Supplementary Information

Activation of Nrf2 by sulfuretin stimulates chondrocyte differentiation and increases bone lengths in zebrafish

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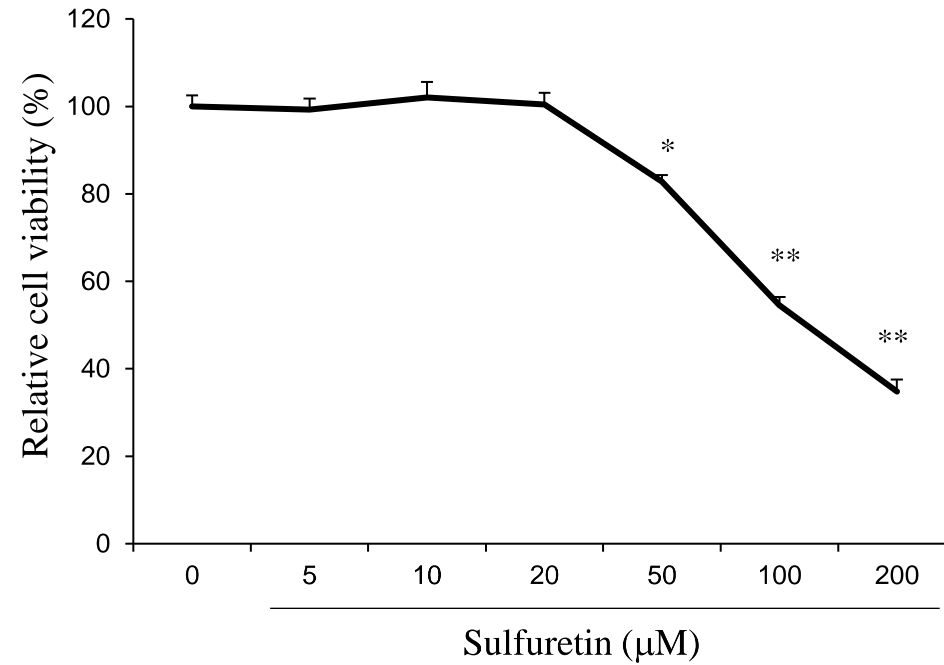

Figure S1. Effects of sulfuretin on cell viability. C3H10T1/2 cells were treated with sulfuretin at indicated concentrations for 48 hours. Cell viability was then determined by MTT assays. Data are presented as mean ± standard error of the mean (SEM) from three independent experiments. Statistical significance was determined relative to the control using Student's *t*-test (*$P < 0.05$, **$P < 0.01$).

