Supplemental Information

mTOR inhibition prevents epithelial stem cell senescence and protects from radiation-induced mucositis

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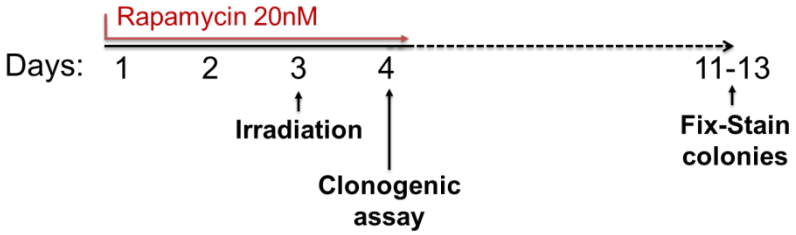
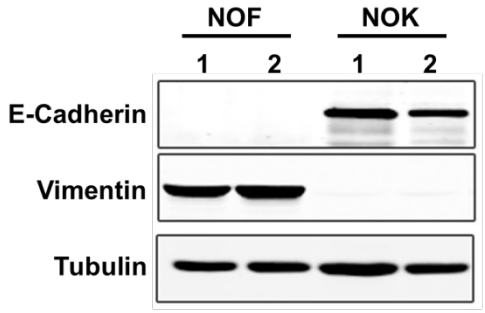
I- SUPPLEMENTAL FIGURES

Figure S1: Fractionated radiation and drug treatment scheme for HNSSC and NOK cells and characterization of primary human NOK cells. (A) Cells were plated and treated with vehicle or 20nM rapamycin for 3 days. Cells were then irradiated with 0 (control), 3 or 8 Gy, and maintained in vehicle or rapamycin for 24 hs. Afterwards, cells were subjected to the clonogenic assay. Note that cells under all conditions were plated in rapamycin free media during the clonogenic assay. See details in the Methods section. **(B)** Western blot analysis of the epithelial marker E-cadherin and the mesenchymal marker vimentin in two independent isolations of human oral primary normal oral fibroblasts (NOF) and keratinocytes (NOK). **(C)** Representative bright field image (left) and actin (green) nuclei (blue) (left) staining of primary NOK. **(D)** Three-dimensional organotypic culture of primary normal oral keratinocytes and oral fibroblasts. H&E staining shows the different layers from the basal proliferative cells to the parakeratinized (differentiated) keratinocytes in the upper layers. Organotypic culture was performed according to (Gangatirkar et al., 2007; Leelahavanichkul and Gutkind, 2012). **(E)** Upper view (left) and 3D reconstruction (right) of the grafting of primary oral keratinocytes expressing histone 2B fused to GFP in the back of a nude mouse, detected by two photon microscopy. Grafting was performed according to (Jensen et al., 2010). Briefly, NOK were infected with lentiviruses expressing histone 2B fused to GFP and incubated for 24 hs. NOK were then harvested along with human oral primary fibroblasts and mixed in a ratio $1x10^6$ of each one and used for the grafting procedure. 15 days after grafting, grafts were removed from the animal and analyzed by two photon microscopy for the presence of surviving NOK (detected by the fluoresce of GFP) in the different layers of the epidermis. Related to Figure 1.

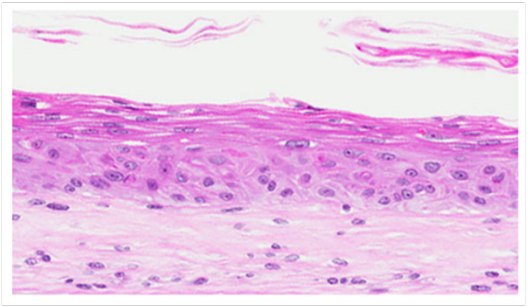
A


Rapamycin 20nM
Days:
1
2
3
4
11-13
Irradiation
Clonogenic assay
Fix-Stain colonies

B


NOF
NOK
1
2
1
2
E-Cadherin
Vimentin
Tubulin

D



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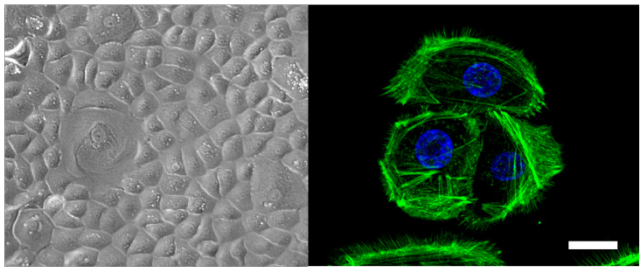

