Title

CERKL is Upregulated in Cutaneous Squamous Cell Carcinoma and Maintains Cellular Sphingolipids and Resistance to Oxidative Stress

Authors

Meyer, JM
Lee, E
Celli, A
et al.

Publication Date

2020-12-03

DOI

10.1111/bjd.19707

Peer reviewed

HHS Public Access

Author manuscript

Br J Dermatol. Author manuscript; available in PMC 2022 June 03.

CERKL is Upregulated in Cutaneous Squamous Cell Carcinoma and Maintains Cellular Sphingolipids and Resistance to Oxidative Stress

J.M. Meyer[1], **E. Lee**[1], **A. Celli**[1], **K. Park**[1], **R. Cho**[2], **W. Lambert**[3], **M. Pitchford**[1], **M. Gordon**[1], **K. Tsai**[5], **J. Cleaver**[4], **S.T. Arron**[1], **T.M. Mauro**[1]

[1]Dermatology Service, VA Medical Center and Department of Dermatology, UC San Francisco, CA

[2]Department of Dermatology, UC San Francisco, CA

[3]Pathology and Laboratory Medicine, Rutgers University, Newark, NJ

[4]Departments of Dermatology and Pharmaceutical Chemistry, UC San Francisco, CA

[5]Moffitt Cancer Center, Tampa, FL

Abstract

Background: Ceramide Kinase-Like Protein (CERKL) was originally described in retinal tissue. CERKL has been shown to protect cells from oxidative stress, and mutations in CERKL underlie the inherited disease, retinitis pigmentosa. CERKL expression maintains cellular sphingolipids via an unknown mechanism.

Objectives: To determine whether CERKL is expressed in epidermis and cutaneous squamous cell carcinoma (cSCC) and whether CERKL expression affects cSCC sphingolipid metabolism and susceptibility to oxidative stress.

Methods: CERKL expression was determined by RNA-Seq, qPCR and immunohistochemistry. CERKL was knocked down in cSCC cells using siRNA. Sphingolipid content was analyzed by liquid chromatography-mass spectrometry (LC-MS). Oxidative stress was induced by treatment with H_2O_2, and apoptosis was measured using flow cytometry to determine annexin v binding.

Results: CERKL mRNA and protein are highly expressed in actinic keratosis and cSCC in comparison with normal epidermis. CERKL also is expressed in metabolically active epithelial cells in normal hair bulbs and sebaceous glands. CERKL knockdown in cultured cSCC cells reduces cellular sphingolipid content and enhances susceptibility to oxidative stress.

Conclusions: These findings suggest that CERKL may be important in cSCC progression and could lead to novel strategies for prevention and treatment of cSCC.

Corresponding Author: Theodora M. Mauro, MD, thea.mauro@ucsf.edu.

CONFLICT OF INTEREST

The authors have no conflict of interest relevant to this work to report.

INTRODUCTION

Cancer is associated with oxidative stress and increased levels of reactive oxygen species (ROS) [1]. While modestly elevated ROS promote cancer initiation by enhancing mutagenesis and activating cell proliferation and survival responses, excessive oxidative damage can also kill cancer cells and thus hinder cancer progression [2]. Therefore, cancer cells require protective mechanisms to limit or reduce ROS, especially during invasion and metastasis [3]. This is accomplished in part by modified cellular metabolism, including adaptations in energy substrates, metabolic intermediates and organelles (e.g., autophagy**)**. Additional research is needed to determine how cancer cells control these pathways, which could provide new strategies for cancer treatment.

Ceramide Kinase-Like Protein (CERKL) is an oxidative stress-associated protein, first described in retinal tissue. Loss-of-function mutations in CERKL underlie retinitis pigmentosa (RP), an autosomal recessive disease with progressive vision loss due to photoreceptor apoptosis [4]. CERKL expression is normally restricted to retina, neural tissues, kidney, trachea, testis and lung [5]. CERKL expression has been reported in malignant cell lines [5], but it has not previously been linked *in vivo* with cutaneous squamous cell carcinoma (cSCC) or other cancers. Although CERKL is structurally related to ceramide kinase enzymes, it appears to protect cells from oxidative stress-induced apoptosis via a mechanism unrelated to kinase activity [6–8]. cSCC growth and survival must proceed in the face of increased oxidative stress. We therefore examined whether CERKL might be upregulated in cSCC, as a mechanism for enhanced survival in the face of oxidative stress.