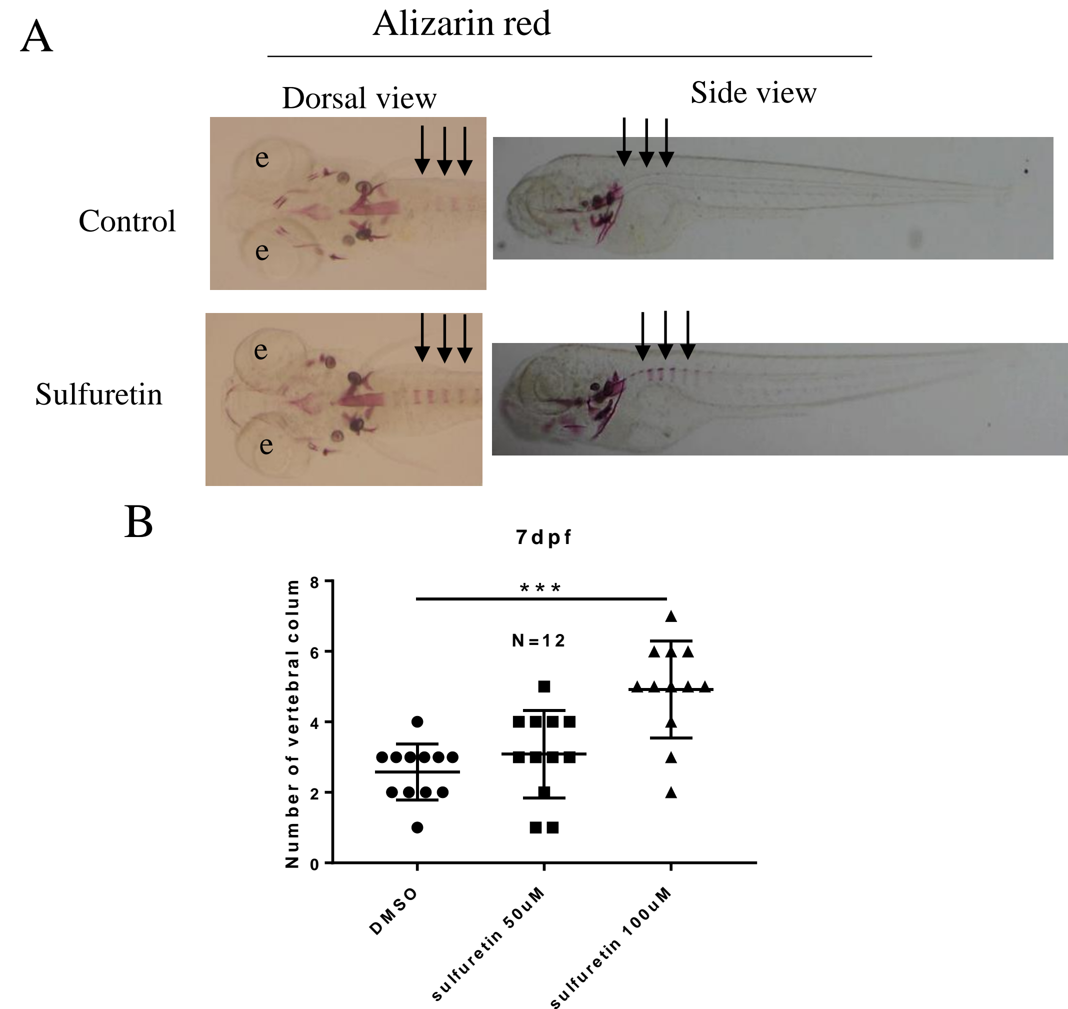

Figure S2. Nrf2 activation by sulfuretin promotes bone formation in zebrafish.

(A–B) Sulfuretin treatment promotes numbers of vertebral columns in zebrafish larvae. (A) Zebrafish at 1dpf were treated with sulfuretin at 500 μM or 100 μM for 6 days. Alizarin red staining was then performed. Arrows indicate vertebral columns. (B) Quantification of increased numbers of vertebral columns by sulfuretin. Data are presented as mean ± standard error of the mean (SEM) (n = 12). Statistical significance of sulfuretin group was determined by comparison with the control using Student's *t*-test (*** $P \langle$ 0.001).

