

Reduced Sod2 Expression Promotes the Development of Atypical Flat Lesions and Dysplasia during Pancreatic Ductal Adenocarcinoma Progression

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Abstract

Mitochondrial reactive oxygen species (mROS) promote acinar-to-ductal metaplasia (ADM), an early step in the development of pancreatic ductal adenocarcinoma (PDAC). Manganese superoxide dismutase (SOD2, also known as MnSOD), the primary mitochondrial ROS detoxifier, shows reduced mitochondrial activity linked to higher PDAC risk, highlighting its clinical importance. Nevertheless, the precise function of SOD2 in PDAC development and progression remains unclear. We investigated SOD2's involvement in PDAC onset and progression by crossing Sod2^{fl/fl} mice with the p48Cre; LSL-KrasG12D (KC) model. Additional experiments used primary mouse acinar cells and macrophages to study ADM, ROS production, and early events that suppress Sod2. Clinical relevance was evaluated using human expression datasets and tissue samples. SOD2 levels decline in low-grade lesions, yet its deletion by itself does not trigger lesion development. When combined with oncogenic Kras, Sod2 knockout enhances dysplasia and cancer stem cell emergence within the standard PDAC progression route. It also elevates the occurrence of oxidative stress-resistant atypical flat lesions (AFLs), an understudied direct precursor to PDAC. Oncogenic Kras impairs mitohormesis—the adaptive response in which mitochondrial stress triggers protective mechanisms that improve cell survival. Signals from inflammatory macrophages lower Sod2 expression, elevating ROS and driving ADM and canonical PanIN progression. In contrast, atypical flat lesions (AFLs) upregulate compensatory antioxidants such as GPX4 and NRF1, which reduce senescence and lipid peroxidation. Additional inflammatory pressure increases dysplasia, hastening tumor development, while AFLs contribute to more poorly differentiated tumor regions. This holds clinical importance because poorly differentiated human PDAC is associated with worse survival and exhibits reduced SOD2 expression.

Keywords: PDAC, Oxidative stress, MnSOD, Initiation, Pancreatic cancer

Introduction

Mitochondrial reactive oxygen species (mROS) are key contributors to the formation and advancement of pancreatic cancer precursor lesions [1-4]. Macrophage-

derived factors such as TNF α and CCL5/RANTES, for instance, generate mROS that trigger acinar-to-ductal metaplasia (ADM), the initial event leading to precancerous pancreatic intraepithelial neoplasia (PanIN) [5]. ROS levels further influence oncogene-driven PanIN cells, where additional increases—arising from NADPH oxidases (NOX) and mitochondria—can induce senescence [6-8] or promote cancer stem cell properties [9]. PanIN cells may also counteract excessive ROS and avoid senescence by elevating stress-responsive and antioxidant regulators, including nuclear factor erythroid 2-related factor 2 (Nrf2) and nuclear protein 1 (Nupr1) [4, 10, 11]. Senescent cells, ROS-resistant

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PanIN cells, and cancer stem cells each potentially play distinct roles in pancreatic tumor development.

Healthy cells defend against oxidative injury from free radicals through the induction of antioxidant defenses [12, 13]. Superoxide dismutases (SODs) are essential antioxidant enzymes that convert the highly reactive superoxide radical ($O_2^{\bullet-}$) into less damaging hydrogen peroxide (H_2O_2). Among the SOD isoforms, SOD2 (MnSOD) is uniquely positioned in the mitochondrial matrix, where it safeguards cells from superoxide produced by the electron transport chain (ETC) during metabolism. In contrast, SOD1 is mainly cytosolic, and SOD3 is extracellular. Impaired SOD2 function causes mitochondrial defects and cellular injury, resulting in various disease states in tissues with high metabolic demand. Complete Sod2 knockout in mice leads to neonatal death [14].

Owing to its vital role in neutralizing free radicals, SOD2 is abundantly expressed in normal pancreatic tissue [15] but decreases in high-grade (HG)-PanIN lesions relative to low-grade (LG)-PanIN [16]. SOD2 expression is further reduced or absent in tumors [15, 17]. Consequently, genetic variants impairing SOD2 function raise pancreatic cancer susceptibility. The SOD2 A16V polymorphism, which influences mitochondrial targeting, confers roughly double the risk (odds ratio 1.96, $P = 0.04$) in individuals with the homozygous V/V genotype [18]. Likewise, the 1221G >A regulatory polymorphism modestly elevates risk (odds ratio 1.18; $P = 0.002$) in people with low vitamin E intake, which normally limits the formation of hydroxyl radicals and lipid peroxidation [19]. Consistent with clinical observations, studies in pancreatic cancer cell lines indicate that reduced SOD2 promotes cell proliferation and xenograft tumor growth in nude mice [15, 20], whereas SOD2 overexpression reverses malignant traits [15, 21]. Proposed mechanisms for SOD2 reduction in pancreatic cancer include promoter hypermethylation [22], direct mRNA suppression by miR-301a [17, 23], or indirect effects through miR-155-mediated inhibition of FOXO3a [24]. To date, however, no *in vivo* animal models have examined how SOD2 loss affects pancreatic cancer initiation or progression.

In the present study, we demonstrate that pancreas-specific Sod2 deletion, when combined with oncogenic Kras, augments ADM frequency, LG-PanIN formation, and abnormal tissue changes. It also induces atypical flat lesions (AFLs), which are direct, senescence-resistant precursors to PDAC [25]. We further show that, in the

presence of added inflammatory stimuli, this results in expanded poorly differentiated tumor regions and shortens median mouse survival by about 50%.

Materials and Methods

Mouse lines, treatment and endpoint

Animal sex was not factored into analyses, as lesion development in KC mice does not differ by sex.

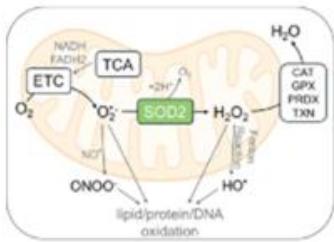
Transgenic mice — Bi-transgenic p48Cre; KrasG12D (KC) mice on the C57BL/6J background were produced by crossing LSL-KrasG12D mice (NCI Mouse Repository; MMHCC) with p48Cre mice (provided by Dr. Pinku Mukherjee, University of North Carolina) as previously described [26, 27]. B6-Sod2fl/fl mice (C57BL/6J background), originally generated by Dr. Takahiko Shimizu (Tokyo Metropolitan Institute of Gerontology) and obtained through Dr. Manisha Patel (University of Colorado Denver), are described in Ikegami *et al.* [28]. KC; Sod2^{-/-} mice were generated via the following breeding strategy: (1) p48Cre/+ × Sod2fl/fl → p48Cre/+; Sod2+/fl (2) p48Cre/+; Sod2+/fl × Sod2fl/fl → p48Cre/+; Sod2^{-/-} (3) LSL-KrasG12D × Sod2fl/fl → LSL-KrasG12D; Sod2+/fl (4) LSL-KrasG12D; Sod2+/fl × Sod2fl/fl → LSL-KrasG12D; Sod2fl/fl (5) p48Cre/+; Sod2^{-/-} × LSL-KrasG12D; Sod2fl/fl → p48Cre/+; LSL-KrasG12D/+; Sod2^{-/-} (KC; Sod2^{-/-}).

Caerulein treatment and humane endpoint — Each caerulein cycle involved eight hourly intraperitoneal (IP) injections (0.05 mg/kg) administered on two successive days. Treatment began at 2 months of age with the first cycle, followed by a 2-week interval; this regimen was repeated for 3 total rounds (**Figure 5b**). Mice were monitored closely and euthanized upon reaching humane endpoints. All procedures complied with institutional guidelines of the Mayo Clinic IACUC (an AAALAC-accredited facility), including criteria such as tumors ≥ 2 cm in longest dimension, tumors ≥ 10% of body weight, interference with vital functions, or ≥ 20% body weight loss.