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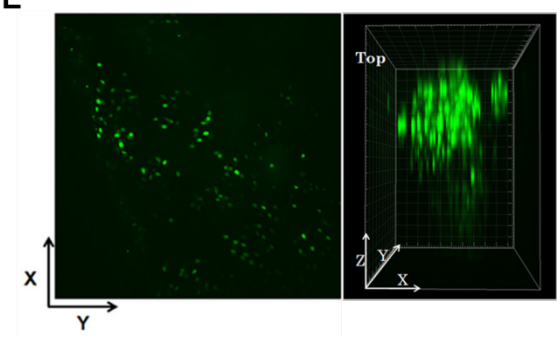
Top
X
Y
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Figure S2: Proliferation levels after rapamycin treatment. Labeling of individual NOK proliferating cells by EdU staining (red) after 72 hs of rapamycin treatment, counterstained with nuclei (blue). Bar: 100µm. Related to Figure 2.

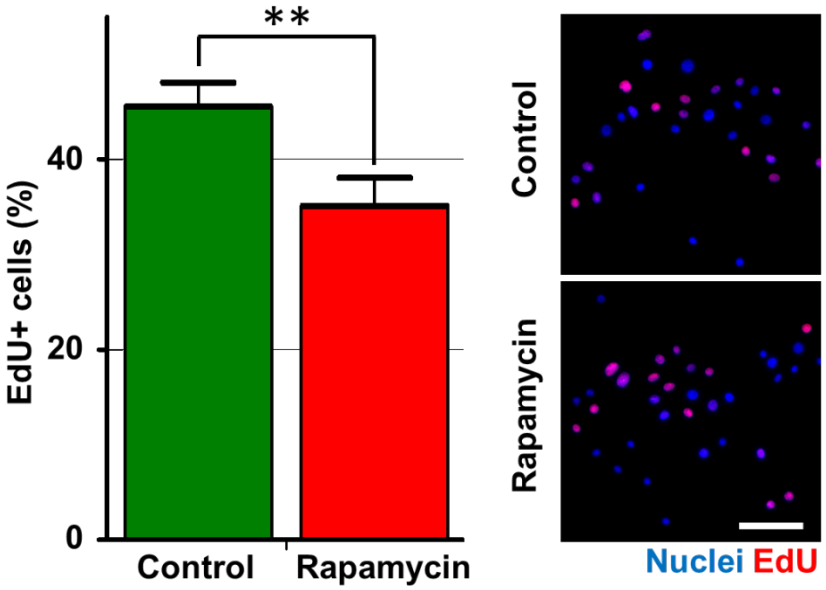

Figure S3: Ratio of reduced/oxidized GSH, SOD mRNA expression levels and MnSOD acetylation levels. (A) Ratio of reduced/oxidized GSH (GSH/GSSG ratio) in NOK and HN12 cells treated (Rap) or not (Con) with rapamycin for 72 hs **(B)** mRNA levels of cytoplasmic SOD (Cu-ZnSOD) and mitochondrial SOD (MnSOD) in NOK treated or not (Control) with rapamycin for 72 hs. **(C)** MnSOD was immunopurified (IP) from NOK treated for 3 days with media containing or not rapamycin (Rap). Its acetylation status was detected by immune-Western blotting (IB) with acetyl-lysine antibody (α-AcK). Rapamycin increased the amount of both total (IB: α-MnSOD) and acetylated (IB: α-AcK) MnSOD. Related to Figure 4.