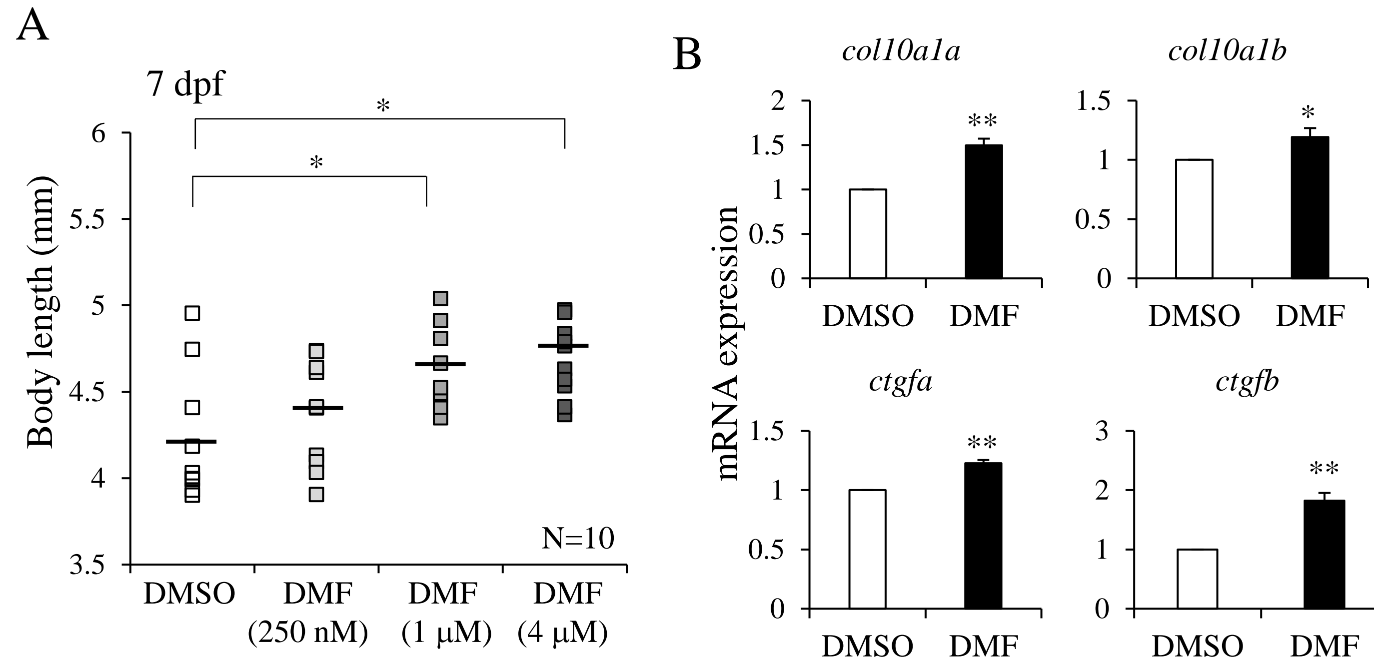

Figure S3. Pharmacological Nrf2 activation increases body length in zebrafish.

(A) Zebrafish at 1 dpf were treated with DMF at 250 nM, 1 μM, and 4 μM for 6 days and body lengths were measured. (B) Zebrafish at 1 dpf were treated with DMF at 4 μM for 6 days and expression levels of *Col10a1, Col10a1b, Ctgfa,* and *Ctgfb* were measured by real-time PCR. Data are presented as mean ± standard error of the mean (SEM) (n = 10). Statistical significance of the sulfuretin group was determined by comparison with the control group using Student's *t*-test (* $P < 0.05$; ** $P < 0.01$).

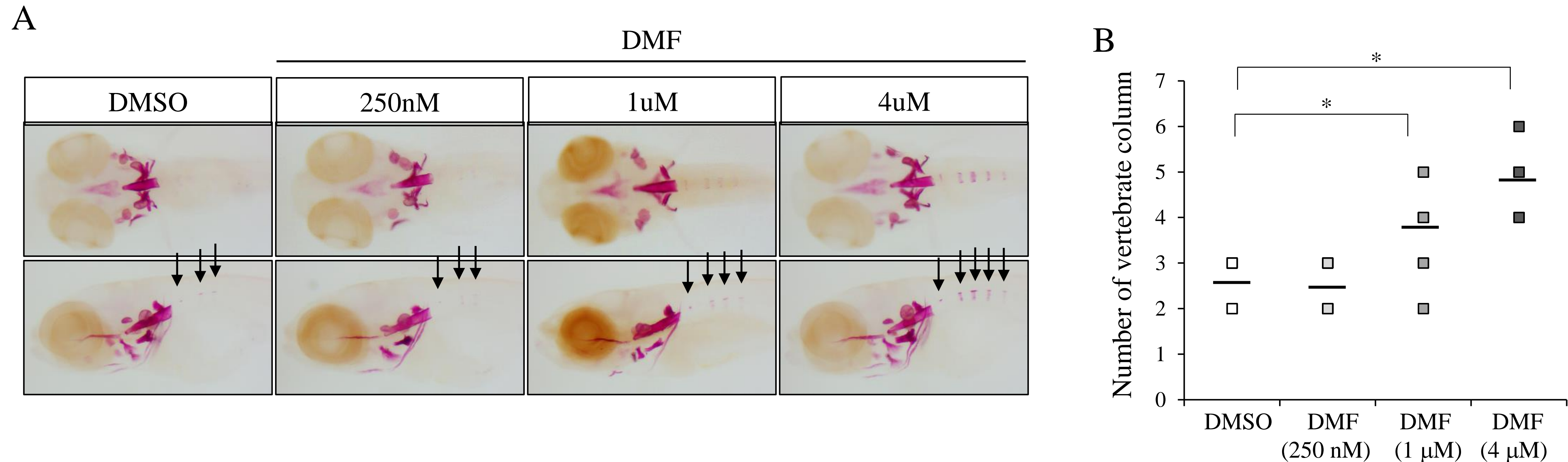

Figure S4. Pharmacological Nrf2 activation by DMF promotes bone formation in zebrafish.

