

# Nuclear Factor Erythroid 2–Related Factor 2 Depletion Sensitizes Pancreatic Cancer Cells to Gemcitabine via Aldehyde Dehydrogenase 3a1 Repression

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## ABSTRACT

As the central regulator of the oxidative stress response, nuclear factor erythroid 2–related factor 2 (Nrf2) is attracting great interest as a therapeutic target for various cancers, and the possible clinical applications of novel Nrf2 inhibitors have been explored in Nrf2-activated cancers. In the present study, we specifically investigated halofuginone, which is derived from a natural plant alkaloid. We found that halofuginone administration decreased the number of pancreatic intraepithelial neoplasias in pancreas-specific *Kras* and *p53* mutant (KPC) mice. In Nrf2-activated pancreatic cancer cell lines established from KPC mice, halofuginone rapidly depleted Nrf2 in Nrf2-activated cancer cells. Both in vitro and in vivo, it sensitized Nrf2-activated pancreatic cancer cells to gemcitabine, which is the first-line chemotherapy in clinical practice. In our mechanistic study, we found that halofuginone downregulated aldehyde dehydrogenase 3a1 (ALDH3A1) in

mouse pancreatic cancer cells. The Nrf2 inducer diethyl maleate upregulated ALDH3A1, and knockdown of *Aldh3a1* sensitized Nrf2-activated cancer cells to gemcitabine, strongly suggesting that ALDH3A1 is regulated by Nrf2 and that it contributes to gemcitabine resistance. The current study demonstrated the therapeutic benefits of halofuginone in Nrf2-activated pancreatic cancers.

## SIGNIFICANCE STATEMENT

We identified nuclear factor erythroid 2–related factor 2 (Nrf2) and its downstream target aldehyde dehydrogenase 3a1 (ALDH3A1) as novel therapeutic targets in pancreatic cancer. They negatively affect the efficacy of a conventional chemotherapeutic agent, gemcitabine. We confirmed that Nrf2 plays a pivotal role in the induction of ALDH3A1.

## Introduction

Pancreatic cancer is an intractable gastrointestinal malignancy with poor prognosis. Therapeutic options for unresectable pancreatic cancers have improved in the last decade, but the median survival time remains shorter than 12 months (Okusaka and Furuse, 2020). Intrinsic and acquired resistance to chemotherapeutic agents promotes tumor regrowth. Numerous molecules, signaling pathways, and adaptive mechanisms are involved in this process, and all could be novel therapeutic targets in pancreatic cancer. Previous studies have clarified some of these mechanisms using in vitro approaches with cultured cells. For example, the Msh homeobox 2 transcription factor, which induces epithelial-to-mesenchymal transition, determines the chemosensitivity of pancreatic cancer cells by upregulating the ATP-binding cassette (ABC) subfamily G

member 2 transporter (Hamada et al., 2012). Prolonged gemcitabine treatment led to resistant pancreatic cancer cell lines with increased cytidine deaminase activity, resulting in enhanced drug detoxification (Samulitis et al., 2015).

We have investigated the role of the Kelch-like erythroid cell-derived protein with Cap'n'Collar homology-associated protein 1 (KEAP1)–nuclear factor erythroid 2–related factor 2 (Nrf2) pathway in pancreatic cancer cells (Hamada et al., 2017). The KEAP1–Nrf2 pathway functions as a major adaptive mechanism for oxidative and electrophilic stress by inducing antioxidant and detoxifying enzymes that result in cell survival and organ protection (Yamamoto et al., 2018; Baird and Yamamoto, 2020). Nrf2 also protects cancer cells from reactive oxygen species and xenobiotics, including chemotherapeutic agents (Taguchi and Yamamoto, 2020). To examine the contribution of Nrf2 to the development of pancreatic cancers, we introduced an *Nrf2*-null background into *Kras*<sup>G12D/+</sup>::*p53*<sup>R172H/+</sup>::*Pdx-1-Cre* (KPC) mice, which spontaneously develop invasive pancreatic cancer driven by mutant *Kras* and *p53* (Hingorani et al., 2005).

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**ABBREVIATIONS:** ABC, ATP-binding cassette; ALDH, aldehyde dehydrogenase; Aldh3a1, aldehyde dehydrogenase 3a1; DEM, diethyl maleate; eIF2 $\alpha$ , eukaryotic translation initiation factor 2A; HO-1, heme oxygenase 1; KEAP1, Kelch-like ECH-associated protein 1; KPC, *Kras* and *p53* mutant; KPCN, *Nrf2*-null KPC; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; Nrf2, nuclear factor erythroid 2–related factor 2; PanIN, pancreatic intraepithelial neoplasia; PCR, polymerase chain reaction; shRNA, short hairpin RNA.

The loss of Nrf2 function in these mice resulted in fewer precancerous lesions, pancreatic intraepithelial neoplasias (PanINs), and invasive cancers (Hamada et al., 2017), indicating that Nrf2 is required to establish pancreatic cancer.

To gain further insights into how Nrf2 contributes to pancreatic cancer development, we established pancreatic cancer cell lines from *Nrf2*-null KPC (KPCN) mice, dubbing them KPCN cells. These cell lines have much higher sensitivity to the electrophilic stress inducer diethyl maleate (DEM), as well as to gemcitabine, a standard chemotherapeutic agent for pancreatic cancer (Hamada et al., 2017), suggesting that Nrf2 elicits the expression of cytoprotective and chemoresistant genes in KPC cells. However, the Nrf2-target genes responsible for gemcitabine resistance have not yet been identified.

Since Nrf2 plays a crucial role in cancer cell survival, its inhibition is likely an effective therapy (Yamamoto et al., 2018; Taguchi and Yamamoto, 2020). Indeed, the small-molecule Nrf2 inhibitor ML385 selectively showed antitumor activity in combination with carboplatin in non-small cell lung cancer cells harboring constitutive Nrf2 activation (Singh et al., 2016). A plant alkaloid-derived Nrf2 inhibitor, halofuginone, has been identified in high-throughput drug screening using a luciferase reporter cell line (Tsuchida et al., 2017). Halofuginone acts as a prolyl-transfer RNA synthetase inhibitor that suppresses protein synthesis and triggers amino acid depletion (Keller et al., 2012). Halofuginone sensitizes Nrf2-addicted cancer cell lines to chemotherapeutic agents.

In the present study, we addressed whether halofuginone could be used to treat pancreatic cancers. Using KPC mice and cell lines, we found that halofuginone pretreatment markedly sensitized pancreatic cancer cells to gemcitabine. Comprehensive analysis of differentially expressed genes after halofuginone pretreatment identified the downregulation of *aldehyde dehydrogenase (ALDH) 3a1* gene expression. A series of mechanistic analyses revealed that halofuginone pretreatment intensifies the efficacy of gemcitabine in Nrf2-activated cancers and that ALDH3A1 is a key target molecule in the function of halofuginone. We believe that this regulatory mechanism is a novel therapeutic target for pancreatic cancer.