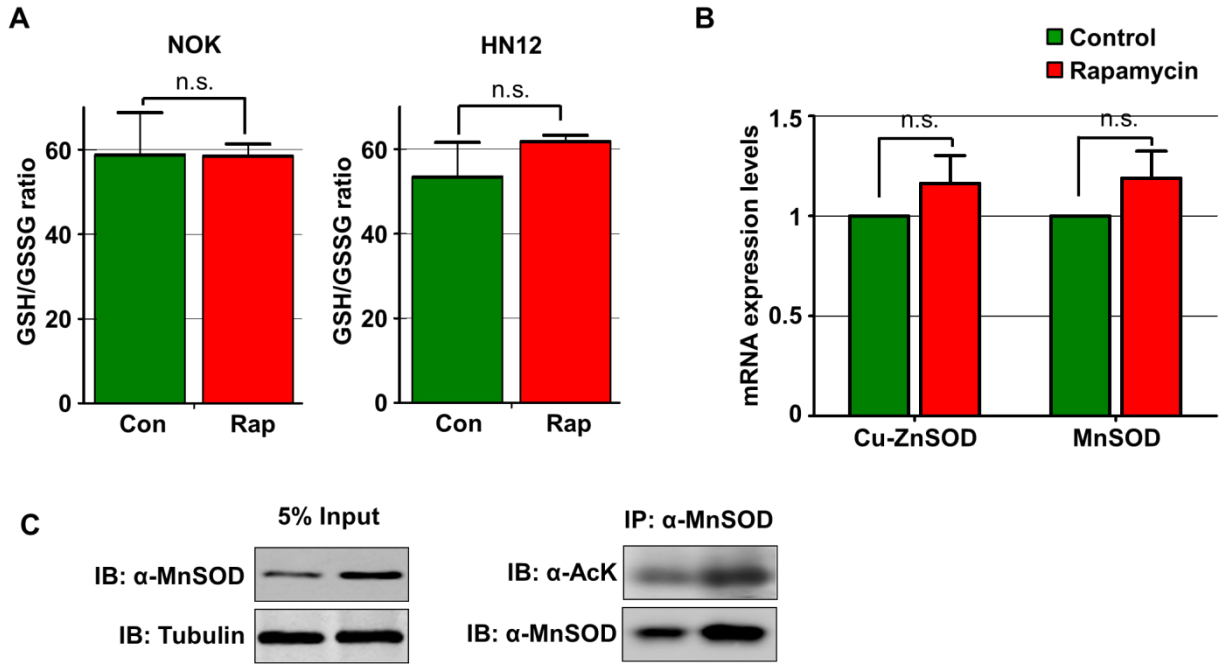

Figure S4: Histology of non-irradiated rapamycin-treated mice and rapamycin effect in irradiated mice with high intensity in a single dose. (A) Representative histology of the tongue from a non-irradiated rapamycin-treated mouse 5 days after the last dose of rapamycin. Mice received the same treatment scheme as shown in Figure 5A, except that mice were not irradiated. Small panels show details at higher magnification. Tongue shows normal dorsal and ventral epithelial morphology. **(B)** Single dose radiation and drug treatment scheme for C3H mice. **(C)** Quantification of tongue ulceration stained with toluidine blue from irradiated animals on day 5 following radiation, receiving vehicle (control) or rapamycin at the moment of radiation. Quantification represents at least 6 animals per group. Related to Figure 5.