(A-B) DMF treatment promotes numbers of vertebral columns in zebrafish larvae. (A) Zebrafish at 1dpf were treated with DMF at 250 nM, 1 μM, or 4 μM for 6 days and stained with Alizarin Red. Arrows indicate vertebral columns. (B) Quantification of increased numbers of vertebral columns by DMF. Data are presented as mean ± standard error of the mean (SEM) (n = 12). Statistical significance of sulfuretin group was determined by comparison with the control using Student's *t*-test (* *P* 〈 0.05).

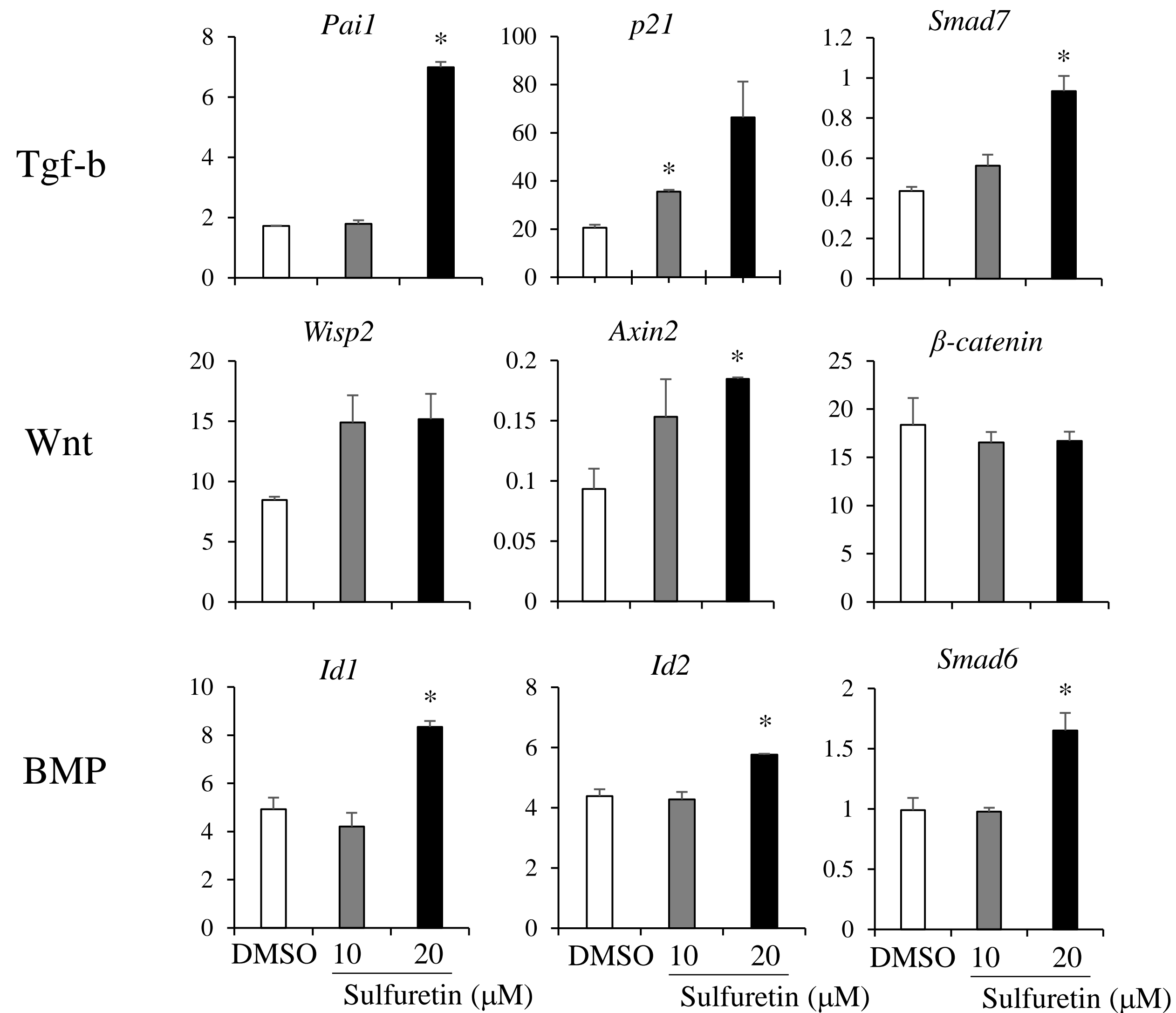

Figure S5. Effects of sulfuretin on expression of Tgf-β, Wnt, and BMP selective genes in chondrocytes. C3H10T1/2 cells were differentiated into chondrocytes in the presence of DMSO or sulfuretin (20 μM) for 12 days. Relative expression levels of Tgf-β (Pai I, P21, and Smad7), Wnt (Wisp2, Axin2, and β-catenin), and BMP ((Id1, Id2, and Samd6) selective genes were measured by real-time PCR. Data are presented as mean ± standard error of the mean (SEM). Statistical significance of sulfuretin treatment was determined by comparison with the control using Student's t-test (ns, not significant; * $P < 0.05$).

SUPPLEMENTARY MATERIALS AND METHODS

Reagents

Sulfuretin (〉98% purity), dimethylformamide (DMF), and tert-butylhydroquinone (tBHQ) was purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM, FBS, and penicillin/streptomycin were purchased from Hyclone (Logan, UT, USA). Insulin, human transferrin, and sodium selenite were from Sigma. The scramble control and Nrf2-specific small interfering RNA (siRNA) were synthesized from Genolution Pharmaceuticals, Inc. (Seoul, Korea) and RNAiMAX was from Invitrogen (Carlsbad, CA, USA). Real-time PCR-related reagents, including cDNA synthesis kit and THUNDERBRID® SYBR® qPCR Mix were purchased from TOYOBO (Osaka, Japan). TRIzol reagent was obtained from Invitrogen and realtime-PCR primers were purchased form IDT (Coralville, IA, USA).