## Materials and Methods

**Materials.** Gemcitabine and DEM were purchased from Wako (Osaka, Japan); 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Dojindo (Kumamoto, Japan); halofuginone and anti-tubulin antibody (T5168) from Sigma-Aldrich (St. Louis, MO); anti-ALDH3A1 antibody (ab76976), anti-heme oxygenase 1 (HO-1) antibody (ab137749), and peroxidase-conjugated anti-rat antibody (ab6734) from Abcam (Cambridge, UK); and anti-caspase-3 antibody (9662), anti-eukaryotic translation initiation factor 2A (eIF2 $\alpha$ ) antibody (5324), anti-phosphorylated eIF2 $\alpha$  antibody (3398), and peroxidase-conjugated anti-rabbit antibody (7074) from Cell Signaling Technology (Beverly, MA). The anti-Nrf2 antibody has been described previously (Maruyama et al., 2008). Peroxidase-conjugated anti-mouse antibody (NA931) was purchased from GE Healthcare (Piscataway, NJ).

**Mice.** Mice were handled according to the Guidelines for Proper Conduct of Animal Experiments of the Ministry of Education, Culture, Sports, Science, and Technology of Japan and Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health. All animal experiments were performed according to the regulations for animal experiments and related activities at Tohoku University (Article No. 2019MdA-154 and 2018MdA-127-03, approved by the institution's animal care and use committee).

*Pdx-1-Cre* transgenic mice, *LSL-K-ras<sup>G12D</sup>* mice, and *LSL-p53<sup>R172H</sup>* mice were obtained from the NCI Mouse Repository (Frederick, MD) (Hingorani et al., 2005; Jackson et al., 2001; Olive et al., 2004). Mice were back-crossed with the C57BL/6J strain for more than five generations. KPC mice were obtained by crossing the mice. *Nrf2*-knockout KPCN (*Kras<sup>G12D/+</sup>::p53<sup>R172H/+</sup>::Pdx-1-Cre::Nrf2<sup>-/-</sup>*) mice were also obtained, as described previously (Hamada et al., 2017). At the age of 5 weeks, KPC mice received intraperitoneal injections (five times per week) of halofuginone (0.25 mg/kg) or saline over 4 weeks and were then euthanized. The total number of PanINs within the pancreas was calculated at 9 weeks.

**Immunohistochemistry.** Tissues from mice were fixed in 4% paraformaldehyde (Wako) and paraffin-embedded for H&E staining or immunohistochemistry. For mouse Aldh3a1 and HO-1, slides were boiled for 10 minutes in target retrieval solution (Dako, Glostrup, Denmark) and incubated with the primary antibody overnight. The visualization of immunohistochemistry was performed using Histofine MOUSESTAIN KIT (Nichirei Biosciences Inc., Tokyo, Japan) and diaminobenzidine.

**Cell Viability Assay.** Cells were seeded in 96-well plates at 5000 cells per well and treated using 0–10  $\mu$ M gemcitabine for 48 hours, with or without 100 nM halofuginone pretreatment for 24 hours. Cell viability was measured using the MTT assay, wherein the cells were treated using 5  $\mu$ g/mL of MTT for 1 hour and then solubilized in dimethyl sulfoxide. The optical density was measured using a spectrophotometer at a wavelength of 570 nm, with a reference wavelength of 690 nm.

**Cell Culture.** Pancreatic cancer cell lines were established from either KPC or *Nrf2*-knockout KPC mice (KPC and KPCN cell lines, respectively), as described previously (Hamada et al., 2017). To establish stable *Aldh3a1* knockdown cell lines, KPC cell line 1 was transfected with the predesigned short hairpin RNA (shRNA) expression vector pLKO.1-puro for mouse *Aldh3a1* (TRCN0000438676; Sigma Aldrich), and the knockdown cells were selected. To establish the control line, KPC line 1 was transfected with predesigned nontargeting shRNA expression vector pLKO.1-puro (nonmammalian shRNA control plasmid; Sigma Aldrich), and the transfected cells were selected. Cells were maintained at 37°C in a humidified incubator with 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics.

**RNA Extraction and Quantitative Reverse Transcription-Polymerase Chain Reaction.** Total RNA was extracted from cells using the RNeasy kit (Qiagen, Valencia, CA). Reverse transcription-polymerase chain reaction (PCR) was performed using 1  $\mu$ g of RNA and the SuperScript VILO Master Mix (Thermo Fisher Scientific, Waltham, MA). The expression of each gene was quantified using the StepOnePlus real-time PCR system (Thermo Fisher Scientific) and Fast SYBR Green Master Mix (Thermo Fisher Scientific), with the following primers (Wang et al., 2012):  $\beta$ -actin, forward 5-GGCTGTATCCCTC-CATCG-3', reverse 5-CCAGTTGGTAACAATGCCATGT-3'; *Aldh3a1*, forward 5-TGGCAAAGACTCGTCAGACC-3', reverse 5-AGTTCCAAG-CACCTATGACAAG-3'.

**Microarray.** Total RNA samples from cells were subjected to microarray analysis in duplicate using whole mouse genome Oligo DNA Microarray version 2.0 (Agilent Technologies, Santa Clara, CA) and the G2539A microarray scanner system (Agilent). Data were acquired using GeneSpring GX software (Agilent). To identify up- or downregulated genes and compare the control and experimental samples, we calculated Z-scores and ratios (non-log-scaled fold change) from the normalized signal intensities of each probe (Quackenbush, 2002). We then established the following criteria for regulated genes: upregulated genes, Z-score  $\geq 2.0$  (ratio  $\geq 1.5$ -fold); downregulated genes, Z-score  $\leq -2.0$  (ratio  $\leq 0.66$ ). The raw data are available at the Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>) (accession no. GSE172046).

**Western Blot.** The cells were lysed in radioimmunoprecipitation buffer to obtain total proteins. Lysates were subjected to electrophoresis using NuPAGE 8% Bis-Tris gel (Life Technologies) and transferred

onto Immobilon-P membranes (Merck Millipore, Billerica, MA). The membranes were incubated overnight at 4°C with primary antibodies. After incubation with peroxidase-conjugated secondary antibody for 1 hour at room temperature, reactive bands were detected using enhanced chemiluminescence Western blotting detection reagents (GE Healthcare, Buckinghamshire, UK). Densitometry analysis was performed using ImageJ (<http://rsbweb.nih.gov/ij/index.html>).