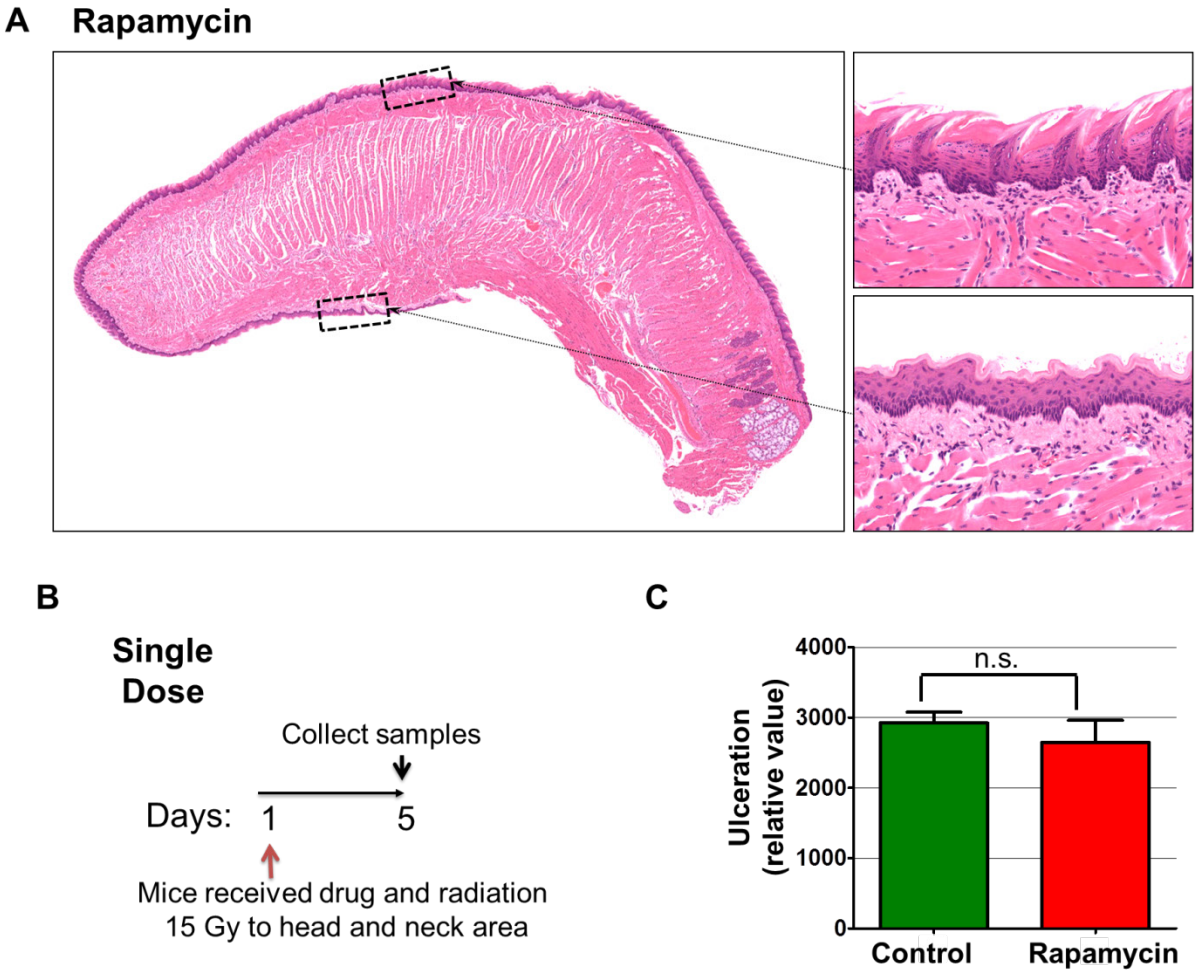

Figure S5: Rapamycin treatment increases the expression of p63 in the epithelial stem cell compartment *in vivo*. Expression levels of the stem cell marker p63 in tongues from non-irradiated animals treated or not with rapamycin at the end of the treatment period (day 5) or 5 days after the last dose of rapamycin (day 10) (See Figure 5 for treatment scheme). Graph shows the average fluorescence value per nucleus in the basal layer, expressed in arbitrary units and quantified as described in the methods section. Related to Figure 6.