RESULTS

To investigate whether CERKL is expressed in cSCC, we first quantified CERKL gene expression in a set of patient-matched normal skin, pre-cancerous actinic dysplasia (actinic keratosis, AK) and cSCC tumors. CERKL expression was significantly greater in AK and cSCC relative to patient-matched normal skin (Figure 1). Immunohistochemistry confirmed CERKL protein expression in cSCC and demonstrated that CERKL was highly expressed in cSCC near areas of necrosis (Figure 2A). CERKL was also expressed in AKs (Figure 2B) and in metabolically active sites such as normal hair bulbs (Figure 2C) and sebaceous glands (Figure 2D), but CERKL expression was not seen in normal interfollicular epidermis adjacent to cSCC (Figure 2E). Consistent with previous reports [8], CERKL was localized to the cytoplasm of cells rather than the nucleus or plasma membrane.

In order to investigate functional roles for CERKL in cSCC, we treated the SCC cell line SCC12F2 with anti-CERKL siRNA, which reduced CERKL expression by ~40% without any increase in apoptosis or loss of cell viability (data not shown). Similar to previous reports in mouse retina[8], we observed that CERKL gene silencing reduced SCC12F2 sphingolipid content, as determined by liquid chromatography-mass spectrometry (Figure 3). All analyzed sphingolipids were significantly reduced by siCERKL compared to control scrambled siRNA, but reductions were greatest for dihydroceramide, ceramide 1-phosphate, and sphingoid bases (including sphingosine, sphinganine, sphingosine 1-phophate and dihydrosphingosine 1-phosphate), while ceramide and sphingomyelin were only modestly

reduced. Sphingolipid reductions were not specific to N-acyl chain length, as all species within each class were reduced similarly (Supplemental Figures 1–5). We then tested whether CERKL is required for cSCC resistance to oxidative stress. When CERKL expression was reduced by siRNA, SCC12F2 cells were more susceptible to H_2O_2-induced apoptosis (Figure 4, scatter plots shown in Supplemental Figure 6). These findings suggest that CERKL contributes to maintenance of cSCC sphingolipids and resistance to oxidative stress.

DISCUSSION

This report is the first to show that CERKL is expressed in cSCC and protects cultured cSCC cells from oxidative stress-induced cell death. Our results are consistent with those reported in retinas of fish, mice and humans as well as diverse cultured cell lines [6, 7, 9], suggesting that CERKL can protect against oxidative stress in a variety of contexts. CERKL upregulation in cSCC might be an adaption to the increased oxidative stress in tumors and could promote cancer progression. Since CERKL protein is expressed in both cSCC tumors and pre-cancerous AKs, but not in normal interfollicular epidermis, CERKL inhibition could be an effective approach for prevention or treatment of cSCC.

Several mechanisms have been proposed for CERKL-mediated protection from oxidative stress, including its role as a reducing agent for the mitochondrial antioxidant TRX2 [10], and by promoting autophagy [11]. Autophagy protects against cell death by converting damaged organelles into additional energy [12]. CERKL has recently been shown to control autophagy by direct molecular interaction with the autophagy regulator, SIRT1 [11]. Since S1P and ceramide are also essential regulators of autophagy [13], CERKL could also modulate autophagy indirectly via changes in sphingolipid metabolism. Indeed, we found that CERKL knockdown broadly reduced sphingolipid concentration in cSCC, similar to that reported in CERKL knockdown in retina [14]. Unlike ceramide kinases, CERKL is not required for ceramide phosphorylation during C1P and S1P synthesis [15], and how CERKL affects sphingolipid metabolism is unclear. In addition to regulating autophagy and cell death, sphingolipid metabolites have been shown to control keratinocyte cell-to-cell adhesion via endoplasmic reticulum Ca2+ stores [16], and thus abnormal CERKL function may lead to acantholysis, a common pathological finding in SCC. Further studies are needed to determine how CERKL carries out its function in cSCC and other tissues.