**Subcutaneous Implantation Model.** Male CD1-*Foxn1*<sup>nu</sup> mice at 5 weeks of age were used in the subcutaneous implantation model. The mice received a bilateral dorsal injection of  $1 \times 10^6$  cells per injection of KPC cell line 2. After 1 week, they received intraperitoneal injections of saline for 3 weeks, twice weekly gemcitabine (50 mg/kg), five times weekly halofuginone (0.25 mg/kg), or a combination of gemcitabine and halofuginone. Tumor size was calculated 3 weeks after treatment using the following formula:  $V = 0.5 \times \text{width}^2 \times \text{length}$  (mm<sup>3</sup>). The mice were then euthanized, and the subcutaneous tumors were subjected to histologic analysis.

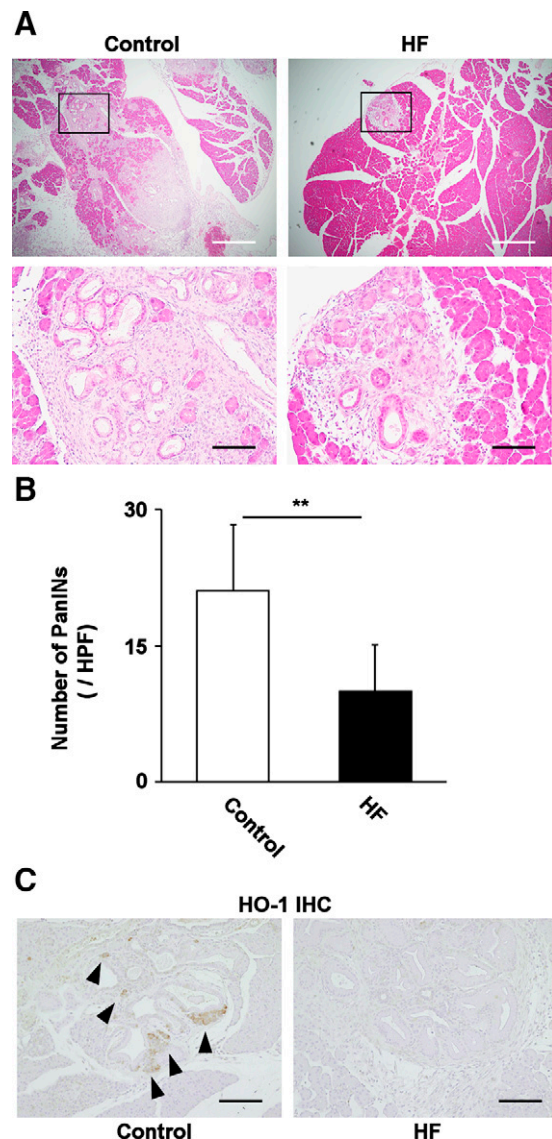
**Statistical Analysis.** All data are presented as means  $\pm$  S.D. Statistical analysis was performed using JMP Pro version 15.0.0 (SAS Institute, Cary, NC). Differences between the two factors were assessed using Student's *t* test, whereas differences between more than two groups were assessed using the Tukey-Kramer method. Statistical significance was set at  $P < 0.05$ .

## Results

**Halofuginone Treatment Decreased the Number of Precancerous Lesions in KPC Mice.** To confirm the anticancer effects of halofuginone on pancreatic cancer in vivo, we administered halofuginone to 5-week-old KPC mice, which were expected to develop Nrf2-activated PanINs at 13 weeks of age (Hamada et al., 2017). Administration of halofuginone for 4 weeks did not cause adverse effects, such as loss of body weight or weakness. At 9 weeks of age, vehicle-treated KPC mice developed precancerous lesion PanINs, but halofuginone treatment significantly decreased the number of PanINs in KPC mice. Typical histologic images of vehicle-treated and halofuginone-treated mouse pancreas are shown in Fig. 1A, with lower and higher magnifications (top and bottom panels, respectively). The number of PanINs was significantly lower in halofuginone-treated mice than in vehicle-treated mice (Fig. 1B). These results indicate that halofuginone attenuates the formation of PanINs in KPC mice without eliciting unwanted adverse effects. We also assessed HO-1 expression in the PanINs of vehicle-treated and halofuginone-treated mice by immunohistochemistry. Halofuginone decreased HO-1 expression in the PanINs (Fig. 1C).

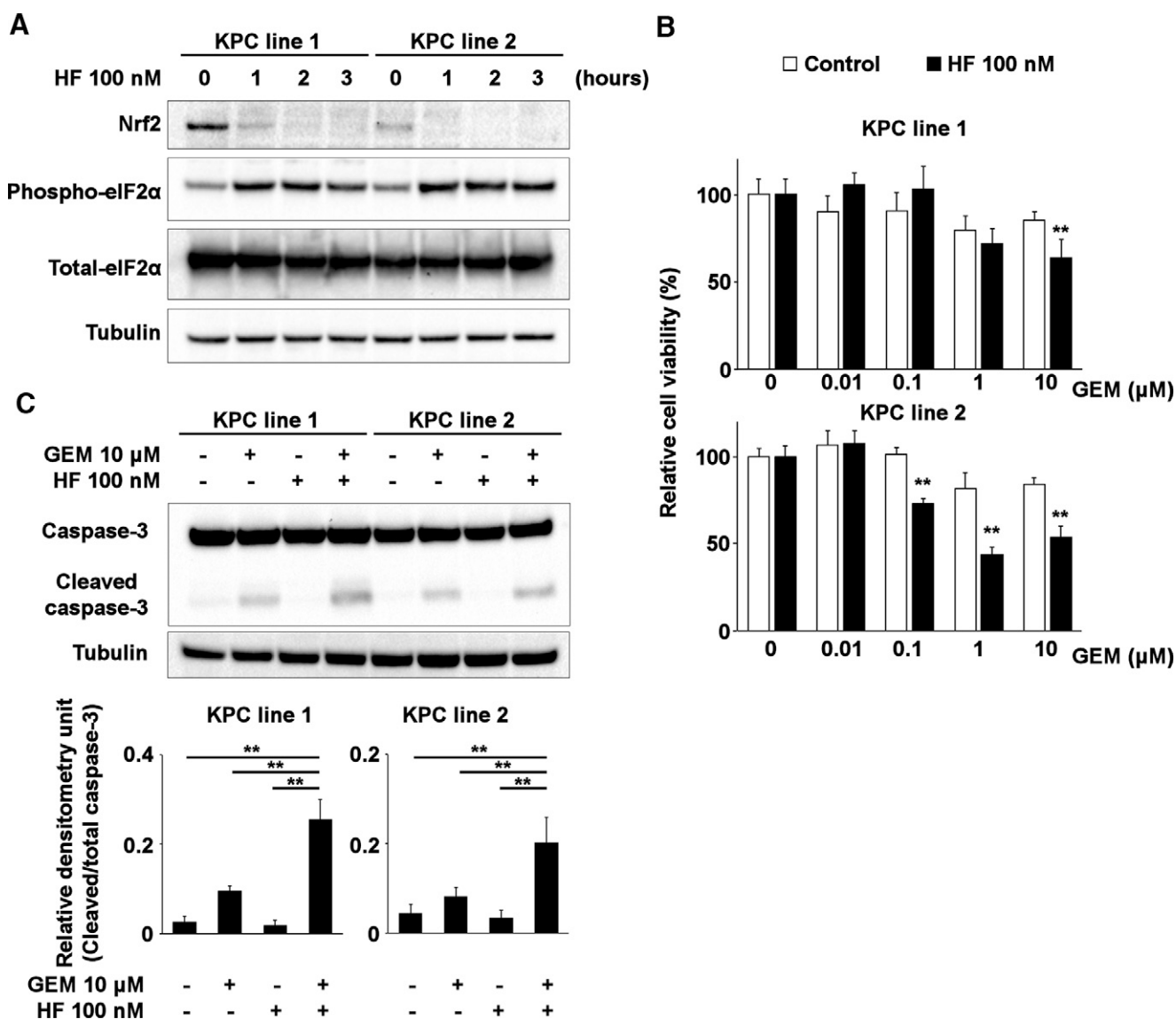
**Halofuginone Depletes Nrf2 and Sensitizes Pancreatic Cancer Cells to Gemcitabine.** We next explored the mechanisms by which halofuginone inhibits Nrf2 in Nrf2-activated pancreatic cancer cell lines established from KPC mice (Hamada et al., 2017). To this end, we used two KPC cell lines that expressed Nrf2 abundantly (KPC cell line 1) and moderately (KPC cell line 2). Treating these cell lines with halofuginone (100 nM) for 1–3 hours markedly depleted Nrf2 expression (Fig. 2A). This rapid decrease in Nrf2 expression was accompanied by an increase in phosphorylated eIF2 $\alpha$ . This is consistent with the known functions of halofuginone, which inhibits prolyl-transfer RNA, leading to amino acid starvation and thus repressing protein synthesis. Nrf2 in particular is severely affected by halofuginone because it is a very short-lived protein (Tsuchida et al., 2017).