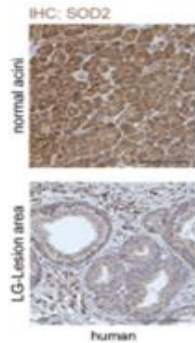
Human pancreatic tissue samples

Tissue microarrays (TMAs) were sourced from US Biomax Inc. (Rockville, MD). TMA BIC14811b was used for **Figure 1b** and PA805c for **Figures 5e and 5f**. All samples were deidentified and not classified as human subject research. Tissue quantified in **Figure 1c**

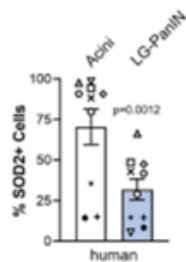
was deidentified and analyzed under Mayo Clinic IRB 19–012566.



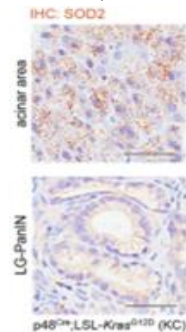
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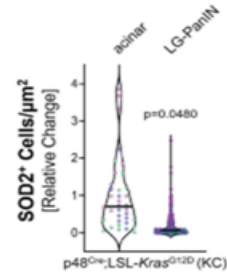
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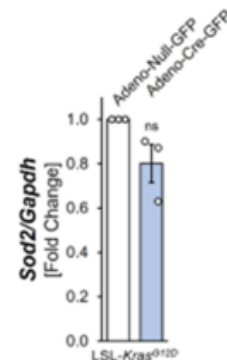
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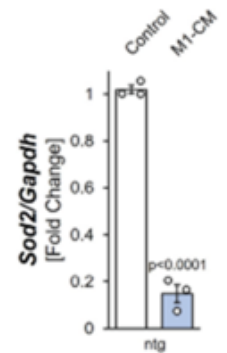
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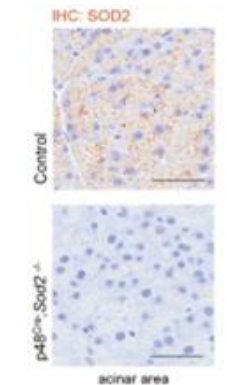
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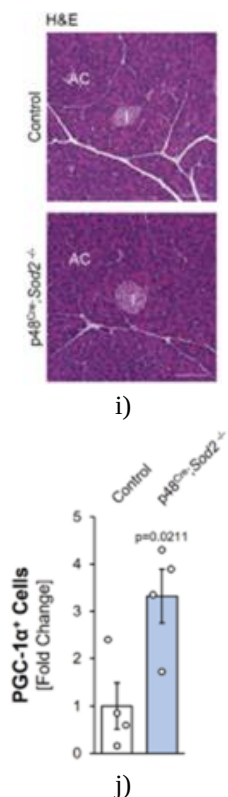


Figure 1. Levels of SOD2 decline within low-grade pancreatic precursor lesions. However, removing Sod2 alone does not cause lesion development.

(a) Overview diagram depicting SOD2's function in clearing reactive oxygen species. The mitochondrial electron transport chain (ETC) produces superoxide ($O_2^{\bullet-}$), which can interact with nitric oxide ($NO^{\bullet}$) to create peroxynitrite ($ONOO^-$) or be dismutated into hydrogen peroxide (H_2O_2) through the action of mitochondrial superoxide dismutase (MnSOD/SOD2). Subsequently, H_2O_2 can participate in the Fe^{2+} -mediated Fenton reaction to yield the extremely reactive hydroxyl radical ($OH^{\bullet}$), or it can be safely reduced to water (H_2O) by cellular antioxidant enzymes and molecules such as catalase (CAT), glutathione peroxidases (GPXs), peroxiredoxins (PRDXs), or thioredoxin (TXN). (b) Images from immunohistochemistry showing SOD2 protein in regions of normal human acinar tissue (top panel) compared with a characteristic low-grade PanIN (LG-PanIN) lesion. Scale bars represent 100 μm . (c) Analysis of the proportion of SOD2-positive cells in normal acinar tissue adjacent to lesions versus LG-PanIN areas from tissue samples of 10 patients. Individual symbols correspond to each patient. Error bars denote the standard error of the

mean (SEM), with significance determined by a paired t-test. (d and e) Example photomicrographs (d) and quantitative assessment (e) of SOD2 immunostaining intensity in acinar compartments or LG-PanIN structures in pancreata from 14-week-old p48Cre; LSL-KrasG12D (KC) mice. Scale bar = 50 μm in panel D. Each colored symbol in the graph indicates data from a separate mouse ($n = 3$ mice total). A statistical comparison was performed using a paired t-test with a ratio, showing significance (e). (f) Relative Sod2 mRNA expression measured in LSL-KrasG12D-derived acinar cells following infection with control Adeno-Null-GFP or Adeno-Cre-GFP vectors. Results are from three independent biological replicates. Error bars show SEM. Paired t-test revealed no statistically significant difference (ns). (g) Sod2 transcript levels in primary acinar cells obtained from non-transgenic (ntg) mice and incubated with either control medium or conditioned medium from M1-polarized macrophages (M1-CM). Data represent three separate experiments. Error bars indicate SEM; a paired t-test confirmed statistical significance. (h) Typical SOD2 immunohistochemistry photomicrographs of pancreatic sections from 14-week-old control animals and p48Cre; Sod2^{-/-} mice. Scale bars = 50 μm . (i) Hematoxylin and eosin-stained sections from 14-week-old control or p48Cre; Sod2^{-/-} mice. Labels indicate 'AC' for acinar regions and 'I' for islets. Scale bars = 100 μm . (j) Graph displaying fold-change in the percentage of PGC-1 α -positive cells among total cells counted. Each data point represents one mouse ($n=4$ per group); error bars reflect SEM. Statistical significance was assessed by t-test.

Histology, imaging, and quantification

Immunohistochemistry — Staining was carried out on tissue fixed in formalin and embedded in paraffin. After deparaffinization and rehydration steps performed according to prior methods [4], antigen unmasking occurred in either 10 mM sodium citrate (pH 6.0) or Tris/EDTA (pH 9.0) buffer. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 5 minutes. Sections were rinsed in 0.5% Tween-20/PBS and then blocked for 5 minutes at room temperature using Protein Block Serum-Free Solution (Agilent Technologies, Inc./Dako, Santa Clara, CA). Primary antibodies along with fluorescent secondary reagents were diluted in Antibody Diluent Background Reducing Solution (Agilent Technologies, Inc./Dako). Immunofluorescent detection involved incubation with

donkey-anti-goat IgG (H+L) at 1:500 in the presence of DAPI (1:1000, Sigma-Aldrich, St. Louis, MO) for 1 hour at room temperature. For chromogenic DAB development, the EnVision Plus Anti-Rabbit Labeled Polymer Kit (Agilent Technologies, Inc./Dako) or Rat-on-Mouse HRP-Polymer (Biocare Medical, Concord, CA) was applied.

Special stains — Routine Hematoxylin and Eosin (H&E) staining and Masson's Trichrome were executed following established procedures [29]. Senescence-associated β -galactosidase activity was detected on 10 μ m fresh-frozen cryosections using a kit from Cell Signaling Technology. Cryosections were fixed for 15 minutes at room temperature with the kit's 1x Fixative Solution, rinsed twice in 1x PBS, and incubated in 1x β -Gal Staining Solution for 48 hours at 37 °C inside a sealed, non-CO₂ chamber. After incubation, slides were washed three times in 1x PBS and counterstained with Nuclear Fast Red for 5 minutes.

Imaging and quantification — Digitization of stained slides was achieved with the Aperio AT2 whole-slide scanner (Leica Biosystems, RRID: SCR_021256). Quantitative analysis used the positive pixel algorithm in Aperio ImageScope (Leica Biosystems) or the positive cell detection module in QuPath (RRID: SCR_018257).

Isolation of primary cells and experimental manipulation

Peritoneal macrophage isolation and polarization — Thioglycolate (5% aged broth, administered at 8% of body weight volume) was injected intraperitoneally into non-transgenic (ntg) mice aged 10–20 weeks. Peritoneal cells were harvested four days post-injection according to detailed protocols [29, 30]. In brief, following a midline incision that preserved the peritoneal lining, 5–7 mL of ice-cold RPMI 1640 containing 10% FBS and 1% Penicillin-Streptomycin was introduced into the cavity. After gentle massage, the lavage fluid was aspirated and the wash repeated. Collected cells were centrifuged (37 × g, 10 min, 4 °C), plated, and allowed to adhere. Non-adherent cells were removed by repeated washes, after which fresh medium was supplied, and cells were equilibrated overnight. All cultures were kept at 37 °C in 5% CO₂. Polarization toward the M1 state was induced by 24-hour treatment with lipopolysaccharide (LPS, 10 ng/ml) plus interferon-gamma (IFN γ , 20 ng/ml) before harvesting conditioned supernatants [31].