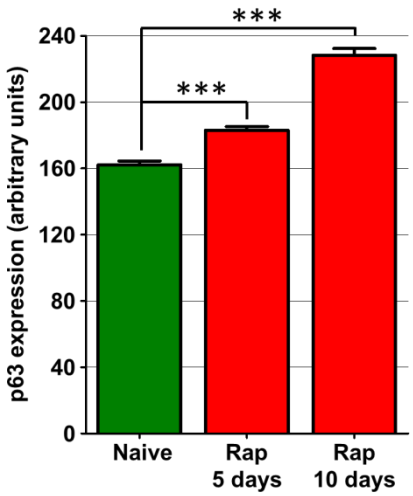

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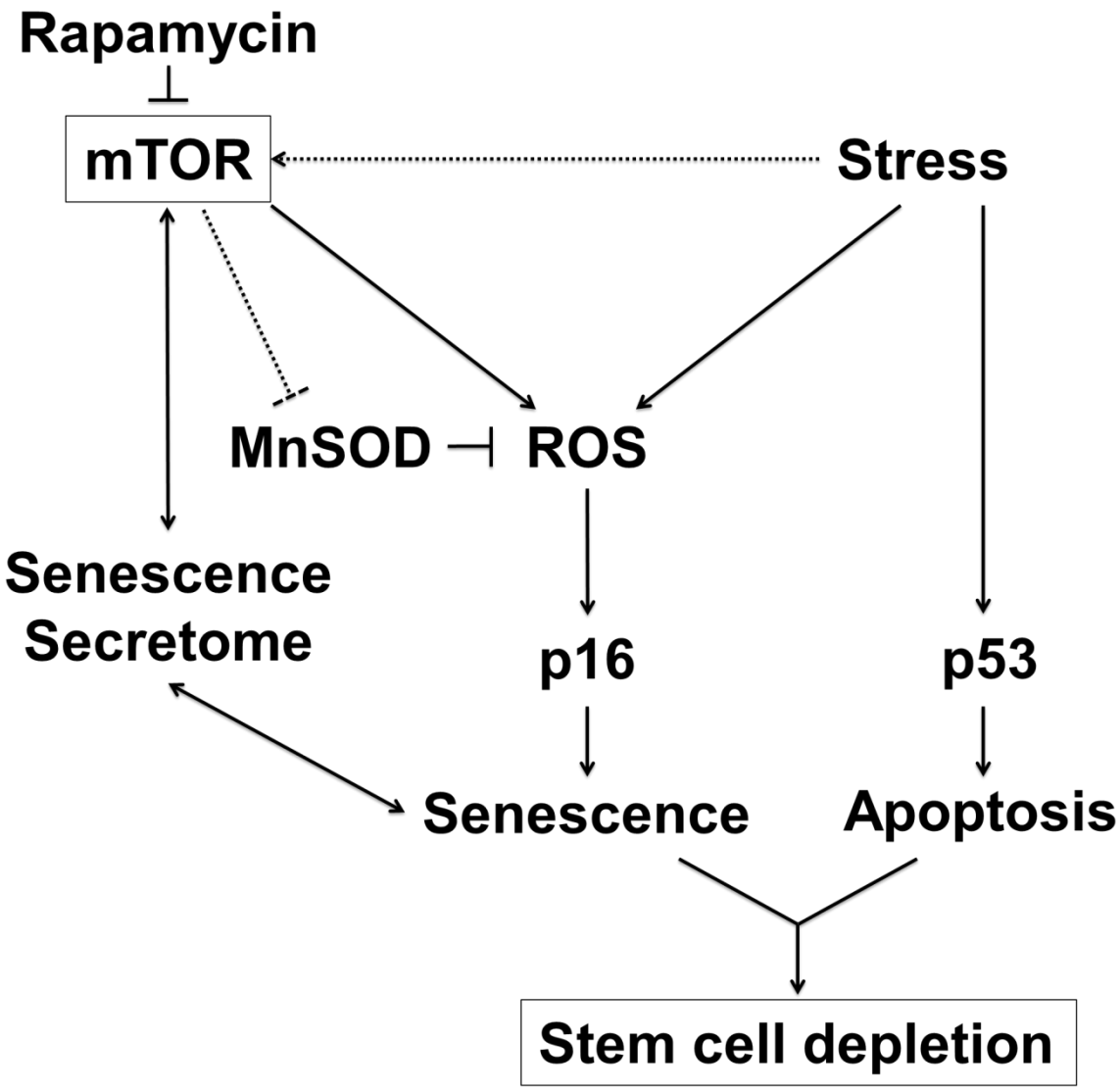
Figure S6: Schematic model of the proposed mechanisms by which mTOR inhibition prevents epithelial stem cell depletion. The emerging picture is that inhibition of mTOR by rapamycin protects normal epithelial stem cells from entering senescence and terminal differentiation programs. This process involves the increased expression of mitochondrial superoxide dismutase (MnSOD) upon rapamycin treatment, thereby reducing ROS-induced DNA damage and oxidative stress, and the consequent induction of p16 expression and irreversible cell senescence. In parallel, rapamycin decreases the release of multiple cytokines (collectively referred to as the senescence secretome), which may act as part of a positive feedback loop accelerating the senescence of the epithelial stem cells and their adjacent cells in an autocrine and paracrine fashion. While rapamycin does not prevent the radiation-induced accumulation of p53 and apoptosis in normal cells, the inhibition of mTOR may protect from the depletion of tissue repopulating epithelial stem cells by preventing ROS accumulation and the release of a pro-senescent and pro-inflammatory secretome, ultimately protecting from radiation-induced mucositis. Related to Figure 6.


Rapamycin
mTOR
Stress
MnSOD
ROS
Senescence
Secretome
p16
p53
Senescence
Apoptosis
Stem cell depletion

II- SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell Culture and siRNA transfections

NOK isolation and culture was performed as described (Leelahavanichkul and Gutkind, 2012). Briefly, small (0.1-0.3 cm) gingival biopsies obtained from healthy volunteers under an NIH-approved clinical protocol (NIH-NIDCR, protocol 06D0144) were rinsed with phosphate buffer saline (PBS) and incubated with trypsin (0.25%) solution overnight at 4^{o}C. Next day the epithelium was peeled and scraped with forceps, finely minced with scalpels and passed through a cell strainer (100 μm). Cells were pelleted at 125g and plated in 60-mm dish coated with 0.3mg/ml of collagen I in 1% acetic acid (BD Biosiences). NOK were maintained in defined keratinocyte serum free media (KSFM) (Invitrogen) supplemented with antibiotics at 37°C in the presence of 5% CO_2 and passed every 3-4 days. NOK between passages 2 to 4 were used for the experiments. HN12 and Cal27 cells were maintained in DMEM (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics, at 37°C in the presence of 5% CO_2. siRNAs were purchased from Thermo-Dharmacon (siGENOME SMARTpool siRNA for MnSOD, catalog M-009784-02-0005; p16, catalog M-011007-03-0005; and non-targeting control siRNA, catalog D-001206-13) and transfected at a final concentration of 80 nM using Lipofectamine RNAiMAX (Invitrogen), according to manufacturer's instructions.