Next, we examined KPC cell viability after treatment with halofuginone (100 nM) and an incremental amount of



**Fig. 1.** Decreased precancerous lesion PanINs in KPC mice treated with halofuginone. (A) H&E staining of the pancreas in KPC mice injected intraperitoneally with control (saline) or halofuginone (HF; 0.25 mg/kg) five times per week for 4 weeks. White bar shows 500  $\mu$ m, and black bar shows 100  $\mu$ m. (B) Average number of PanINs in four random high-power fields (HPFs) in the pancreases of four KPC mice that underwent control treatment and four that received HF ( $N = 16$ ;  $^{**}P < 0.01$ ). Error bars show S.D. (C) Immunohistochemistry for HO-1 expression in KPC mice injected intraperitoneally with control (saline) or HF (0.25 mg/kg) five times per week for 4 weeks. Black bar shows 100  $\mu$ m.

gemcitabine (0.01 to 10  $\mu$ M). We treated the KPC cell lines first with halofuginone for 24 hours and then with gemcitabine for 48 hours. Halofuginone pretreatment significantly enhanced the cytotoxic effect of gemcitabine, compared with cells treated with gemcitabine alone (Fig. 2B). This synergistic effect of halofuginone and gemcitabine was reproducible in the two KPC cells, although KPC cell line 1, which had higher Nrf2 expression, showed weaker sensitivity than the moderate Nrf2-expressing KPC cell line 2. We also examined the expression of cleaved caspase-3 to determine whether apoptosis increased. The expression of cleaved caspase-3 was induced by 10  $\mu$ M gemcitabine and promoted by halofuginone pretreatment in both KPC cell lines (Fig. 2C), suggesting that combined gemcitabine



**Fig. 2.** Nrf2-inhibitory effects of halofuginone in KPC cell lines. (A) Western blot for Nrf2 depletion and eIF2α phosphorylation indicating amino acid starvation after halofuginone (HF; 100 nM) treatment. Total eIF2α and α-tubulin are displayed as loading controls. (B) MTT assay for cell viability 48 hours after gemcitabine (GEM; 0.01–10 μM) treatment, with or without HF pretreatment for 24 hours (5000 cells per well;  $N = 6$ ,  $**P < 0.01$  compared with control at the same concentration of GEM). The error bars show S.D. (C) Western blot for expression of cleaved caspase-3 after gemcitabine treatment with or without HF pretreatment. α-Tubulin is displayed as a loading control. Lower panel shows the result of densitometry analysis ( $N = 3$ ,  $**P < 0.01$ ).

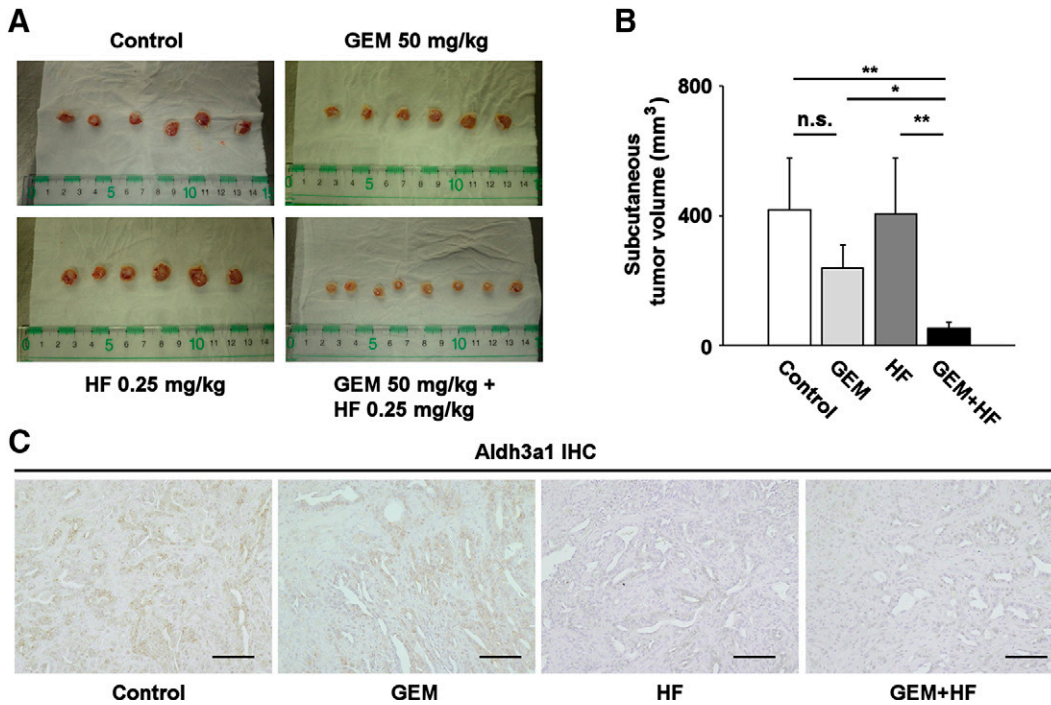
and halofuginone increased apoptosis. KPC cell line 1 treated with halofuginone and gemcitabine expressed more cleaved caspase-3 than KPC cell line 2 (Fig. 2C). These differences between KPC cell lines 1 and 2 might be due to additional factors induced by Nrf2, sensitizing cancer cells to gemcitabine-induced apoptosis, which were depleted by halofuginone. These results indicate that halofuginone depletes Nrf2 in pancreatic cancer cells and enhances their sensitivity to gemcitabine.