MATERIALS AND METHODS

Tissue acquisition and cell culture

Skin tumors and adjacent normal skin were obtained from surgery during cSCC excisions. The cSCC cell line SCC12F2 was cultured in DMEM with 10% fetal bovine serum, 2% glutamine and 1% penicillin/streptomycin. To induce oxidative stress, SCC12F2 cells were seeded into 6-well culture plates at a density of 25K cells/cm^2, and treated on day 4 with 250 mM H_2O_2 for 18 hours. Cells were then rinsed with PBS, collected by dissociation with trypsin/EDTA, and apoptosis was quantified as annexin-V staining by flow cytometry [17], using the Annexin kit (Thermo-Fisher) according to the manufacturer's instructions. Flow cytometry on control and experimental cells was done using the CANTO instrument.

Gene expression analysis

RNA expression levels in patient-matched primary human SCC, AK, and normal skin were derived from a previously published dataset using RNA-Seq [18]. Relative mRNA expression was assessed by quantitative RT-PCR (qRT-PCR) using SensiMix™ SYBR PCR Master Mix (Bioline, Bioline, Taunton, MA) as described previously [19]. Total RNA was extracted from cells in RLT buffer with 1% BME, and isolated using RNeasy mini kit (Qiagen, Germantown, MD), followed by preparation of cDNA using SensiFAST™ cDNA synthesis kit (Bioline). The following primer sets were used: ceramide kinase like (CERKL), 5'-CAGGGATCTCCCAAATCTGA-3' and 5'-AGCCTCTAGGTGCCACTGAA-3'; human glyceraldehyde 3'-phosphate dehydrogenase (GAPDH), 5' -GGAGTCAACGGATTTGGTCGTA-3' and 5'-GCAACAATATCCACTTTACCAGAGTTAA-3'. The thermal cycling conditions were 95°C for 10 min, 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s, repeated 40 times on ABI Prism 7900 (Applied Biosystems, Foster City, CA). mRNA expression was normalized to levels of GAPDH, used as a control. Values shown represent mean (±SD) for three independent assays.

After harvest, cells were lysed using the QIA shredder kit and purified following the protocols of the RNeasy kit by QIAGEN. The amount of RNA produced is then quantified using a nanodrop.

qPCR was run with the cDNA using CERKL primers:

qPCR was run with the cDNA using CERKL primers:

hGAPDHF for Value 5 24 651 1, GAAGGTGAAGGTCGGAGTC

hGADPHR for Value 5 24 16 1, GAAGATGGTGATGGGATTTC

hCERKLF for Value 5 24 16 1, CAGGGATCTCCCAAATCTGA

hCERKLR for Value 5 24 16 1, AGCCTCTAGGTGCCACTGAA

Immunohistochemistry

8 um skin sections were obtained from paraffin embedded tissue, and immunostained with an antibody to CERKL (ab198918, rabbit, abcam) at a concentration of 1:100 or 1:200, with a secondary antibody of biotinylated goat anti-rabbit IgG [Vector BA-1000]). Samples without the secondary antibody and normal interfollicular skin were used as controls. Sections were counterstained with hematoxylin and eosin. Samples were photographed on a Leica dm4000b microscope.

Gene expression knockdown using siRNA

On day 2 of culture, SCC12F2 cells were transfected using Lipofectamine RNAiMAX with 20 nM anti-CERKL or scrambled siRNA, obtained as Silencer Pre-designed siRNA from Ambion (Life Technologies). The sequence for the Cerkl siRNA was: Sense (5′ -> 3′): GCAUCAGAGGUCCAUAUUAtt; Antisense (5′ -> 3′):

UAAUAUGGACCUCUGAUGCaa. Control experiments demonstrated approximately 40% CERKL knockdown in cSCC expression after siRNA, and did not show an increase in apoptosis after CERKL siRNA treatment alone.