Primary acinar cell isolation, adenoviral transduction, and ADM assays — The full procedure for acinar cell preparation, viral infection, and three-dimensional

collagen culture for acinar-to-ductal metaplasia studies is available in Fleming Martinez and Storz [32]. In summary, the pancreas was excised, chopped into small pieces, and subjected to enzymatic digestion using 2 mg/ml collagenase (15 min, 37 °C, 220 rpm shaking). Dispersed cells underwent three washes in HBSS supplemented with 5% FBS (931 × g, 2 min, 4 °C), sequential filtration through 500 μ m and 105 μ m meshes, and resuspension in HBSS containing 30% FBS. Following centrifugation (233 × g, 2 min, 4 °C), cells were placed in complete Waymouth's medium (Waymouth MB 752/1 base plus 0.1 mg/ml soybean trypsin inhibitor, 1 μ g/ml dexamethasone, and 1% FBS). Adenoviral infection with Adeno-Null-GFP or Adeno-Cre-GFP (Vector Biolabs) was performed at 10⁷ pfu/ml, with gentle agitation for the first hour. For collagen-embedding experiments, infection was maintained for 3 hours before cells were mixed with collagen. Culture plates were pre-coated with collagen I, and acinar cells were suspended within a collagen–medium gel. After polymerization, Waymouth's complete medium was added to the gel and renewed daily as specified. When testing N-acetylcysteine (NAC), 5 mM NAC or vehicle (water) was included in both the embedding mixture and the overlay medium.

RNA isolation and quantitative real-time PCR

RNA was purified with the miRNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription to cDNA used the High-Capacity cDNA RT Kit (Applied Biosystems, Bedford, MA). Real-time PCR reactions employed TaqMan Fast Mix 2x (Applied Biosystems) with predesigned assays for Gapdh (Mm99999915_g1) and Sod2 (Mm01313000_m1). Amplification and detection occurred on a QuantStudio 7 Flex instrument (Applied Biosystems). Target gene expression was normalized to Gapdh, and relative quantities were calculated using the $\Delta\Delta$ CT method.

ROS detection assay

Harvested cells were suspended in phenol red-free HBSS and incubated with H2DFFDA probe (45 min, 37 °C, 5% CO₂). Cells were washed twice (233 × g, 2 min) and resuspended in phenol red-free complete Waymouth's medium. Parallel aliquots for cell counting were fixed with 4% paraformaldehyde (15 min, room temperature), washed (500 × g, 2 min), stained with DAPI (1:500, 10 min, room temperature), and washed again. Samples were transferred to black-walled, clear-bottom 96-well

plates. Fluorescence intensity was recorded on a BioTek Synergy HT reader (Winooski, VT) maintained at 37 °C with shaking. DAPI signal was acquired once at the start of the experiment; H2DFFDA fluorescence was measured every 15 minutes for 12 hours. Settings were: DAPI (gain 75; excitation 360/40 nm, emission 460/40 nm) and H2DFFDA (gain 55; excitation 485/20 nm, emission 528/20 nm).

Statistics and reproducibility

All quantitative histology assessments were performed on tissue from 3 to 5 mice per experimental condition. Mice were randomly assigned to groups, maintaining roughly equal sex ratios. No blinding was applied during the conduct of experiments or data collection. All obtained data were included in analyses. Quantification of immunohistochemical staining was performed using Aperio Positive Pixel Count or QuPath software. Unless otherwise specified, error bars represent mean $\pm$ standard deviation (SD). Statistical analyses were performed in GraphPad Prism (RRID: SCR_002798; GraphPad Inc., La Jolla, CA), with the specific tests indicated in the figure legends. Differences reaching $P < 0.05$ were regarded as statistically significant.

Results and Discussion

Expression of SOD2 decreases markedly in low-grade pancreatic lesions. However, targeted depletion of Sod2 by itself does not lead to the development of any lesions. Heightened generation of reactive oxygen species inside mitochondria (mROS) of acinar cells is capable of triggering acinar-to-ductal metaplasia [4, 5] along with the appearance of low-grade PanIN lesions (PanIN1A/B). These changes mark the beginning of the pathway that eventually gives rise to pancreatic cancer [33]. The enzyme manganese superoxide dismutase (MnSOD, also called SOD2) serves as the main mitochondrial defense mechanism that removes dangerous radicals produced during oxidative phosphorylation (**Figure 1a**). When SOD2 function is compromised, it can produce a variety of harmful effects in tissues with high energy demands [34, 35].

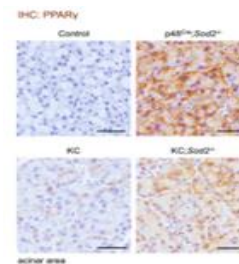
Evaluation of samples from patients with PDAC showed reduced SOD2 staining in LG-PanIN areas situated next to cancerous tissue, in contrast to nearby normal acinar regions (**Figures 1b and 1c**). The same reduction was evident in the KC mouse model (p48Cre; KrasG12D), which develops precancerous lesions and displayed clear

downregulation of SOD2 within LG-PanIN structures (**Figures 1d and 1e**).

Further experiments examined whether the presence of oncogenic KrasG12D, known to initiate PanIN formation, is responsible for reduced SOD2 levels. Direct comparison between non-transgenic control (ntg) mice and KC mice revealed no change in SOD2 levels within acinar cells due to KrasG12D. Parallel in vitro studies using primary acinar cells isolated from LSL-KrasG12D mice and activated via Adeno-Cre-GFP infection supported this conclusion (**Figure 1f**).

Given that macrophages activated by inflammation can induce oxidative stress within acinar cells and promote ADM without requiring oncogenic Kras [5, 36], we examined the effects of their secreted factors. Conditioned medium collected from M1-polarized macrophages reduced Sod2 expression in cultured acinar cells by roughly 80% (**Figure 1g**).

To explore whether eliminating Sod2 expression is enough on its own to cause ADM or LG-PanIN development, we produced mice carrying an acinar-cell-restricted Sod2 knockout (p48Cre; Sod2^{-/-}). This was achieved with a previously established conditional Sod2^{fl/fl} strain [28]. Complete loss of SOD2 protein occurred in acinar cells (**Figure 1h**), accompanied by elevated ROS as expected. Nevertheless, the overall pancreatic architecture stayed normal at both 14 weeks and 24 weeks, with no detectable abnormal regions or lesions (**Figure 1i**). This absence of pathology suggested the possible involvement of mitohormesis, a protective adaptation that activates when SOD2 is reduced or impaired [37]. Such a response enables cells to better tolerate ongoing mitochondrial stress by upregulating antioxidant defenses through low-level stimulation of PGC-1 α and PPAR γ pathways [37-39]. Supporting this mechanism, the acinar-specific Sod2 knockout mice exhibited an average 3.3-fold higher PGC-1 α expression (**Figure 1j**) as well as an average 183-fold increase in PPAR γ (**Figures 2a and 2b**).



a)