**Halofuginone Potentiates the Antitumor Effect of Gemcitabine In Vivo.** To validate the in vitro results with KPC cells, we examined whether concomitant halofuginone improves the anticancer activity of gemcitabine in vivo. To this end, we subcutaneously implanted KPC cell line 2 into male immunodeficient CD1-*Foxn1*<sup>nu</sup> mice at 5 weeks of age to generate tumor-bearing mice, which were then treated using halofuginone (0.25 mg/kg i.p.; five times

per week for 3 weeks) and/or gemcitabine (50 mg/kg i.p.; twice per week for 3 weeks).

Treatment with gemcitabine alone tended to reduce the size of subcutaneous tumors in immunodeficient mice injected with KPC cell line 2, but there was no significant difference between the vehicle control and gemcitabine (Fig. 3, A and B). Halofuginone alone had no substantial effect on tumor size. In contrast, the combination of gemcitabine and halofuginone significantly reduced the tumor size. None of the groups showed any adverse effects, such as body weight loss or weakness. These results demonstrate that halofuginone sensitizes pancreatic cancer cells to gemcitabine, both in vitro and in vivo.

**Halofuginone Reduces ALDH3A1 Expression Both In Vitro and In Vivo.** To gain mechanistic insights, we next attempted to identify the downstream target genes of Nrf2 that are responsible for the sensitization to gemcitabine. For



**Fig. 3.** Effects of combination treatment with gemcitabine and halofuginone (HF) in the subcutaneous implantation model. Macroscopic view (A) and the quantified size (B) of subcutaneous tumors in immunodeficient mice injected with the KPC cell line 2 ( $1 \times 10^6$  cells) 3 weeks after treatment with saline, gemcitabine (GEM; 50 mg/kg i.p.; twice weekly), HF (0.25 mg/kg i.p.; five times weekly), or the combination of GEM and HF ( $N = 6-8$ ; \* $P < 0.05$ , \*\* $P < 0.01$ ; n.s., not significant). Error bars show S.D. (C) Immunohistochemistry (IHC) for ALDH3A1 in subcutaneous tumors with control treatment, GEM, HF, and GEM+HF. Black bar shows 100  $\mu$ m.

this purpose, we conducted microarray analyses using KPC cell lines 1 and 2 treated with or without halofuginone (100 nM, 24 hours). We identified a number of downregulated genes in individual cell lines and selected nine genes that were commonly downregulated by halofuginone treatment in cells from both KPC cell line 1 and KPC cell line 2 (Table 1). In addition to the canonical Nrf2-target genes, such as the glutathione S-transferase family and ABC transporter family, we identified *Aldh3a1*. A previous study showed that Nrf2 silencing in pancreatic cancer cells led to reduced ALDH1A1 and ALDH3A1 and resulted in enhanced sensitization to 5-fluorouracil (Duong et al., 2017). We hypothesized that *Aldh3a1* might be an important Nrf2-target gene in pancreatic KPC cells, so we conducted a series of experiments to clarify whether *Aldh3a1* is responsible for gemcitabine sensitivity in an Nrf2-dependent manner.

We first confirmed that halofuginone alone and the combination of gemcitabine and halofuginone reduced *Aldh3a1* expression in subcutaneous tumors (Fig. 3C). Administration of halofuginone alone had little effect on the size of subcutaneous tumor, suggesting additional mechanisms supporting tumor growth besides *Aldh3a1* in in vivo setting. We next assessed ALDH3A1 expression after halofuginone treatment

(100 nM; 24 hours) in KPC cell lines 1 and 2. Halofuginone treatment significantly reduced ALDH3A1 mRNA expression (Fig. 4A). Western blotting experiments revealed that ALDH3A1 protein levels were also markedly decreased by halofuginone treatment (Fig. 4B).

We then asked whether halofuginone treatment actually reduced ALDH3A1 protein levels in KPC tumor cells. To investigate this, we treated KPC mice with a series of intraperitoneal injections of halofuginone (0.25 mg/kg per injection; five times per week for 4 weeks) and analyzed ALDH3A1 protein expression using immunohistochemical staining. The results revealed that halofuginone led to significantly lower ALDH3A1 expression in PanINs than in control KPC mice (Fig. 4C). Taken together, these results demonstrate that halofuginone can repress ALDH3A1 expression both in vitro and in vivo.

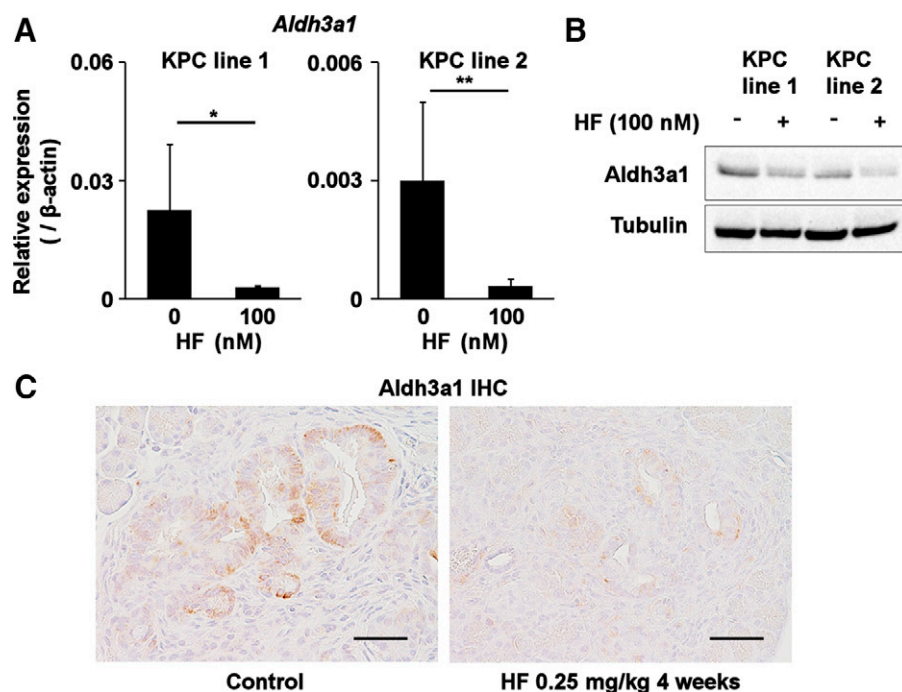
**Nrf2 Deletion Suppresses ALDH3A1 Induction via the Electrophilic Stress Inducer DEM.** Thus far, the results strongly suggest that ALDH3A1 is regulated by Nrf2. To confirm this, we next examined whether ALDH3A1 can be induced by the electrophilic Nrf2 inducer DEM and whether Nrf2 depletion can reverse this induction. We found that treating KPC cell line 1