Sphingolipid analysis by liquid chromatography tandem-mass spectrometry

To assess cellular sphingolipid levels, cells were harvested 2 days after siRNA treatment using a cell scraper, lysed in RIPA buffer, followed by extraction of sphingolipids, as reported [20]. Extracted lipids, dried using a vacuum system (Vision, Seoul, Korea), were re-dissolved in methanol and analyzed by liquid chromatography-electrospray ionization-tandem-mass spectrometry (API 3200 QTRAP mass; AB/SCIEX, Concord, ON, Canada) by selective ion monitoring mode. Ceramide tandem-mass spectrometry transitions (m/z) were 510→264 for C14-ceramide, 538→264 for C16-ceramide, 552→264 for C17-ceramide, 566→264 for C18-ceramide, 594→264 for C20-ceramide, 648→264 for C24:1-ceramide, and 650→264 for C24-ceramide, respectively. The sphingoid bases tandem-mass spectrometry transitions (m/z) were 286→238 for C17 sphingosine as an internal standard, 300→252 for C18 sphingosine, and 302→60 for C18 sphinganine. Data were acquired using Analyst 1.4.2 software (Applied Biosystems).

Statistical Analysis

Data was analyzed using unpaired, 2-tailed student's T-tests.

DATA AVAILABILITY

RNA sequencing data has previously been deposited in NCBI/GEO with SuperSeries accession code GSE84194. Additional data in this paper is available upon request from the corresponding author (T.M.M).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the editorial assistance of Ms. Joan Wakefield. This work was supported by NIH grants R01AR051930 and R01AG028492, which were administered by the Northern California Institute for Research and Education, and with resources of the Research Service, Department of Veterans Affairs. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. These sponsors had no role in writing this article or in the decision to submit it for publication. We wish to thank the Laboratory for Cell Analysis (LCA) for the use of their microscopy equipment. We also wish to thank the staff at the UCSF Benioff Children's Hospital for their help in collecting foreskins used to isolate primary keratinocytes for cell culture.

REFERENCES

1. Reczek CR, Chandel NS. ROS Promotes Cancer Cell Survival through Calcium Signaling. Cancer Cell 2018; 33(6): 949–951. [PubMed: 29894695]
2. Sabharwal SS, Schumacker PT. Mitochondrial ROS in cancer: initiators, amplifiers or an Achilles' heel? Nat Rev Cancer 2014; 14(11): 709–21. [PubMed: 25342630]
3. Gill JG, Piskounova E, Morrison SJ. Cancer, Oxidative Stress, and Metastasis. Cold Spring Harb Symp Quant Biol 2016; 81(163–175. [PubMed: 28082378]