Irradiation and clonogenic assays

Cells were plated in 3.5 cm dishes and treated with vehicle or 20nM rapamycin (LC Laboratories) for 3 days, and then γ-irradiated with 0 (control), 3 or 8 grays (Gy), and kept with

vehicle or rapamycin for 24 hours (hs) (see treatment scheme in Figure S1A). Afterwards, cells were trypsinized, counted with an automated cell counter (Scepter, Millipore) and replated in duplicate in 6 well plates at 400, 800 or 1200 cells per well. Cells were grown for further 7 to 10 days and the resulting colonies were fixed in 3.2% paraformaldehyde and stained with 0.5% of crystal violet in PBS for 30 min at room temperature. Plates were washed with running water to remove excess of labeling and scanned. Colonies were counted and measured with calibrated images in ImageJ with the analyze particles function. Surviving fraction was calculated as previously described (Franken et al., 2006) relative to the respective non-irradiated cells. Colony forming efficiency was determined using the same protocol but expressed as the proportion of plated cells that formed colonies relative to the number of colonies formed by non-irradiated control cells. For siRNA experiments cells were transfected with the corresponding siRNA 24 hs before rapamycin treatment as described above.

Immunoblot Analysis

Cells were treated and irradiated as described above and harvested 30 min or 24 hs after radiation. Cells were lysed at 4 °C in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40) supplemented with protease (protease inhibitor cocktail, Sigma) and phosphatase inhibitors (1mM Na_3VO_4 and 1mM NaF). Equal amounts of total cell lysate proteins were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Bands were detected by using near-infrared fluorescence (Odyssey LI-COR Biotechnology) with goat anti-mouse coupled to IRDye700CW (LI-COR Biotechnology) or goat anti-rabbit coupled to Alexa Fluor 680 (Invitrogen). The relative contribution of individual bands was calculated using the Odyssey Application Software v3 (LI-COR Odyssey). Primary

antibodies used were: phospho Akt 473 and 308 (1:500, Cell Signaling), Akt (1:500, Cell Signaling), S6 (1:500, Cell Signaling), phospho S6 (1:500, Cell Signaling), 4EBP (1: 500, Cell Signaling), GAPDH (1:1000, Cell Signaling), γH2AX (1:500, Millipore), p16 (1:50, Santa Cruz), p63 (1:500, Santa Cruz), α-tubulin (1:2000, Sigma), involucrin (1:2000, Sigma), human p53 (1:2000, Dako), MnSOD (1:150, Santa Cruz), Cu-ZnSOD (1:150, Santa Cruz), Catalase (1:150, Santa Cruz).

Cytokine measurements

Cells were plated in 3.5 cm dishes and treated with vehicle or 20nM rapamycin for 3 days in complete KSFM. 24 hs previous to cytokine measurement, media was changed to KSFM without supplements. At the end of the 24 hs period media and cells were harvested. Media was filtered through 0.45µm PVDF low protein binding filter, and cytokines were analyzed by the Cytokine Core Laboratory, University of Maryland, using the Luminex Multianalyte System. The cell lysates were used to quantify cellular proteins. Values of cytokines were corrected to the protein and expressed as percentage of the control. Media that was not conditioned was completely absent of cytokines. Each value corresponds to duplicate measurements of 3 independent samples of NOK. Concentration of cytokines for cells under control conditions were (expressed as pg. of cytokine/ml/10µg protein ± SE): CXCL1 (GROα), 67±3; IL1β, 1.0±0.2; IL6, 41±7; IL8, 11.8±0.6; VEGFA, 12±2; TNF-α, 0.54±0.04.

Cell proliferation

Cell proliferation was evaluated using incorporation of 5-ethynyl-2′-deoxyuridine (EdU) with the Click-iT EdU Cell Proliferation Assay Kit (Invitrogen). Briefly, cells were treated with rapamycin 20nM or vehicle for 3 days and then incubated with 10 μM EdU for 6 hs before fixation, permeabilization, and EdU staining, which were carried out according to the kit's protocol. Cell nuclei were stained with Hoechst 33342 (Invitrogen). The proportion of cells incorporating EdU was determined by fluorescence microscopy and quantified using ImageJ "nucleus counter" plugin as described below. Eight different fields containing approximately 60 cells each were quantified per condition.