**TABLE 1**  
Commonly downregulated genes in halofuginone-treated KPC cell line 1 and 2

Gene symbol	Fold change in KPC cell line 1	Description
<i>Aldh3a1</i>	0.14	Aldehyde dehydrogenase family 3, subfamily A1
<i>Gstm1</i>	0.12	Glutathione S-transferase, mu 1
<i>Gstm2</i>	0.09	Glutathione S-transferase, mu 2
<i>Gstm3</i>	0.13	Glutathione S-transferase, mu 3
<i>Abcc5</i>	0.14	ATP-binding cassette, subfamily C (CFTR/MRP <sup>a</sup> ), member 5
<i>Gstt1</i>	0.05	Glutathione S-transferase, theta 1
<i>Gsta2</i>	0.22	Glutathione S-transferase, alpha 2
<i>Gsta3</i>	0.11	Glutathione S-transferase, alpha 2
<i>Gsta4</i>	0.11	Glutathione S-transferase, alpha 2

<sup>a</sup>CFTR/MRP, cystic fibrosis transmembrane conductance regulator/multidrug resistance-associated protein.

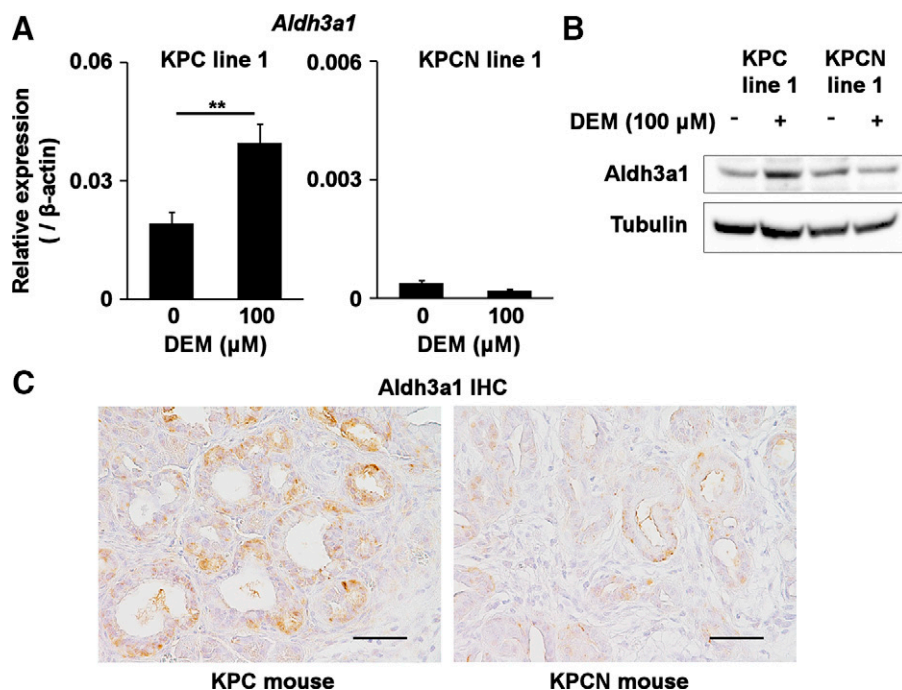
**Fig. 4.** Decreased expression of ALDH3A1 after halofuginone (HF) treatment. (A) Quantitative reverse transcription PCR for ALDH3A1 mRNA expression 24 hours after treatment with HF (100 nM;  $N = 6$ ;  $*P < 0.05$ ;  $**P < 0.01$ ). Error bars show S.D. (B) Western blot for ALDH3A1 expression 24 hours after treatment with HF (100 nM).  $\alpha$ -Tubulin is displayed as a loading control. (C) Immunohistochemistry (IHC) for ALDH3A1 expression in KPC mouse pancreas 4 weeks after HF treatment (0.25 mg/kg per injection i.p.; five times per week). Black bar shows 100  $\mu$ m.

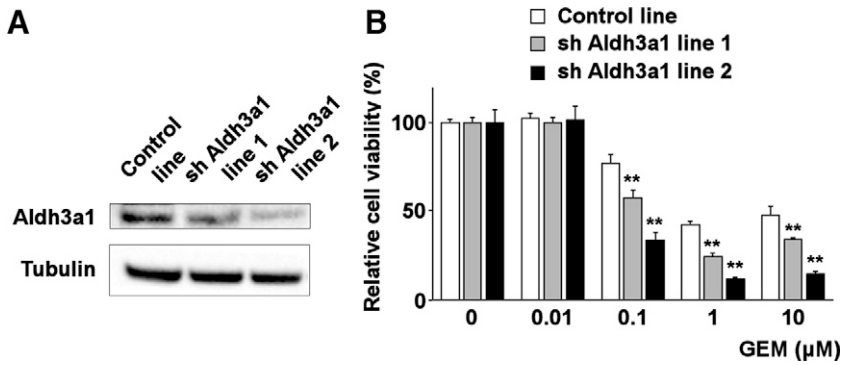


with DEM (100  $\mu$ M, 24 hours) resulted in increased ALDH3A1 expression at both the mRNA and protein levels (Fig. 5, A and B). In contrast, the cell line established from Nrf2-knockout mice, which we refer to as KPCN cell line 1, showed no increase in ALDH3A1 after DEM treatment (Fig. 5, A and B). We also assessed ALDH3A1 expression in the PanIN lesions of KPC and KPCN mice using immunohistochemistry. In KPCN mice, ALDH3A1 expression was markedly lower than that in KPC mice (Fig. 5C).

**Alldh3a1 Knockdown Sensitized Pancreatic Cancer Cells to Gemcitabine.** We addressed whether loss of ALDH3A1 function in KPC cells contributes to the increase in gemcitabine sensitivity. To answer this, we performed *Aldh3a1* knockdown in KPC cell line 1 and examined the relationship between knockdown and gemcitabine sensitivity. We established two cell lines stably expressing shRNA for ALDH3A1, with each cell line showing a distinct reduction in ALDH3A1 expression (Fig. 6A). Both showed reduced viability after gemcitabine treatment; the reduction correlated with the

**Fig. 5.** Effects of Nrf2 deletion on the expression of ALDH3A1. (A) Quantitative reverse transcription PCR for ALDH3A1 mRNA expression 24 hours after DEM (100  $\mu$ M) treatment in KPC cell line 1 and KPCN cell line 1 ( $N = 6$ ,  $**P < 0.01$ ). Error bars show S.D. (B) Western blot for *Aldh3a1* expression after DEM (100  $\mu$ M, 24 hours) treatment in KPC cell line 1 and KPCN cell line 1.  $\alpha$ -Tubulin is displayed as a loading control. (C) Immunohistochemistry (IHC) for ALDH3A1 expression in KPC and KPCN mouse pancreas at 90 days after birth. Black bar shows 100  $\mu$ m.