4. Tuson M, Marfany G, Gonzalez-Duarte R. Mutation of CERKL, a novel human ceramide kinase gene, causes autosomal recessive retinitis pigmentosa (RP26). Am J Hum Genet 2004; 74(1): 128–38. [PubMed: 14681825]
5. Bornancin F, Mechtcheriakova D, Stora S, Graf C, Wlachos A, Devay P, et al. Characterization of a ceramide kinase-like protein. Biochim Biophys Acta 2005; 1687(1-3): 31–43. [PubMed: 15708351]
6. Tuson M, Garanto A, Gonzalez-Duarte R, Marfany G. Overexpression of CERKL, a gene responsible for retinitis pigmentosa in humans, protects cells from apoptosis induced by oxidative stress. Mol Vis 2009; 15(168–80. [PubMed: 19158957]
7. Li C, Wang L, Zhang J, Huang M, Wong F, Liu X, et al. CERKL interacts with mitochondrial TRX2 and protects retinal cells from oxidative stress-induced apoptosis. Biochim Biophys Acta 2014; 1842(7): 1121–9. [PubMed: 24735978]
8. Chen J, Liu F, Li H, Archacki S, Gao M, Liu Y, et al. pVHL interacts with Ceramide kinase like (CERKL) protein and ubiquitinates it for oxygen dependent proteasomal degradation. Cell Signal 2015; 27(11): 2314–23. [PubMed: 26296657]
9. Riera M, Burguera D, Garcia-Fernandez J, Gonzalez-Duarte R. CERKL knockdown causes retinal degeneration in zebrafish. PLoS One 2013; 8(5): e64048. [PubMed: 23671706]
10. Fathinajafabadi A, Perez-Jimenez E, Riera M, Knecht E, Gonzalez-Duarte R. CERKL, a retinal disease gene, encodes an mRNA-binding protein that localizes in compact and untranslated mRNPs associated with microtubules. PLoS One 2014; 9(2): e87898. [PubMed: 24498393]
11. Hu X, Lu Z, Yu S, Reilly J, Liu F, Jia D, et al. CERKL regulates autophagy via the NAD-dependent deacetylase SIRT1. Autophagy 2019; 15(3): 453–465. [PubMed: 30205735]
12. Filomeni G, De Zio D, Cecconi F. Oxidative stress and autophagy: the clash between damage and metabolic needs. Cell Death Differ 2015; 22(3): 377–88. [PubMed: 25257172]
13. Young MM, Wang HG. Sphingolipids as Regulators of Autophagy and Endocytic Trafficking. Adv Cancer Res 2018; 140(27–60. [PubMed: 30060813]
14. Garanto A, Mandal NA, Egido-Gabas M, Marfany G, Fabrias G, Anderson RE, et al. Specific sphingolipid content decrease in Cerkl knockdown mouse retinas. Exp Eye Res 2013; 110(96–106. [PubMed: 23501591]
15. Graf C, Niwa S, Muller M, Kinzel B, Bornancin F. Wild-type levels of ceramide and ceramide-1-phosphate in the retina of ceramide kinase-like-deficient mice. Biochem Biophys Res Commun 2008; 373(1): 159–63. [PubMed: 18555012]
16. Celli A, Mackenzie DS, Zhai Y, Tu CL, Bikle DD, Holleran WM, et al. SERCA2-controlled Ca(2)+-dependent keratinocyte adhesion and differentiation is mediated via the sphingolipid pathway: a therapeutic target for Darier's disease. J Invest Dermatol 2012; 132(4): 1188–95. [PubMed: 22277942]
17. Abu-Yousif AO, Smith KA, Getsios S, Green KJ, Van Dross RT, Pelling JC. Enhancement of UVB-induced apoptosis by apigenin in human keratinocytes and organotypic keratinocyte cultures. Cancer Res 2008; 68(8): 3057–65. [PubMed: 18413777]
18. Chitsazzadeh V, Coarfa C, Drummond JA, Nguyen T, Joseph A, Chilukuri S, et al. Cross-species identification of genomic drivers of squamous cell carcinoma development across preneoplastic intermediates. Nat Commun 2016; 7(12601.
19. de Feraudy S, Boubakour-Azzouz I, Fraitag S, Berneburg M, Chan L, Chew K, et al. Diagnosing xeroderma pigmentosum group C by immunohistochemistry. Am J Dermatopathol 2010; 32(2): 109–17. [PubMed: 19915453]
20. Park K, Elias PM, Shin KO, Lee YM, Hupe M, Borkowski AW, et al. A novel role of a lipid species, sphingosine-1-phosphate, in epithelial innate immunity. Mol Cell Biol 2013; 33(4): 752–62. [PubMed: 23230267]

What's already known about this topic?

Ceramide Kinase-Like Protein (CERKL) is thought to protect cells from oxidative stress, and mutations in CERKL underlie the inherited disease, retinitis pigmentosa.

What does this study add?

We found that CERKL is upregulated in cutaneous squamous cell carcinoma (cSCC) compared to normal epidermis. CERKL protects cSCC cells from oxidative stress and maintains cellular sphingolipids.

What is the translational message?

These findings demonstrate that CERKL may be important in cSCC progression by increasing the resistance of tumors to oxidative stress. Further investigation of CERKL could lead to novel strategies for prevention and treatment of cSCC.