Cell apoptosis and senescence

NOK were plated in collagen coated LabTek chamber slides and treated with vehicle or 20nM rapamycin for 3 days. Cells were then γ-irradiated or not, and further treated with vehicle or rapamycin for 24 hs. For TUNEL assay cells were processed for staining immediately, for SA-β-gal cells were passed and assessed 4 days after plating. Apoptosis was detected using the Click-iT TUNEL Alexa Fluor 594 Imaging Assay (Invitrogen) and SA-β-gal activity was measured using the SA-β-gal kit (Cell Signaling Technology) according to the manufacturer's instructions. The proportion of positive cells was determined by fluorescence or transmission microscopy and quantified using ImageJ. Eight different fields containing approximately 60 cells each were quantified per condition. For the calculation of population doublings cells were cultured as described and treated with vehicle or 20nM rapamycin. Population doublings were calculated every passage using the formula x = [log10(NH/N1)]/log10(2)] (Cristofalo et al., 1998), where

N1 is the inoculum cell number and NH the cell harvest number. To yield the cumulated doubling doublings, the population doublings for each passage was calculated and then added to the population doubling levels of the previous passages.

ROS and GSH/GSSG measurement

ROS measurement was performed by incubating cells with Dihydroethidium (hydroethidine) (5mM stock solution stabilized in DMSO, Molecular Probes) at a final concentration of 1.5 µM in culture media without supplements or FBS for 10 min at 37 °C. Afterwards cell were washed with PBS, detached and analyzed by fluorescent activated cell sorting (FACS). FACS analysis was performed in a FACSCalibur flow cytometer (BD Bioscience). GSH and GSSG were measured in NOK and HN12 cells treated for 3 days with vehicle or 20nM of rapamycin with the GSH/GSSG-Glo™ Assay kit (Promega) according to manufacturer's instructions.

MnSOD quantitative PCR and immunopurification

Total RNA was isolated from cultures and processed as described (Martin et al., 2007). One µg of cDNA was used as template for quantitative PCR using iQ SYBR Green Supermix (Bio-Rad). Samples were analyzed using a Bio-Rad iCycler iQ multicolor real-time PCR detection system. Oligonucleotides used for amplification were from Quiagen for human MnSOD (catalog 330001 PPH01716B) and CuZnSOD (catalog 330001 PPH00234B). For immunopurification, proteins were extracted in lysis buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 10% glycerol, 2 mM $MgCl_2$, and 1% NP40) supplemented with a complete protease inhibitor cocktail (SIGMA). Protein extracts were subjected to centrifugation at 14,000 rpm for 10 min and then immunoprecipitated with specified antibodies overnight. Immunoprecipitates were extensively

washed with lysis buffer and eluted with loading buffer. Antibodies used were: Acetyl-lysine antibody (Cell Signaling) and MnSOD (Santa Cruz Biotechnology).

Animal irradiation

Female C3H mice, bred in the National Cancer Institute Animal Production Area (Frederick, MD), were used for this study. The mice were 7–9 weeks of age at the time of experimentation and weighed between 20 and 30 grams. All experiments were carried out under the aegis of a protocol approved by the National Cancer Institute Animal Care and Use Committee and were in compliance with the Guide for the Care and Use of Laboratory Animal (1996), National Research Council. The study was conducted in a blinded fashion. The head and neck area was irradiated by placing each animal in a specially built Lucite jig in such a way that the animal could be immobilized without the use of anesthetics (Vitolo et al., 2004). Additionally, the jig was fitted with a Lucite cone that surrounded the head and prevented head movement during radiation exposure. For single-dose radiation, mice were once injected intraperitoneally with rapamycin and irradiated at 15 Gy (see treatment scheme in Figure S4B). For fractionated radiation, mice were injected intraperitoneally every day for 5 days with rapamycin (5mg/kg) and irradiated at 6 Gy/day (see treatment scheme in Figure 5A). Ionizing radiation was delivered with a Therapax DXT300 X-ray irradiator (Precision X-ray) by using 2.0 mm Al filtration (300 kVp) at a dose rate of 1.9 Gy/minute. After radiation, animals were removed from the jig, housed (4 or 5 animals per cage) in a climate- and light-controlled environment, and allowed free access to food and water. On day 5 following the final radiation dose, animals were sacrificed. Some tongues were stained in a solution of 1% toluidine blue in 10% acetic acid and analyzed macroscopically. Repeated wiping with gauze soaked in acetic acid was continued until there

was no further recovery of dye. A negative result is indicated by no dye uptake or light, diffusely stippled uptake of dye. A positive result, identified as lack of epithelium and therefore an ulcer, is indicated by deep, royal blue staining in epithelium defects. Image quantifications were performed with NIH Image J software; tongues were selected, an automatic threshold was applied and blue staining was quantified as the average staining intensity value per tongue (expressed in arbitrary units). Result corresponds to 6 animals and is representative of 3 independent experiments. Finally, tongues were processed and embedded in paraffin, 3-μm sections were stained with H&E and microscopic analysis was conducted. Stained slides were scanned at 40x using an Aperio CS Scanscope (Aperio, CA, USA). For immunofluorescence staining, tongues were embedded in OCT and kept at -80^{o}C for cryosection.