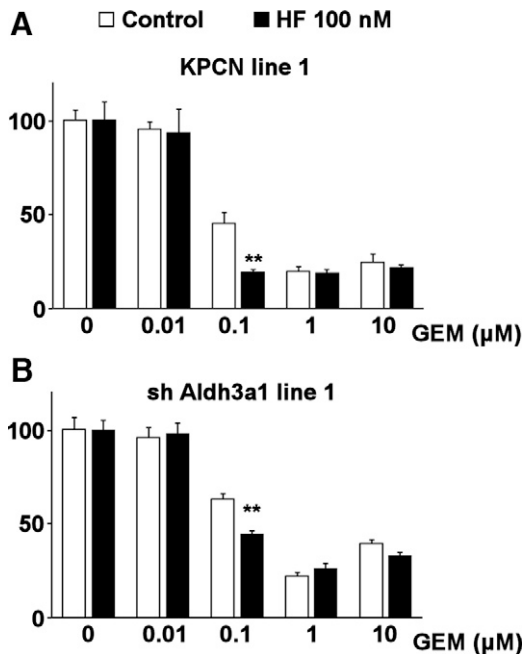




**Fig. 6.** Establishment of *Aldh3a1* knockdown (sh *Aldh3a1*) cell lines and gemcitabine (GEM) sensitivity. (A) Western blot for ALDH3A1 expression in stable *Aldh3a1* knockdown cell lines.  $\alpha$ -Tubulin is displayed as a loading control. (B) MTT assay for cell viability of stable *Aldh3a1* knockdown cell lines 48 hours after GEM treatment (0–10  $\mu$ M; 5000 cells per well,  $N = 6$ , \*\*  $P < 0.01$ ). Error bars show S.D.

decreased ALDH3A1 expression in both cell lines and was significant compared with the control cell line (Fig. 6B). In conclusion, these results strongly suggest that ALDH3A1 reduction is responsible for the gemcitabine sensitization induced by Nrf2 depletion.

**Nrf2 and Aldh3a1 Are Responsible for Sensitization to Gemcitabine.** Finally, we assessed whether loss of ALDH3A1 or NRF2 in KPC cells attenuates the sensitization to gemcitabine caused by halofuginone treatment. Pretreatment with halofuginone (100 nM) for 24 hours did not sensitize stable *Aldh3a1* knockdown cell line 1 and KPCN cell line 1 to gemcitabine at 1 or 10  $\mu$ M (Fig. 7, A and B). These results indicate that halofuginone sensitizes pancreatic cancer cells to gemcitabine via depletion of NRF2/ALDH3A1 to certain extent.



**Fig. 7.** Effects of halofuginone in *Nrf2*-deleted and *Aldh3a1* knockdown (sh *Aldh3a1*) cell lines. MTT assay for cell viability of *Nrf2*-deleted KPCN cell line 1 (A) and stable *Aldh3a1* knockdown cell line 1 (B) at 48 hours after gemcitabine (GEM; 0.01–10  $\mu$ M) treatment, with or without HF pretreatment for 24 hours (5000 cells per well;  $N = 6$ , \*\* $P < 0.01$  compared with control at the same concentration of GEM). The error bars show S.D.

## Discussion

In this study, we found that the Nrf2 inhibitor halofuginone sensitized pancreatic cancer cells to gemcitabine. Through a study exploring halofuginone activity, we discovered that halofuginone reduces the expression of ALDH3A1. Furthermore, by establishing stable *Aldh3a1* knockdown cell lines, we confirmed that ALDH3A1 decrease potentiates the sensitivity of pancreatic cancer cells to gemcitabine treatment. We also found that halofuginone has suppressive effects on ALDH3A1 expression in vivo in mice with Nrf2-activated pancreatic cancers. The PanINs of KPCN mice showed low ALDH3A1 expression, and DEM treatment failed to induce ALDH3A1 in KPCN cell lines, indicating that Nrf2 contributes to ALDH3A1 expression. Notably, the PanINs of KPC mice that had received halofuginone administration showed markedly decreased ALDH3A1 expression, suggesting that halofuginone combined with gemcitabine could be used in pancreatic cancer therapy.

ALDH3A1 plays pivotal roles in other types of cancers. For instance, *Aldh3a1* knockdown potentiates the sensitivity of human pancreatic cancer cells to 5-fluorouracil (Duong et al., 2017). Higher expression of ALDH3A1 in the cancer stem cell fraction has also been reported in prostate cancer, along with elevated expression of the cancer stem cell marker ABCG2 (Wang et al., 2020b). Pharmaceutical inhibition of ALDH3A1 by shikonin, a natural naphthoquinone, sensitizes cancer cells to the chemotherapeutic agent cabazitaxel (Wang et al., 2020b). Irradiated lung cancer cells release ALDH3A1-containing exosomes, which increase the invasive capacity of cancer cells receiving them (Wang et al., 2020a). In cancer cells that receive ALDH3A1-containing exosomes, glycolysis is accelerated, suggesting that metabolic reprogramming occurs. The KEAP1-Nrf2 system also affects the metabolic status of cancer cells by inducing metabolic reprogramming (Mitsuishi et al., 2012). In one study, comprehensive metabolome analysis in non-small cell lung cancer cells enabled the activation status of Nrf2 to be classified based on characteristic metabolites of Nrf2, which could be used as diagnostic markers (Saigusa et al., 2020).

The expression levels of ALDH3A1 in surgically resected cancer tissues correlate with the prognosis of patients with gastric cancer. Combined with other differentially expressed genes, ALDH3A1 shows diagnostic efficacy in patients with gastric cancer (Zhao et al., 2017). The impact of ALDH3A1 expression status on the prognosis of pancreatic cancers needs further validation in relationship with therapeutic intervention such as adjuvant chemotherapy. Several studies have targeted multi-isoform ALDH, including ALDH3A1. Newly

synthesized ALDH inhibitors, such as 2-[4-(2,3-dioxo-5-trifluoromethyl-2,3-dihydroindol-1-ylmethyl)benzyl] isothiourea hydrobromide, have shown anticancer effects in melanoma cells, colon cancer cells, and multiple myeloma cells (Dinavahi et al., 2020). This cytotoxic effect caused by ALDH loss was accompanied by increased reactive oxygen species and lipid peroxidation, indicating greater oxidative stress. Based on the present study and these reported studies, we propose that ALDH3A1 plays a pivotal role as a target gene of Nrf2 in the response to oxidative stress in pancreatic cancers.