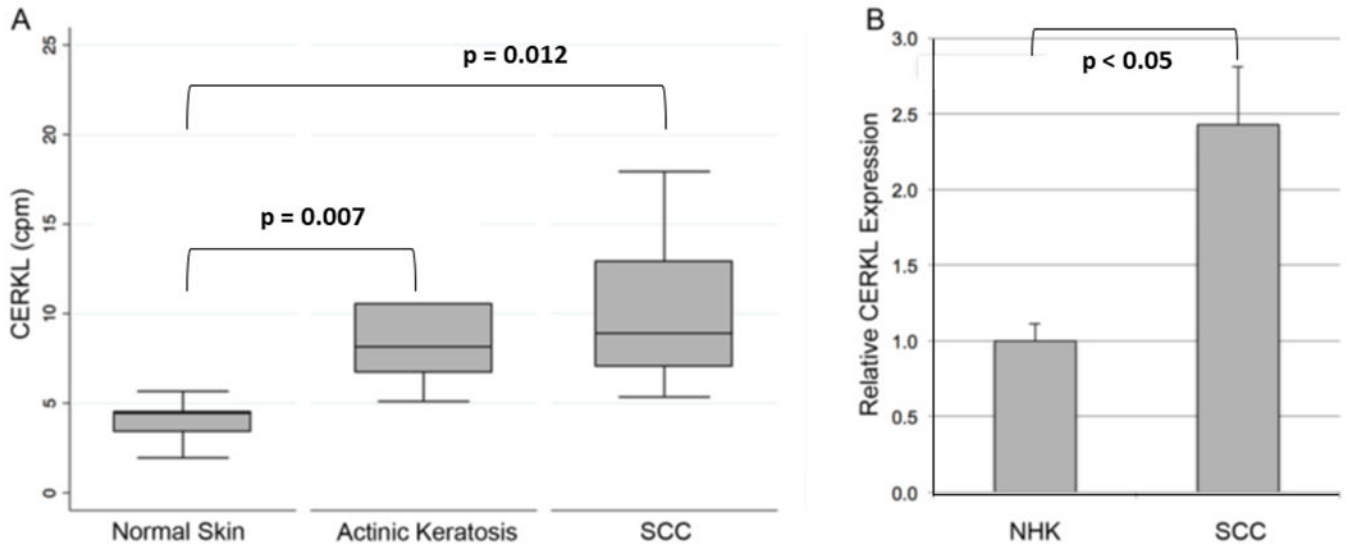

Figure 1. CERKL gene expression is upregulated in AK and cSCC.

CERKL gene expression is upregulated in actinic keratosis and cutaneous squamous cell carcinoma, compared to patient-matched normal skin. RNAseq expression data for CERKL from 9 sets of patient-matched normal skin (N=7), AK (N=10), and cSCC (N=9). Mean counts per million mapped reads for normal=4.1, AK=10.01 (p=0.007); cSCC=11.24 (p=0.012). There was no difference in expression between AK and SCC (p=0.6).