Immunofluorescence

NOK were seeded on the coverslips coated with collagen, treated and irradiated as described above. One hour after radiation cells were washed with ice-cold PBS and fixed with 3.2% paraformaldehyde in PBS. After washing three times with PBS, cells were permeabilized with TritonX100 0.1% in glycine 200mM in PBS and nonspecific binding was blocked with 3% of bovine serum albumin (BSA) in PBS for 1 h. Fixed cells were incubated with the primary antibody (anti γH2AX; 1: 800) overnight at 4^{o}C, followed by 1.5 hs incubation with the secondary antibody (goat anti-mouse Alexa Fluor 488, Invitrogen). Then nuclei were stained with Hoechst 33342 (1:2000 Invitrogen) and actin was stained with Alexa 546-phalloidin (Invitrogen) according to the manufacturers' instructions. For tissue immunostaining, cryosections were mounted in silanated slides and fixed with 3.2% paraformaldehyde in PBS.

After washing three times with PBS, cells were permeabilized with TritonX100 0.5% in glycine 200mM in PBS and non-specific binding was blocked with 3% of BSA or 10% FBS in PBS for 1 h. Slides were then incubated with the primary antibody overnight at 4°C, followed by a 1.5 h incubation with the secondary antibody (goat anti-mouse and goat anti-rabbit Alexa Fluor 488 or 546, 1:800; Invitrogen). Then nuclei were stained with Hoechst 33342 and actin was stained with Alexa Fluor 546 or 488-phalloidin (Invitrogen) according to manufacturers' instructions. Primary antibodies used were γH2AX (1:800, Millipore), γH2AX-FITC (1:400, Millipore), pS6 (1: 500, Cell Signaling), mouse p53 (1:100, Dako), mouse Ki-67 (1:100, Dako), MnSOD (1:100, Santa Cruz). Images were taken using Zeiss Axio Imager Z1 microscope equipped with an Apotome device (Carl Zeiss) and a motorized stage. Tissue images are stitched from 3 to 5 different images taken with a Zeiss Plan APOCHROMAT 20x/0.8na objective using AxioVision 4.8 software with MosaiX (Carl Zeiss). For p63 (Fig 4C), Z scan was performed and final images are maximum intensity projections (MIP) from 4 to 5 focal planes. Final images were stitched and bright contrast adjusted with AxioVision 4.8 (Carl Zeiss).

Image quantifications

Image quantifications were performed with ImageJ with the MBF ImageJ bundle (http://www.macbiophotonics.ca/imagej/installing_imagej.htm). For p53 and γH2AX the intensity per nucleus was counted in epithelial cells in all layers. The epithelia region was selected on the basis of its histological appearance, an automatic threshold was applied and then the "nucleus counter" plugin was used to draw a region of interest (ROI) around each nucleus and the average gray value per nucleus was recorded (expressed in arbitrary units). Values

correspond to the average of approximately 600 cells coming from at least 6 different pictures each from 3 different mice. A similar method was used to count the number of Ki-67 and p63 positive cells. The number of total epithelial cells was calculated in the Hoechst image. The epithelia region (total and basal cells) was selected on the basis of its histological appearance in the Hoechst image and the same region was used to count cells in the Ki-67 and p63 image. Then a threshold was applied and the "nucleus counter" plugin was used to calculate the number of positive and total cells. In every picture the Ki-67 index was calculated as= (number of Ki-67+ cells/ total number of epithelial cells in all layers) x100. In every picture p63+ cell percentage was calculated as= (number of p63+ cells/ total number of basal epithelial cells) x100. Values correspond to the average of at least 6 different pictures each from 3 different mice. For MnSOD the intensity per picture of the basal layer of the epithelia was quantified. Values correspond to the average of at least 5 different pictures each from 3 different mice.

Statistical analysis

All analyses were performed in triplicate or greater and the means obtained were used for independent t-tests. Statistical analyses were carried out using the Prism 5 statistical analysis program (GraphPad, San Diego CA). Asterisks denote statistical significance (NS, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$). All data are reported as mean ± SEM.

III- SUPPLEMENTAL REFERENCES:

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Vitolo, J.M., Cotrim, A.P., Sowers, A.L., Russo, A., Wellner, R.B., Pillemer, S.R., Mitchell, J.B., and Baum, B.J. (2004). The stable nitroxide tempol facilitates salivary gland protection during head and neck irradiation in a mouse model. Clin Cancer Res *10*, 1807-1812.