Protease inhibitor cocktail was purchased from Roche Diagnostics (Manheim, Germany), polyvinylidene difluoride (PVDF) membranes from Bio-Rad Laboratories (Hercules, CA, USA) and western blotting detection reagent from Amersham Biosciences (Little Chalfont, UK). Nrf2 antibodies (sc-13032) and actin antiboies (sc-47778) were from Santa Cruz Biotech Santa Cruz, CA, USA), and horseradish peroxidase (HRP)-conjugated secondary antibodies were from Jackson ImmunoResearch Lab (West grove, PA, USA). Luciferase activities were measured using a Dual-luciferase Reporter Assay System (Promega, Madison, WI, USA). Other chemicals and reagents were purchased from Sigma.

Cell cultures

HEK293T cells and C3H10T1/2 cells were purchased and maintained as described previously (1). Cells of a passage number less than 20 were resuspended at density of 2×10^7cell/ml and one droplet (20 μl) was placed in the center of a 24-well plate. The medium was then replaced with chondrogenic medium containing 10 μg/ml of human insulin, 5.5 μg/ml of human transferrin, and 5 ng/ml of sodium selenite to induce cell differentiation.

Primary prechondrocytes were obtained from 5-7 days old C57BL/6 mice as reported previously (2). Briefly, murine epiphyseal cartilage was isolated from femoral heads and knee joints of newborn mice after digestion with 3 mg/ml

collagenase D for 45 min followed by digestion with 0.5 mg/ml collagenase D overnight at 37℃. Suspended prechondrocytes were passed through a 40 μm cell strainer and collected by centrifugation at 400 x g for 10 min. Cell pellets were washed twice with PBS and mixed with DMEM with 10% FBS and 1% penicillin/streptomycin. Primary prechondrocytes were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin and replaced with fresh media every two days and they were differentiated in the presence of sulfuretin (20 μM) or tBHQ (50 μM) for 2 weeks.