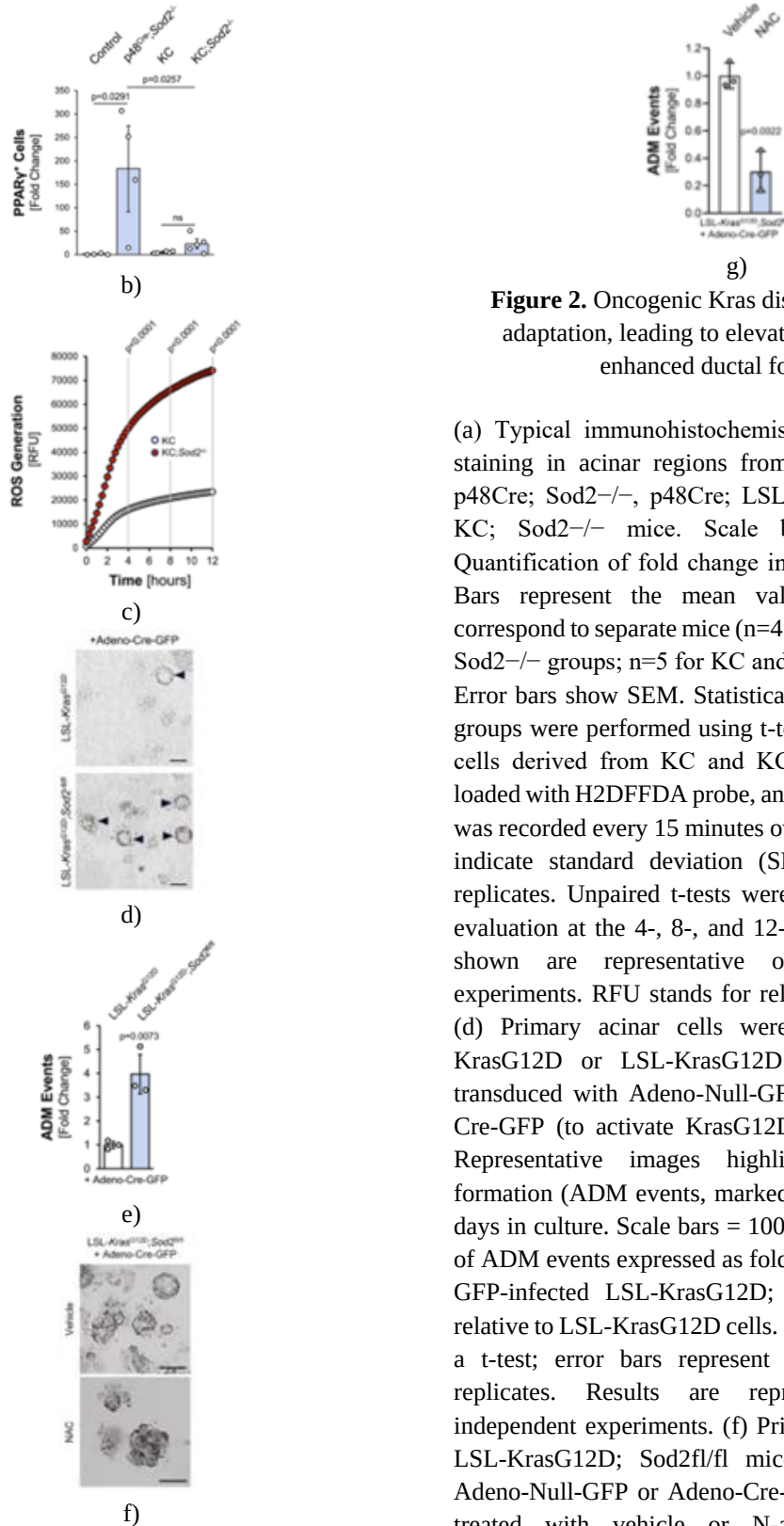


Figure 2. Oncogenic Kras disrupts mitohormetic adaptation, leading to elevated ROS levels and enhanced ductal formation.

(a) Typical immunohistochemistry images of PPAR γ staining in acinar regions from 14-week-old control, p48Cre; Sod2 $^{-/-}$, p48Cre; LSL-KrasG12D (KC), and KC; Sod2 $^{-/-}$ mice. Scale bars = 50 μ m. (b) Quantification of fold change in PPAR γ -positive cells. Bars represent the mean value; individual points correspond to separate mice (n=4 for control and p48Cre; Sod2 $^{-/-}$ groups; n=5 for KC and KC; Sod2 $^{-/-}$ groups). Error bars show SEM. Statistical comparisons between groups were performed using t-tests. (c) Primary acinar cells derived from KC and KC; Sod2 $^{-/-}$ mice were loaded with H2DFFDA probe, and fluorescence intensity was recorded every 15 minutes over 12 hours. Error bars indicate standard deviation (SD) across 5 technical replicates. Unpaired t-tests were applied for statistical evaluation at the 4-, 8-, and 12-hour time points. Data shown are representative of three independent experiments. RFU stands for relative fluorescent units. (d) Primary acinar cells were isolated from LSL-KrasG12D or LSL-KrasG12D; Sod2fl/fl mice and transduced with Adeno-Null-GFP (control) or Adeno-Cre-GFP (to activate KrasG12D and/or delete Sod2). Representative images highlight ductal structure formation (ADM events, marked by arrows) after three days in culture. Scale bars = 100 μ m. (e) Quantification of ADM events expressed as fold change in Adeno-Cre-GFP-infected LSL-KrasG12D; Sod2fl/fl acinar cells relative to LSL-KrasG12D cells. Statistical analysis used a t-test; error bars represent SD from 3 technical replicates. Results are representative of three independent experiments. (f) Primary acinar cells from LSL-KrasG12D; Sod2fl/fl mice were infected with Adeno-Null-GFP or Adeno-Cre-GFP and subsequently treated with vehicle or N-acetylcysteine (NAC). Representative images display ductal structures (ADM events) on day 3. Scale bars = 50 μ m. (g) Quantification

of ADM events shown as fold change in NAC-treated versus vehicle-treated Adeno-Cre-GFP-infected LSL-KrasG12D; Sod2fl/fl cells. t-test was used for statistics; error bars indicate SD from 3 technical replicates. Data are representative of three independent experiments.

Presence of oncogenic Kras suppresses mitohormetic adaptation and increases ROS and formation of ductal structures

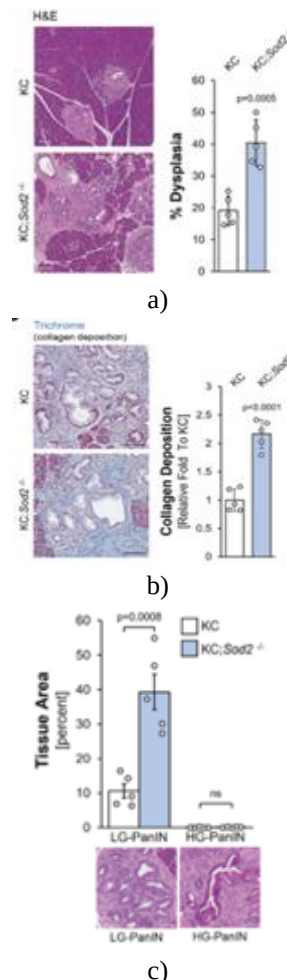
Because Sod2 deletion by itself failed to initiate early pancreatic lesions, we examined whether it could amplify known PDAC-promoting processes when combined with oncogenic Kras. To this end, Sod2fl/fl mice were crossed into the KC background. In the resulting KC; Sod2^{-/-} animals, the mitohormetic response was substantially weaker than in p48Cre; Sod2^{-/-} mice lacking the Kras mutation. PPAR γ expression in KC; Sod2^{-/-} pancreata rose only by a non-significant average of 4.6-fold relative to KC controls (**Figures 2a and 2b**), far below the 183-fold elevation seen in p48Cre; Sod2^{-/-} mice. Similarly, PGC-1 α levels in KC; Sod2^{-/-} versus KC mice increased by a non-significant average of 1.4-fold, in contrast to the 3.3-fold rise observed in p48Cre; Sod2^{-/-} animals.

Due to this diminished adaptive response, acinar cells from KC; Sod2^{-/-} mice exhibited a sharp rise in reactive oxygen species production compared with cells carrying only the KrasG12D mutation (KC) (**Figure 2c**). These observations indicate that SOD2 loss intensifies the metabolic alterations induced by KrasG12D that elevate mitochondrial ROS and promote ADM [4, 5]. To directly assess whether Sod2 knockout together with mutant Kras boosts ADM and ductal structure formation, primary acinar cells from LSL-KrasG12D or LSL-KrasG12D; Sod2fl/fl mice were embedded in collagen gels and transduced with Adeno-Cre-GFP to simultaneously activate KrasG12D and delete Sod2 (**Figure 2d**). In these ex vivo assays, KC; Sod2^{-/-} cells developed approximately four times more ADM lesions than KC cells (**Figure 2e**).