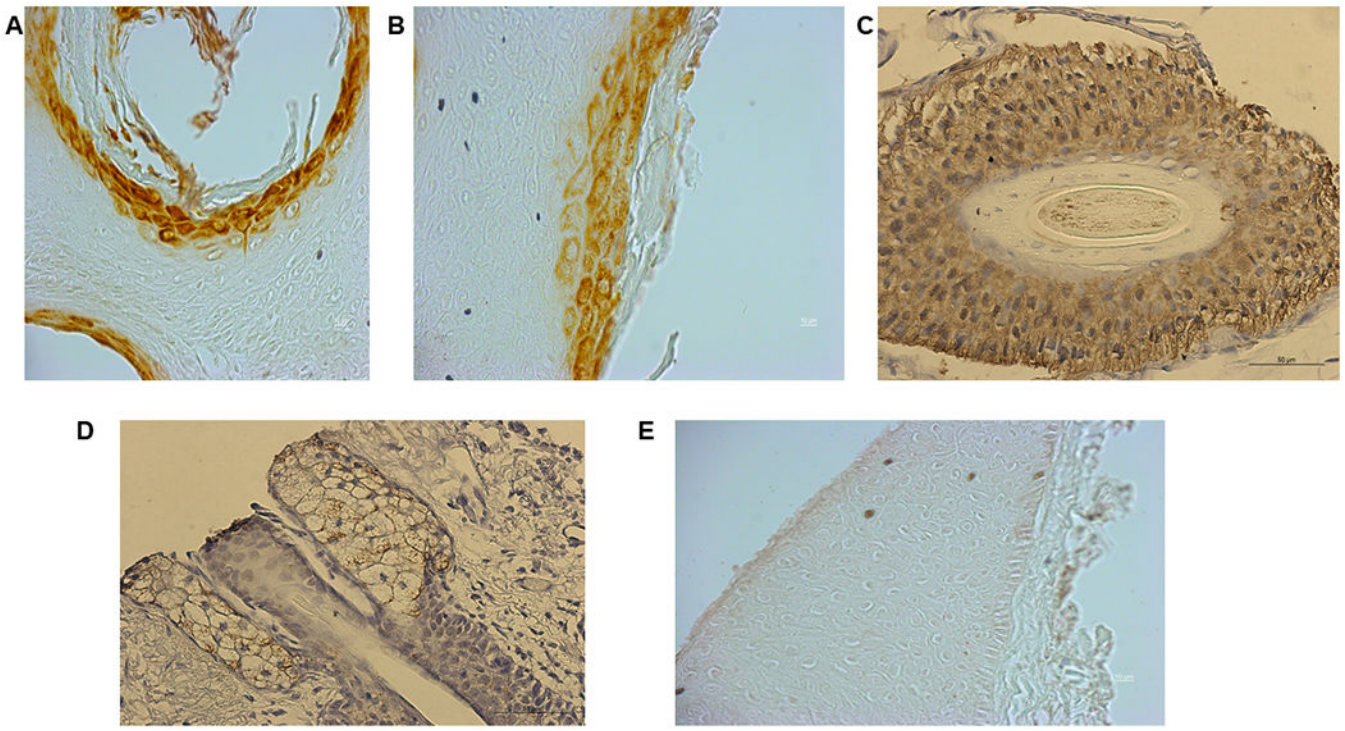

Figure 2. CERKL protein is expressed in cSCC, AKs and appendages but not normal epidermis.
CERKL expression was assessed by immunostaining of cSCC (A, * indicating an area of necrosis), AK (B), hair bulb (C), sebaceous gland (D) and normal interfollicular epidermis (E). Total magnification A-D 400X; E 200X.

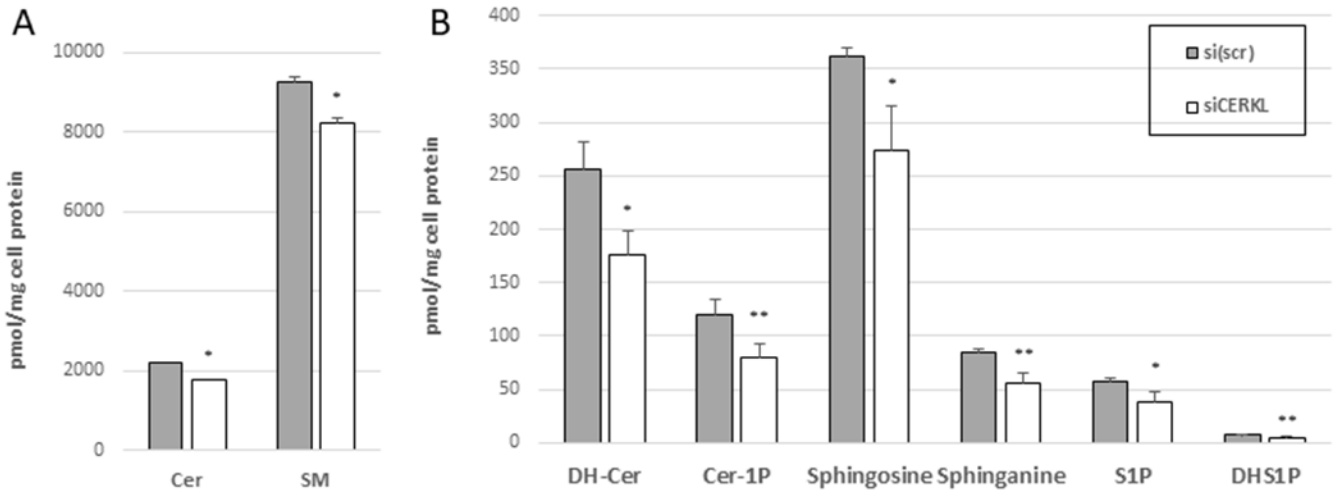

Figure 3. CERKL knockdown reduces cSCC cell sphingolipid content.

SCC12F2 cells were treated with anti-CERKL siRNA for 48 hours, then collected and analyzed for protein (bicinchoninic acid method) and sphingolipid (by liquid chromatography tandem-mass spectrometry) content. Si(scr) = scrambled control siRNA; siCERKL = anti-CERKL siRNA. N=3 for each group. Data are presented as the mean +/− SEM. *, $p<0.05$ vs si(scr); **, $p<0.01$ vs si(scr).

