senescence. Spleens from mice of the indicated genotypes and phenotypes were homogenized, and equal amounts were examined by Western blotting.