To confirm that the excess ROS was responsible for the increased ADM, we applied the antioxidant N-acetylcysteine (NAC), which reduced ADM formation by about 70% (**Figures 2f and 2g**). Taken together, these findings demonstrate that oncogenic Kras and SOD2 depletion act synergistically to drive pancreatic lesion development.

Sod2 knockout accelerates KrasG12D-driven dysplasia and cancer stem cell formation

We subsequently examined the consequences of Sod2 loss on precancerous changes within the KC model in vivo. Acinar-cell-specific Sod2 deletion in KC mice produced a statistically significant roughly two-fold expansion in dysplastic areas (including abnormal tissue, lesions, and fibrosis), rising from approximately 19.1% to 40.4% of total pancreatic area at 14 weeks (**Figure 3a**). This effect became even more evident by 24 weeks. Assessment of collagen deposition via trichrome staining revealed a comparable 2.2-fold increase in fibrotic content (**Figure 3b**). Notably, there was no corresponding rise in activated pancreatic stellate cells, as indicated by stable numbers of smooth muscle actin (SMA)-positive cells. This observation aligned with unchanged counts of Ym1-positive (alternatively activated) macrophages, a subset of M2 macrophages previously associated with stellate cell activation in the KC model [40].



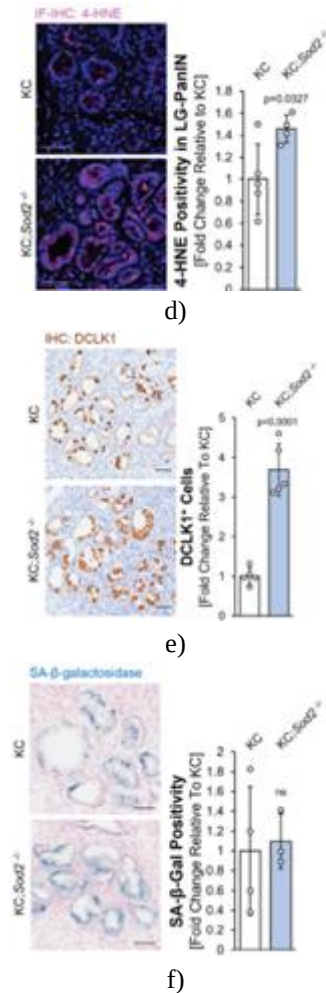


Figure 3. Loss of Sod2 promotes faster dysplasia and cancer stem cell development driven by KrasG12D.

(a) Typical H&E photomicrographs (left) with corresponding measurement of dysplastic tissue proportion (right) in pancreata of 14-week-old p48Cre; KrasG12D (KC) and p48Cre; KrasG12D; Sod2^{-/-} (KC; Sod2^{-/-}) mice (n=5 per group). Scale bars = 100 μ m. Data proportions were arcsine-transformed before t-test evaluation. Error bars reflect SD. (b) Example trichrome images demonstrating collagen (blue staining, left) and its quantification (right) across n=5 mice per group. Positive pixel counts were arcsine-transformed and expressed as fold change. Statistical significance was determined by t-test. Error bars represent SD. Scale bars = 100 μ m. (c) Area percentages occupied by low-grade PanIN (LG-PanIN) and high-grade PanIN (HG-PanIN) in KC versus Sod2^{-/-} mice (n=5 per group). Individual lesion regions were manually delineated to compute

tissue coverage; t-tests were used for comparisons. Error bars show SEM. Representative H&E images illustrate the different lesion categories. Scale bars = 50 μ m. (d) Photomicrographs (left) and quantitative analysis (right) of 4-HNE staining intensity within LG-PanIN lesions (KC n=5, KC; Sod2^{-/-} n=4). Error bars indicate SD; t-test confirmed significance. Scale bars = 50 μ m. (e) Characteristic staining patterns for p16INK4a/CDKN2A. Scale bars = 50 μ m. (f and g) Example images (f) and measured levels (g) of senescence-associated (SA) β -galactosidase staining (KC n=4, KC; Sod2^{-/-} n=3). Scale bars = 50 μ m. Error bars denote SD; t-test found no significant difference. Further assessment of 14-week-old pancreatic samples indicated a statistically meaningful elevation in the fraction of LG-PanIN within abnormal tissue, rising from 10.6% to 39.3% (**Figure 3c**). High-grade PanIN lesions, however, remained infrequent and showed only a modest, non-significant uptick (0.04% in KC mice versus 0.11% in KC; Sod2^{-/-}). While KC; Sod2^{-/-} pancreata contained more total lesions, the relative distribution between ADM and LG-PanIN inside abnormal zones at 24 weeks mirrored that of KC controls (ADM: 68% vs. 67%; LG-PanIN: 32% vs. 33%). Together with findings from the collagen explant experiments (**Figures 2d and 2g**), this points to increased initiation of ADM events in the absence of Sod2, yet comparable progression rates toward LG-PanIN.

In agreement with the pronounced oxidative stress measured in cultured KC; Sod2^{-/-} acinar cells (**Figure 2c**) and the weakened mitohormetic controls seen in whole tissue (**Figures 2a and 2b**), LG lesions in KC; Sod2^{-/-} mice accumulated more of the lipid peroxidation byproduct 4-hydroxy-2-nonenal (4-HNE), signifying greater in vivo ROS activity and cellular damage (**Figure 3d**). Heightened ROS in LG-PanIN is also linked to increased DCLK1-expressing cells [9], which are considered potential cancer stem cells (CSCs) due to their tumor-promoting capacity [41, 42]. LG-PanIN lesions in KC; Sod2^{-/-} mice indeed harbored an average of 3.7-fold more DCLK1-positive cells (**Figure 3e**). Collectively, these changes demonstrate that Sod2 removal hastens KrasG12D-mediated dysplasia and CSC expansion. We also checked whether excess ROS would heighten senescence in PanIN cells by assessing senescence-associated β -galactosidase (SA- β -Gal) activity. LG-PanIN cells in standard KC mice already displayed robust SA- β -Gal positivity, with no

meaningful additional rise in the KC; Sod2^{-/-} setting (**Figure 3f**).

Absence of Sod2 favors the emergence of oxidative stress-resistant atypical flat lesions (AFLs)

Abnormal pancreatic regions in KC; Sod2^{-/-} mice contained roughly 5.5 times more atypical flat lesions (AFLs) than in KC mice (0.5% versus 2.74%) (**Figure 4a**). These AFLs are flat, non-mucinous structures capable of arising straight from acinar cells and serving as immediate precursors to invasive pancreatic cancer (**Figure 4b**) [25, 43-45]. Measurement of surrounding collagen showed notably less deposition around AFLs compared with PanINs in KC; Sod2^{-/-} tissue, consistent with prior characterizations of their loosely organized stroma [25, 43]. Despite lacking SOD2, AFLs exhibited substantially reduced 4-HNE staining and therefore lower lipid peroxidation than PanIN lesions (**Figure 4c**). Correspondingly, AFL cells displayed diminished senescence, evidenced by lower SA- β -galactosidase activity relative to LG-PanIN cells (**Figure 4d**). Because these lesions avoided senescence, we checked for the CSC marker DCLK1. Still, we observed none, fitting with their reduced oxidative stress environment.