Differentiated chondrocytes were washed with PBS and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. After removing fixative solution, fixed chondrocytes were rinsed twice with PBS and then rinse with 0.1 M HCl once. Fixed cells were stained with 1% Alcian blue 8GX solution at room temperature for 1 hour and then washed with 0.1 M HCl to remove background.

C3H10T1/2 cells were seeded to 96-well plates and incubated with sulfuretin at the indicated doses for 48 h. Cells were treated with thiazolyl blue tetrazolium bromide (MTT) for 3 h at 37℃ and then washed with PBS twice. Insoluble formazans were dissolved in dimethyl sulfoxide (DMSO) and the absorbance was measured at 540 nm with a microplate reader (Tecan, Mannedorf, Switzerland).

To measure luciferase activity, 6XARE sequences containing luciferase vector were transfected to 293FT cells using Lipofectamine 2000. 293FT cells were transfected with luciferase vector and/or Nrf2-expressing vector for 48 h, and treated with sulfuretin. Cells were harvested and luciferase activities were measured using a Dual-luciferase Reporter Assay System.

For Nrf2 knockdown experiments, C3H10T1/2 cells in a 6-well plate were transfected with 50 nmol of the scrambled (5'-CCUCGUGCCGUUCCAUCA GGUAGUU-3') or the Nrf2 siRNA (5'-GCACAAUGGAAUUCAAUGAUU-3') using RNAiMAX. After 6 hours, the media was replaced with fresh media and the cells were induced into chondrocytes using a differentiation protocol with or without sulfuretin treatments.

Expression analysis

Total RNAs were isolated using TRIzol reagent and reverse transcribed to cDNAs using a PrimeScript 1st strand cDNA synthesis kit at 42 ˚C for 60 min. Synthesized

cDNAs were heat inactivated at 95 ˚C for 5 min and used for real-time PCR analysis. PCR was performed on the Applied Biosystems QuantStudio 3 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using THUNDERBIRD® SYBR® qPCR Mix. Expression levels were normalized to 36B4 or β-actin. Realtime-PCR primers were shown in **Table S1**.

To detect Nrf2 protein expression levels, C3H10T1/2 cells were treated with sulfuretin for 6 hours were harvested and lysed in RIPA buffer (1 % NP-40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 10 mM NaF) containing a protease inhibitor cocktail. Protein lysates were subjected to SDS-PAGE and transferred to PVDF membranes. These membranes were probed with primary antibodies against Nrf2 or actin and HRP-conjugated secondary antibodies. An enhanced chemiluminescent western blotting detection reagent was used to detect protein expression.

Zebrafish experiments

AB strain zebrafish (*Danio rerio*) was maintained at 28.5 ˚C with 14 h light and 10 h dark cycles constantly. Embryos and larvae were raised in egg water (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4) in Petri dishes at 28.5 ˚C . Embryos were fixed at desired stages with 4 % paraformaldehyde. Zebrafish husbandry was performed in accordance with guidelines from the Korea Research Institute of Bioscience and Biotechnology (KRIBB). This study was approved by KRIBB-IACUC (approval number: KRIBB-AEC-22152). Dechorinated embryos were incubated with sulfuretin (50 and 100 μM) or DMF (0.25, 1, and 4 μM) in Petri dishes from 1 day postfertilization (dpf). Solutions were changed daily until the harvested day.

To transiently knockdown *nrf2* without affecting normal morphological development of larvae, ~4 ng of translation-blocking *nrf2* morpholino (MO) (5'-CAT TTC AAT CTC CAT CAT GTC TCA G-3') was injected into one cell-stage embryo (3). A standard control MO (5'-CCT CTT ACC TCA GTT ACA ATT TAT A-3') was used as a negative control.