In conclusion, the present study demonstrated that the Nrf2 inhibitor halofuginone shows a synergistic anticancer effect with gemcitabine. The suppression of Nrf2 activity led to ALDH3A1 reduction. Nrf2 inhibition and ALDH3A1 reduction could be used to potentiate the conventional chemotherapeutic agent gemcitabine in pancreatic cancers. Halofuginone and/or ALDH inhibitors may be the next important innovation in the treatment of pancreatic cancers, which are an intractable gastrointestinal malignancy.

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#### Authorship Contributions

*Participated in research design:* Hamada, Masamune.

*Conducted experiments:* Matsumoto, Hamada, Tanaka.

*Contributed new reagents or analytic tools:* Taguchi.

*Performed data analysis:* Hamada.

*Wrote or contributed to the writing of the manuscript:* Hamada, Yamamoto, Masamune.

#### References

- Baird L and Yamamoto M (2020) The molecular mechanisms regulating the KEAP1-NRF2 pathway. *Mol Cell Biol* **40**:e00099-e20.
- Dinavahi SS, Gowda R, Bazewicz CG, Battu MB, Lin JM, Chitren RJ, Pandey MK, Amin S, Robertson GP, and Gowda K (2020) Design, synthesis characterization and biological evaluation of novel multi-isoform ALDH inhibitors as potential anticancer agents. *Eur J Med Chem* **187**:111962.
- Duong HQ, You KS, Oh S, Kwak SJ, and Seong YS (2017) Silencing of NRF2 reduces the expression of ALDH1A1 and ALDH3A1 and sensitizes to 5-FU in pancreatic cancer cells. *Antioxidants* **6**:52.
- Hamada S, Satoh K, Hirota M, Kanno A, Umino J, Ito H, Masamune A, Kikuta K, Kume K, and Shimosegawa T (2012) The homeobox gene MSX2 determines chemosensitivity of pancreatic cancer cells via the regulation of transporter gene ABCG2. *J Cell Physiol* **227**:729–738.
- Hamada S, Taguchi K, Masamune A, Yamamoto M, and Shimosegawa T (2017) Nrf2 promotes mutant K-ras/p53-driven pancreatic carcinogenesis. *Carcinogenesis* **38**:661–670.
- Hingorani SR, Wang L, Multani AS, Combs C, Deramaudt TB, Hruban RH, Rustgi AK, Chang S, and Tuveson DA (2005) Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell* **7**:469–483.
- Jackson EL, Willis N, Mercer K, Bronson RT, Crowley D, Montoya R, Jacks T, and Tuveson DA (2001) Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras. *Genes Dev* **15**:3243–3248.
- Keller TL, Zocco D, Sundrud MS, Hendrick M, Edenius M, Yum J, Kim YJ, Lee HK, Cortese JF, Wirth DF et al. (2012) Halofuginone and other febrifugine derivatives inhibit prolyl-tRNA synthetase. *Nat Chem Biol* **8**:311–317.
- Maruyama A, Tsukamoto S, Nishikawa K, Yoshida A, Harada N, Motojima K, Ishii T, Nakane A, Yamamoto M, and Itoh K (2008) Nrf2 regulates the alternative first exons of CD36 in macrophages through specific antioxidant response elements. *Arch Biochem Biophys* **477**:139–145.
- Mitsuishi Y, Taguchi K, Kawatani Y, Shibata T, Nukiwa T, Aburatani H, Yamamoto M, and Motohashi H (2012) Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming. *Cancer Cell* **22**:66–79.
- Okusaka T and Furuse J (2020) Recent advances in chemotherapy for pancreatic cancer: evidence from Japan and recommendations in guidelines. *J Gastroenterol* **55**:369–382.
- Olive KP, Tuveson DA, Ruhe ZC, Yin B, Willis NA, Bronson RT, Crowley D, and Jacks T (2004) Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. *Cell* **119**:847–860.
- Quackenbush J (2002) Microarray data normalization and transformation. *Nat Genet* **32** (Suppl):496–501.
- Saigusa D, Motoike IN, Saito S, Zorzi M, Aoki Y, Kitamura H, Suzuki M, Katsuo F, Ishii H, Kinoshita K et al. (2020) Impacts of NRF2 activation in non-small-cell lung cancer cell lines on extracellular metabolites. *Cancer Sci* **111**:667–678.
- Samulitis BK, Pond KW, Pond E, Cress AE, Patel H, Wisner L, Patel C, Dorr RT, and Landowski TH (2015) Gemcitabine resistant pancreatic cancer cell lines acquire an invasive phenotype with collateral hypersensitivity to histone deacetylase inhibitors. *Cancer Biol Ther* **16**:43–51.
- Singh A, Venkannagari S, Oh KH, Zhang YQ, Rohde JM, Liu L, Nimmagadda S, Sudini K, Brimacombe KR, Gajghate S et al. (2016) Small molecule inhibitor of NRF2 selectively intervenes therapeutic resistance in KEAP1-deficient NSCLC tumors. *ACS Chem Biol* **11**:3214–3225.
- Taguchi K and Yamamoto M (2020) The KEAP1-NRF2 system as a molecular target of cancer treatment. *Cancers (Basel)* **13**:46.
- Tsuchida K, Tsujita T, Hayashi M, Ojima A, Keleku-Lukwete N, Katsuo F, Otsuki A, Kikuchi H, Oshima Y, Suzuki M et al. (2017) Halofuginone enhances the chemosensitivity of cancer cells by suppressing NRF2 accumulation. *Free Radic Biol Med* **103**:236–247.
- Wang C, Xu J, Yuan D, Bai Y, Pan Y, Zhang J, and Shao C (2020a) Exosomes carrying ALDOA and ALDH3A1 from irradiated lung cancer cells enhance migration and invasion of recipients by accelerating glycolysis. *Mol Cell Biochem* **469**:77–87.
- Wang L, Stadlbauer B, Lyu C, Buchner A, and Pohla H (2020b) Shikonin enhances the antitumor effect of cabazitaxel in prostate cancer stem cells and reverses cabazitaxel resistance by inhibiting ABCG2 and ALDH3A1. *Am J Cancer Res* **10**:3784–3800.
- Wang X, Spandidos A, Wang H, and Seed B (2012) PrimerBank: a PCR primer database for quantitative gene expression analysis, 2012 update. *Nucleic Acids Res* **40**:D1144–D1149.
- Yamamoto M, Kensler TW, and Motohashi H (2018) The KEAP1-NRF2 system: a thiol-based sensor-effector apparatus for maintaining redox homeostasis. *Physiol Rev* **98**:1169–1203.
- Zhao L, Lei H, Shen L, Tang J, Wang Z, Bai W, Zhang F, Wang S, and Li W (2017) Prognosis genes in gastric adenocarcinoma identified by cross talk genes in disease-related pathways. *Mol Med Rep* **16**:1232–1240.

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