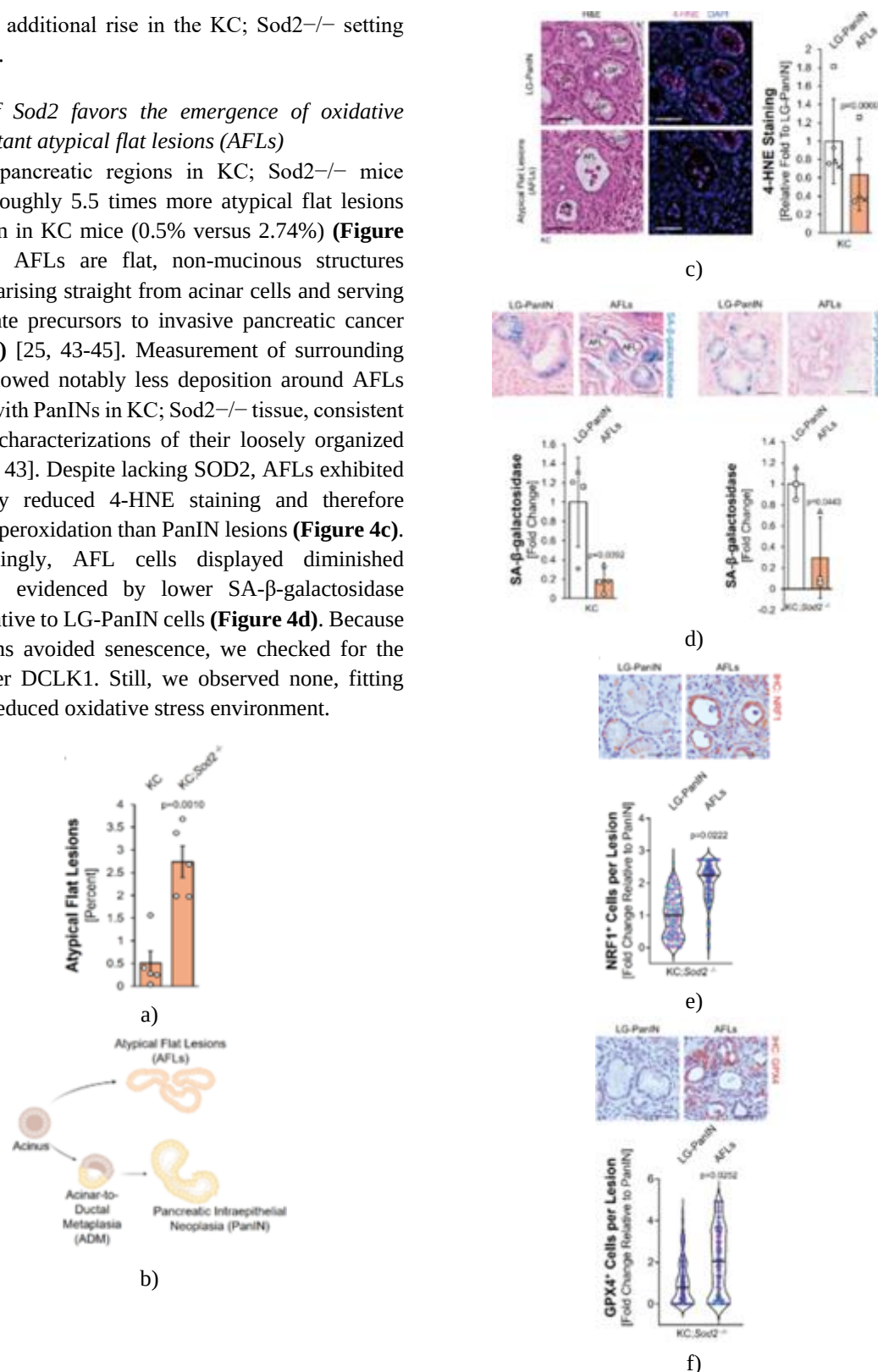


Figure 4. Sod2 deletion elevates the occurrence of oxidative stress-resistant atypical flat lesions (AFLs).

(a) Measurement of AFL percentage in abnormal tissue of 14-week-old KC and KC; Sod2^{-/-} mice. Each data point corresponds to one mouse (n=5 per group). Error bars represent SEM; t-test confirmed statistical significance. (b) Diagram illustrating how AFLs develop directly from acinar cells as an alternative route to pancreatic cancer, bypassing the conventional pathway involving acinar-to-ductal metaplasia (ADM) followed by pancreatic intraepithelial neoplasia (PanIN) lesions. (c) Quantification of 4-HNE levels with representative images in PanIN lesions versus AFLs from KC mice. Symbols represent individual mice (n=5). Error bars show SD; t-test indicated statistical significance. Scale bars = 50 μ m. (d) Example images (upper panels) and quantification (lower panels) of SA- β -galactosidase staining in LG-PanINs and AFLs from KC mice (left, n=4) or KC; Sod2^{-/-} mice (right, n = 3). Each symbol denotes one mouse. Scale bars = 50 μ m. Error bars indicate SD; t-test showed statistical significance. (e) Typical images (upper) and quantification (lower) of NRF1 immunohistochemistry in LG-PanIN versus AFLs in KC; Sod2^{-/-} mice. Scale bars = 50 μ m. Different colors represent data from separate mice (n=3); a paired t-test demonstrated significance. (f) Typical images (upper) and quantification (lower) of GPX4 immunohistochemistry in LG-PanIN versus AFLs in KC; Sod2^{-/-} mice. Scale bars = 50 μ m. Different colors represent data from separate mice (n=3); a paired t-test demonstrated significance.

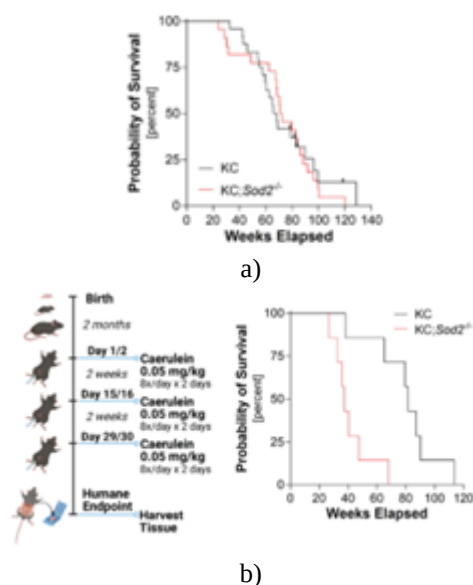
Next, we explored the mechanisms by which AFL cells in KC; Sod2^{-/-} mice offset the absence of SOD2 and limit oxidative injury. Initial analysis focused on PGC-1 α and PPAR γ levels to check for recovery of mitohormetic pathways in AFLs. However, PGC-1 α expression showed no significant elevation in AFLs compared with LG-PanIN, while PPAR γ levels were marginally lower (data not shown). Nuclear factor erythroid 2-related factor 2 (Nrf2), which partners with PGC-1 α and PPAR γ during mitohormesis [37], exhibited only a minor rise in nuclear localization. These observations indicate that mitohormetic adaptation is not reinstated in AFLs relative to PanIN lesions.

Rather than relying on mitohormesis, AFLs showed increased expression of the antioxidant regulator nuclear factor erythroid 2-related factor 1 (Nfe2l1/NRF1) (Figure 4e). NRF1 acts as a transcription factor that governs mitochondrial activity and drives numerous antioxidant genes [46], including glutathione peroxidase 4 (Gpx4) [47]. GPX4 plays a key role in neutralizing

lipid hydroperoxides and preventing lipid peroxidation-induced cell death [48]. Matching the rise in NRF1, GPX4 protein levels were also elevated in AFLs (Figure 4f), pointing to engagement of this protective cascade. Both NRF1 and GPX4 support mitochondrial integrity and curb oxidative stress, and have been linked to reduced senescence [49, 50].

Inflammation in KC; Sod2^{-/-} mice produces poorly differentiated tumor regions and hastens disease progression to endpoint

Given that Sod2 loss in the KC background boosts ADM and PanIN development (Figures 2d, 2e and 3c) while simultaneously generating AFLs (Figure 4a) — structures proposed as direct routes to malignancy [25, 43, 44] — we expected KC; Sod2^{-/-} mice to form pancreatic tumors more rapidly than standard KC animals. Although the resulting tumors retained SOD2 deficiency, overall long-term survival remained equivalent between KC and KC; Sod2^{-/-} cohorts (Figure 5a). Notably, pancreatic tissue collected at humane endpoint from KC; Sod2^{-/-} mice displayed lower lipid peroxidation compared with KC controls. Thus, while Sod2 knockout amplifies KrasG12D-driven dysplasia, it does not, by itself, accelerate the development of overt PDAC.