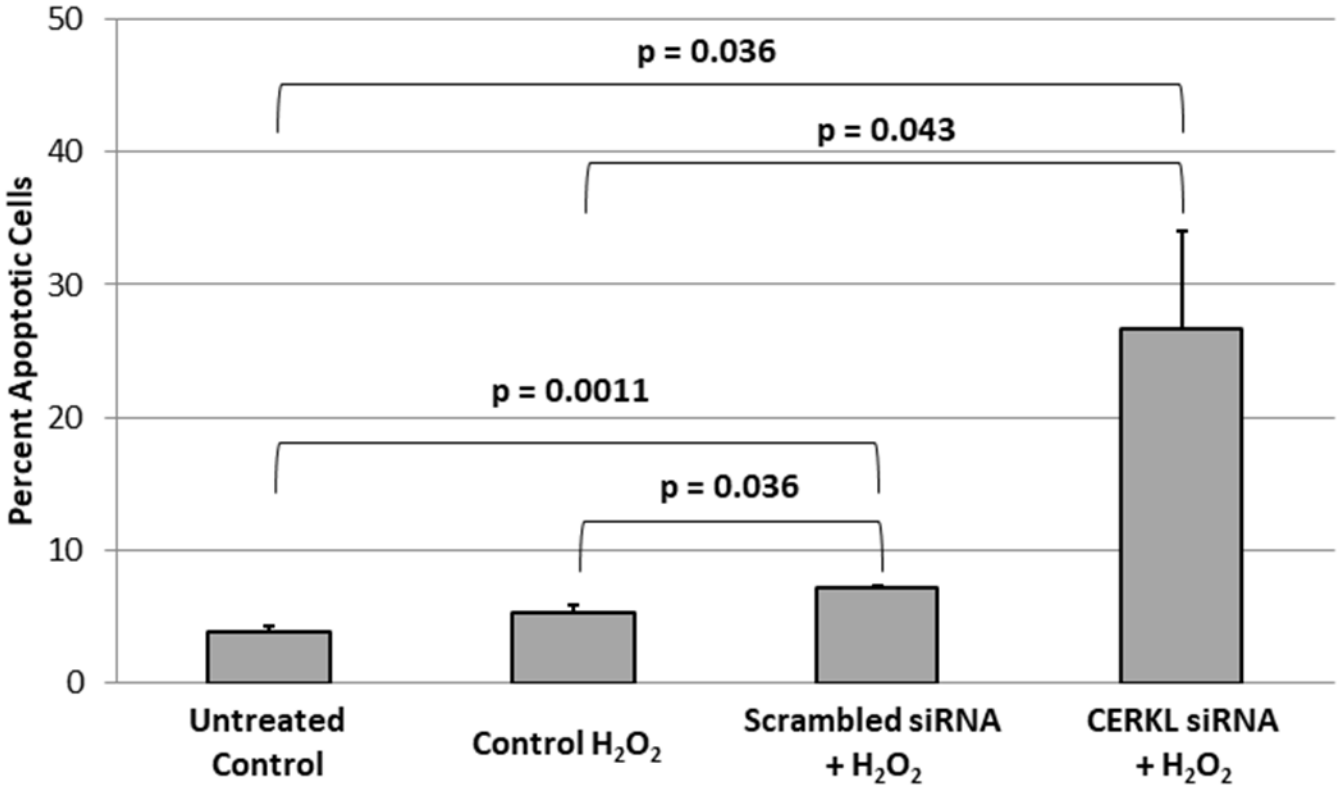

Figure 4. CERKL enhances SCC survival in response to oxidative stress.

SCC12F2 cells were treated with scrambled control or anti-CERKL siRNA for 48 hours, then treated with or without 250 uM H_2O_2 for an additional 18 hours. SCC12F2 cells not treated with siRNA were also treated with H_2O_2 in parallel for comparison. Cells were then collected, and apoptosis was quantified as annexin-V staining by flow cytometry [17]. Data are presented as the mean +/− SEM (N=3 per group).