For alizarin red staining, fixed embryos were washed with 1X phosphate-buffered saline containing 0.1% Tween-20 (PBST) for 10 min three times, followed by washing with 1% KOH in 1X PBST for 10 min and then with 1% KOH for 10 min. Pigmentation of embryos was bleached with 3% H_2O_2 in 1% KOH until embryos

became transparent. Bleached embryos were incubated with 0.005% alizarin red in 1% KOH for 4 h, followed by incubation with 1% KOH for 10 min. They were then incubated with 1% KOH/50% glycerol for 10 min. They were stored in 100% glycerol at 4 ℃.

The Whole-mount *in situ* hybridization process was performed following a previously described protocol (4). In short, DIG-labelled mRNA probes (~1 kb) were made and hybridized with anti-digoxigenin-AP Fab fragments. Staining was then developed by adding BCIP/NTP substrate. Stained embryos were visualized with a Leica M205 FCA. Images were taken with a Leica Flexacam C1 camera.

Statistics

Data are presented as mean ± standard error of the mean (SEM) or standard deviation (SD). Differences in gene expression and mineral accumulation were analyzed using a two-tailed unpaired Student's *t*- test. Statistical significance was considered when a *p*-value was less than 0.05.

Table S1. Real-time PCR primer sequences

Gene	Species	Forward (5'-3')	Reverse (3'- 5')
Nrf2	Mouse	TCTCCTCGCTGGAAAAAGAA	AATGTGCTGGCTGTGCTTTA
Hmox1	Mouse	CCTGAACTTTGAAACCAGCAG	TGCTTTTACAGGCCAGTTTTG
Nqo1	Mouse	TCATTCTCTGGCCGATTCA	CATCCTTCCAGGATCTGCAT
Gclm	Mouse	CCCGAAAGAACTGCTCTCTGA	CCACAGCGGCACCCAA
Srxn1	Mouse	TACCAATCGCCGTGCTCATC	AGGGTCCGCCAGGATCG
Maff	Mouse	GACAAGCACGCACTGAGC	CATTTTCGCAGAAGATGACCT
Pparg	Mouse	CCATTCTGGCCCACCAAC	AATGCGAGTGGTCTTCCATCA
Fabp4	Mouse	CACCGCAGACGACAGGAAG	GCACCTGCACCAGGGC
Cd36	Mouse	GGCCAAGCTATTGCGACAT	CAGATCCGAACACAGCGTAGA
Col10a1	Mouse	GCATCTCCCAGCACCAGAATC	CTTTATGCCTGTGGGCGTTT
Mmp3	Mouse	CCCTGGGACTCTACCACTCA	GCTGTGGGAGTTCCATAGAGG
Mmp13	Mouse	GGAGCCCTGATGTTTCCCAT	GTCTTCATCGCCTGGACCATA
Aggrecan	Mouse	CTTCTGCTTCCGAGGTGTGT	CCACCTGAGTGACGATCCAG
Col2a1	Mouse	CCCGGAAAGTCTGGGGAAAG	GCCTGGGTAACCTCTGTGAC
Sox9	Mouse	GAGCACTCTGGGCAATCTCA	CCCCTCTCGCTTCAGATCAA
Ihh	Mouse	CATGACCCAGCGCTGCAA	GGAGGAGCAAGCAGTCCAAA
36b4	Mouse	AGATGCAGCAGATCCGCAT	GTTCTTGCCCATCAGCACC
mmp13a	zebrafish	TTTGGGCTCTCTATGGCT	TTTCCGCAGACGGAACAT
col10a1a	zebrafish	TTCACCTGCCACATTCCA	ACATGACTGGGTCACCAT
col10a1b	zebrafish	CCAGATCGTGTACAATGG	ATACAGTCCTGGAACCTG
ctgfa	zebrafish	CTGCCTGGTTCAGACTACAG	CGAGACTGCTTCTCAAGGC
ctgfb	zebrafish	CCACTGAATGGAGCGAATGC	GATCATGCACATGCGGGTCT
nqo1	zebrafish	GCCTTCATGGAGACATCA	GTATGCAGGAGACCAGAA
gclc	zebrafish	ATCCCCATGTTGAACAGC	CCTGAAGCTCGTTTCTTG
gclm	zebrafish	ACCTCAGACCTGGACAAA	TGAGATCTGGAGGCATCA

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