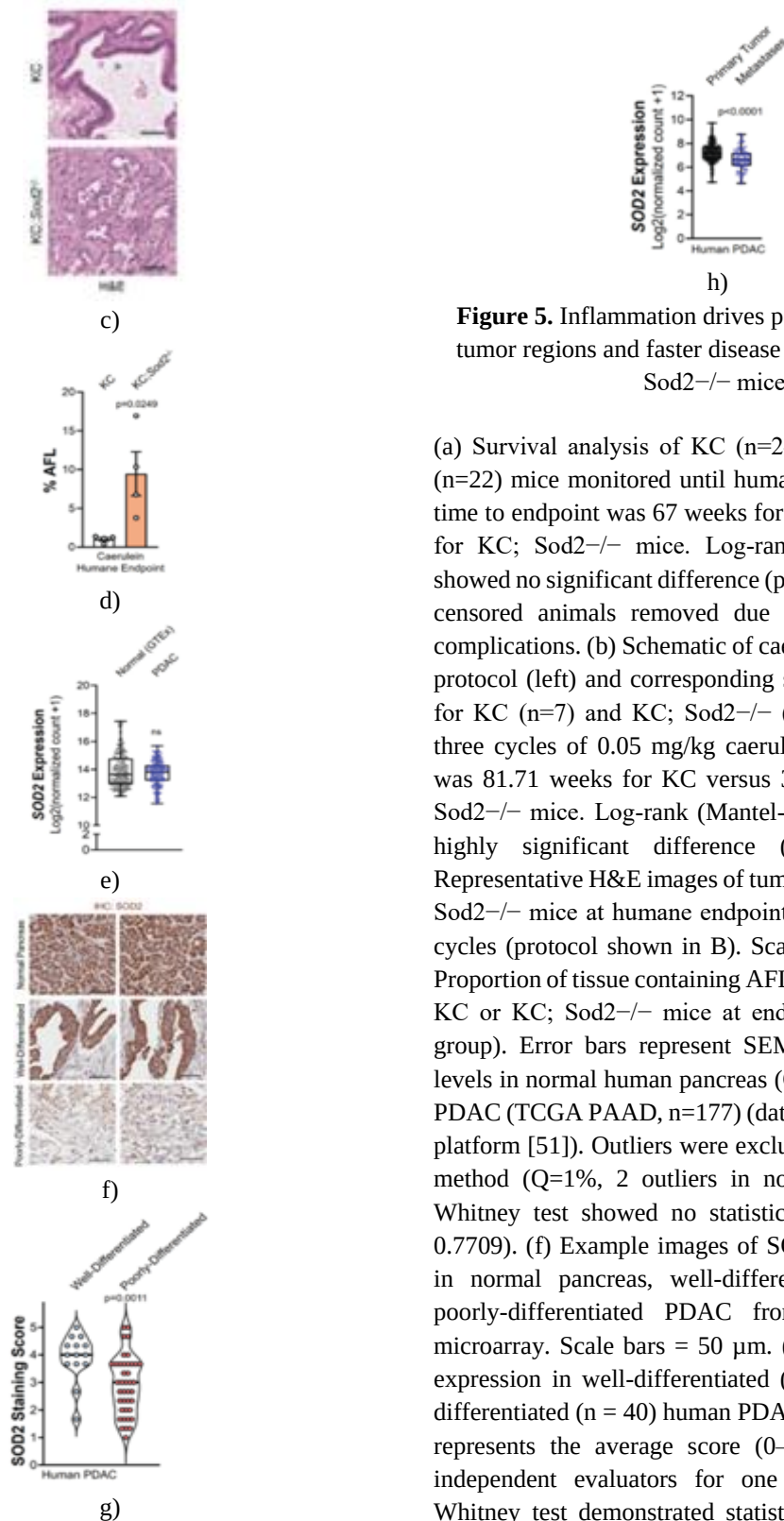


Figure 5. Inflammation drives poorly differentiated tumor regions and faster disease progression in KC; Sod2^{-/-} mice.

(a) Survival analysis of KC (n=24) and KC; Sod2^{-/-} (n=22) mice monitored until humane endpoint. Median time to endpoint was 67 weeks for KC and 71.50 weeks for KC; Sod2^{-/-} mice. Log-rank (Mantel-Cox) test showed no significant difference (p=0.7855). Ticks mark censored animals removed due to non-tumor health complications. (b) Schematic of caerulein administration protocol (left) and corresponding survival curve (right) for KC (n=7) and KC; Sod2^{-/-} (n=7) mice receiving three cycles of 0.05 mg/kg caerulein. Median survival was 81.71 weeks for KC versus 37.29 weeks for KC; Sod2^{-/-} mice. Log-rank (Mantel-Cox) test indicated a highly significant difference (P = 0.0028). (c) Representative H&E images of tumors from KC and KC; Sod2^{-/-} mice at humane endpoint after three caerulein cycles (protocol shown in B). Scale bars = 50 μm. (d) Proportion of tissue containing AFLs in caerulein-treated KC or KC; Sod2^{-/-} mice at endpoint (n=4 mice per group). Error bars represent SEM. (e) SOD2 mRNA levels in normal human pancreas (GTEx, n=167) versus PDAC (TCGA PAAD, n=177) (data obtained from Xena platform [51]). Outliers were excluded using the ROUT method (Q=1%, 2 outliers in normal group). Mann-Whitney test showed no statistical significance (P = 0.7709). (f) Example images of SOD2 immunostaining in normal pancreas, well-differentiated PDAC, and poorly-differentiated PDAC from a human tissue microarray. Scale bars = 50 μm. (g) Scoring of SOD2 expression in well-differentiated (n = 14) and poorly-differentiated (n = 40) human PDAC tumors. Each point represents the average score (0–5 scale) from three independent evaluators for one TMA core. Mann-Whitney test demonstrated statistical significance. (h) SOD2 expression levels in primary human PDAC tumors (n = 218) compared with metastases (n = 71) from the publicly available dataset GSE205154 [52]. t-test

showed statistical significance. No outliers were detected by ROUT ($Q = 1\%$).

Since macrophage populations did not differ between KC and KC; Sod2^{-/-} pancreata, we reasoned that additional inflammatory stimuli might be required for full tumor progression. To test this, chronic pancreatitis was induced via repeated caerulein injections (**Figure 5b**). Under these conditions, KC; Sod2^{-/-} mice reached humane endpoint substantially earlier, with a median survival of 37.29 weeks compared with 81.71 weeks in KC mice (**Figure 5b**). Tumors in KC; Sod2^{-/-} animals at endpoint continued to lack SOD2 and exhibited predominantly poorly differentiated histology with prominent AFL components, in contrast to the more well-differentiated tumors observed in KC controls (**Figure 5c**). Furthermore, AFLs occupied a ten-fold greater fraction of the tissue in KC; Sod2^{-/-} mice at endpoint (**Figure 5d**). Overall senescence levels (assessed by p16INK4a) were comparable between groups at endpoint, but senescent cells were largely confined to LG-PanIN areas and absent from AFLs.

We then examined whether these experimental observations align with SOD2 patterns in human pancreatic cancer. Broad comparison of SOD2 expression revealed no significant difference between normal pancreas and PDAC specimens (**Figure 5e**). However, when tumors were stratified by differentiation status, poorly differentiated PDAC displayed a clear and statistically significant reduction in SOD2 levels relative to well-differentiated cases (**Figures 5f and 5g**). Patients with poorly differentiated tumors are known to have worse survival outcomes than those with well-differentiated disease [53], largely because such tumors are more prone to metastasis [54]. Consistent with this, analysis of circulating tumor cells from patients with localized versus metastatic PDAC [55] showed lower SOD2 expression in metastatic samples, and direct comparison of primary tumors to metastases confirmed reduced SOD2 in metastatic lesions (**Figure 5h**).

Mitochondrial reactive oxygen species (mROS) can drive early neoplastic changes by causing mutations in both mitochondrial and nuclear DNA [56-58]. They also generate lipid peroxidation products such as 4-hydroxynonenal (4-HNE), which further impair mitochondrial respiration [4]. Mitochondrial oxidative stress is centrally involved in pancreatic cancer pathogenesis [1-4]. Earlier studies demonstrated that rising mROS levels trigger the transition of resting acinar cells to a ductal-like state through ADM [3, 4], an early

step in PDAC development [33]. Oncogenic Kras expression in acinar cells produces a modest ROS increase primarily through NADPH oxidases [7] and recruits macrophages [27] that amplify mROS via secreted mediators including TNF α , CCL5/RANTES, and IL6 [5, 59].

SOD2 functions as a key mitochondrial antioxidant protecting cells from metabolic oxidative stress [34], and genetic variants in SOD2 are associated with elevated pancreatic cancer susceptibility [18, 19]. While SOD2 is abundant in normal human pancreas [15], its expression declines in neoplastic lesions of PDAC patients [16]. The current study verifies this reduction from normal acinar tissue to LG-PanIN in human samples (**Figures 1b and 1c**) and in the KC mouse model (**Figures 1d and 1e**). Importantly, the drop in SOD2 is not directly caused by oncogenic Kras (**Figure 1f**) but instead results from inflammatory macrophage-derived signals (**Figure 1g**). This supports the cooperative action of Kras signaling and inflammation in PDAC initiation [60].

The precise pathway by which macrophage factors suppress SOD2 during lesion formation remains to be fully elucidated. In pancreatic cancer cell lines, miR-301a directly targets the SOD2 3'-UTR to inhibit translation [17, 23]. Another possibility involves miR-155-mediated suppression of FOXO3a [24], a transcription factor that upregulates SOD2 in non-proliferating cells [61]. Future research should clarify whether macrophage-secreted molecules influence these microRNAs and how they contribute to early lesion development.

To investigate the functional consequences of SOD2 loss, we generated acinar cell-specific Sod2 knockouts. Although Sod2 deletion caused oxidative stress in isolated cells, it did not induce ADM or lesions in intact pancreatic tissue (**Figure 1i**). This protection arises from mitohormesis, an adaptive mechanism in which mild mitochondrial stress activates protective responses that avert oxidative damage and organelle dysfunction [62]. Mitohormesis involves upregulation of PPAR γ , PGC-1 α , and their partner Nrf2 [37], sustained mitochondrial unfolded protein response (UPRmt), and enhanced expression of antioxidants such as catalase, peroxiredoxins 3 and 5, thioredoxin 2, and thioredoxin reductase [39]. Notably, the presence of oncogenic Kras dampened this mitohormetic adaptation in Sod2-deficient acinar cells (**Figures 2a and 2b**). Consequently, ROS levels rose further (**Figures 2c and 3d**), driving increased ADM in explant cultures (**Figures**

2d and 2g) and more ADM events, LG-PanIN formation, and overall dysplasia in vivo (**Figures 3a–3c**). We also detected a higher number of DCLK1-positive cells within PanIN lesions in KC; *Sod2*^{−/−} mice (**Figure 3e**). These cells can derive from PanIN [63] under high oxidative conditions [9] and possess cancer stem cell features that facilitate tumorigenesis [41, 42]. *Sod2* knockout in the KC model markedly increased atypical flat lesions (AFLs) (**Figure 4a**). AFLs originate directly from acinar cells [45] and are distinguished from ADM/PanIN by their non-mucinous nature and surrounding loose stroma [25, 43]. They represent an alternative direct precursor route to PDAC independent of the classic PanIN pathway [25, 43, 44]. Our characterization shows that AFLs experience less lipid peroxidation and senescence than LG-PanIN lesions (**Figures 4c and 4d**). This resistance is mediated by upregulation of nuclear factor erythroid 2-related factor 1 (Nfe2l1/NRF1) (**Figure 4e**), a regulator of numerous antioxidant genes that counteract lipid peroxidation, senescence, and ferroptosis [46, 47, 49, 50]. One important downstream target, glutathione peroxidase 4 (Gpx4), is correspondingly elevated in AFLs (**Figure 4f**). The reasons why *Sod2* loss leads to this compensatory response and AFL formation in certain contexts are not yet clear. We hypothesize that specific microenvironmental cues, such as hypoxia, nutrient levels (e.g., lactate, glucose), or signals from cancer-associated fibroblasts (CAFs), modulate NRF1. Additional studies are needed to define the unique microenvironment of AFLs compared with LG-PanIN. Cellular senescence acts as a barrier to the progression of pancreatic lesions [64]. Although *Sod2* deletion combined with oncogenic *Kras* increased less-senescent AFLs as well as overall ADM/PanIN and dysplasia, this did not translate into accelerated tumor formation (**Figure 5a**). The absence of expanded activated pancreatic stellate cells/myCAFs and unchanged macrophage infiltration suggested that critical tumor-promoting inflammatory signals were still lacking. Induction of chronic pancreatitis with caerulein in KC; *Sod2*^{−/−} mice overcame this limitation, significantly shortening survival (median 37.29 weeks versus 81.71 weeks in KC); (**Figure 5b**). Prior research indicates that caerulein-driven inflammation can suppress oncogene-induced senescence [60]. While endpoint senescence levels were similar between groups, the proportion of non-senescent AFLs rose dramatically from 1% in KC to

10% in KC; *Sod2*^{−/−} mice (**Figure 5d**), implying that inflammatory stimuli favor AFL expansion.

Earlier reports described high SOD2 in normal pancreas with loss in tumors [15]. However, global comparison in our analysis found no overall difference between normal tissue and PDAC (**Figure 5e**). Only when separating by histologic grade did poorly differentiated tumors show significantly lower SOD2 (**Figures 5f and 5g**). Patients with poorly differentiated PDAC have substantially worse prognosis [53], which aligns with our mouse survival data and the histologic appearance of tumors at endpoint (**Figure 5c**).

In summary, downregulation of SOD2 alongside oncogenic *Kras* represents a key step in PDAC development that promotes early lesion formation (ADM/PanIN) and dysplasia. Compensatory antioxidant pathways can generate non-senescent AFLs. When additional inflammation is present, both lesion types contribute to tumorigenesis, with AFLs preferentially giving rise to poorly differentiated tumor regions, while conventional lesions support well-differentiated areas (**Figure 6**).

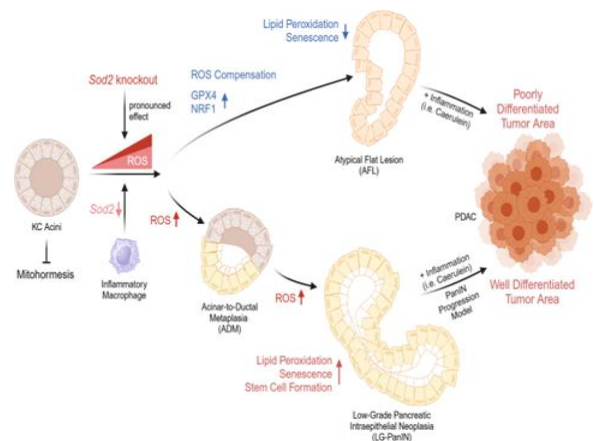


Figure 6. Schematic summary of SOD2's role in the development of atypical flat lesions (AFLs), low-grade PanIN (LG-PanIN), and their advancement to PDAC.

Oncogenic *Kras* expressed in acinar cells inhibits mitohormesis — the adaptive mechanism whereby mild mitochondrial stress triggers protective responses that improve cell survival. When *Sod2* is deleted or suppressed by signals from inflammatory macrophages, ROS levels rise. This drives acinar-to-ductal metaplasia that progresses into LG-PanIN lesions characterized by elevated lipid peroxidation, increased senescence, and higher numbers of stem-like cells (following the standard

ADM/PanIN pathway). In contrast, atypical flat lesions (AFLs) activate compensatory mechanisms by upregulating GPX4 and NRF1. These factors help neutralize excess ROS, thereby reducing lipid peroxidation and senescence. Upon additional inflammatory pressure, overall dysplasia increases, accelerating tumor development. The presence of AFLs contributes to the formation of more poorly differentiated tumor regions.

Conclusion

Oncogenic Kras impairs mitohormesis's control of oxidative stress, thereby facilitating acinar-to-ductal metaplasia as an early initiating step. Additional loss or reduction of Sod2 amplifies ROS production, resulting in greater dysplasia, expanded low-grade PanIN formation, heightened lipid peroxidation, enhanced senescence, and increased stem cell features. At the same time, Sod2 depletion promotes the emergence of atypical flat lesions (AFLs). These AFLs upregulate NRF1 and GPX4 to offset ROS accumulation and exhibit lower levels of lipid peroxidation and senescence compared with conventional low-grade PanIN lesions.

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