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Sirtuin1 and autophagy protect cells from fluoride-induced cell stress

Maiko Suzuki and **John D. Bartlett***
Department of Mineralized Tissue Biology and Harvard School of Dental Medicine, The Forsyth Institute, 245 First Street, Cambridge, MA, 02142, USA

Abstract

Sirtuin1 (SIRT1) is an (NAD^+)-dependent deacetylase functioning in the regulation of metabolism, cell survival and organismal lifespan. Active SIRT1 regulates autophagy during cell stress, including calorie restriction, endoplasmic reticulum stress and oxidative stress. Previously, we reported that fluoride induces endoplasmic reticulum (ER) stress in ameloblasts responsible for enamel formation, suggesting that ER-stress plays a role in dental fluorosis. However, the molecular mechanism of how cells respond to fluoride-induced cell stress is unclear. Here, we demonstrate that fluoride activates SIRT1 and initiates autophagy to protect cells from fluoride exposure. Fluoride treatment of ameloblast-derived cells (LS8) significantly increased *Sirt1* expression and induced SIRT1 phosphorylation resulting in the augmentation of SIRT1 deacetylase activity. To demonstrate that fluoride exposure initiates autophagy, we characterized the expression of autophagy related genes (*Atg*); *Atg5*, *Atg7* and *Atg8/LC3* and showed that both their transcript and protein levels were significantly increased following fluoride treatment. To confirm that SIRT1 plays a protective role in fluoride toxicity, we used resveratrol (RES) to augmented SIRT1 activity in fluoride treated LS8 cells. RES increased autophagy, inhibited apoptosis, and decreased fluoride cytotoxicity. Rats treated with fluoride (0, 50 and 100 ppm) in drinking water for 6 weeks had significantly elevated expression levels of *Sirt1*, *Atg5*, *Atg7* and *Atg8/LC3* in their maturation stage enamel organs. Increased protein levels of p-SIRT1, ATG5 and ATG8/LC3 were present in fluoride-treated rat maturation stage ameloblasts. Therefore, the SIRT1/autophagy pathway may play a critical role as a protective response to help prevent dental fluorosis.

Keywords

Sirt1; autophagy; fluoride; enamel; ameloblast; dental fluorosis

1. Introduction

Fluoride is a specific and effective caries prophylactic and its addition to drinking water at a concentration of 0.7 ppm is recommended by the Centers for Disease Control and Prevention (CDC; 2011). However, chronic ingestion of excess fluoride causes dental fluorosis [1] which is manifested as mottled, discolored, porous enamel that is susceptible to

*Corresponding author at: Department of Mineralized Tissue Biology, The Forsyth Institute, 245 First Street, Cambridge, MA 02142, USA, Tel.: +1-(617) 892-8388; Fax: +1-(617) 892-8510 jbartlett@forsyth.org (J.D. Bartlett).

decay [2]. Compared to normal enamel, fluorosed enamel has a lower mineral content and a higher protein content [3–9] and therefore has reduced hardness. Acute or chronic fluoride over-exposure can also result in skeletal fluorosis [10], renal toxicity [11], epithelial lung cell toxicity [12] and reproductive toxicity [13]. Over-ingestion of fluoride from various sources such as beverages and toothpaste can also cause dental fluorosis, resulting in hypomineralized, stained and brittle teeth. Recent reports indicate that as many as 32% of children in the United States suffer from mild to severe forms of dental fluorosis [14] and that this number is increasing [15].

Fluoride is also an environmental health hazard. On June 8, 1783 the Laki volcanic crater in Iceland erupted and continued erupting until February 1784. This released an estimated 8 million tons of hydrogen fluoride (HF) [16]. Grazing livestock had severe fluorosis symptoms including softening and deformation of bones and joints. In areas thick with fine ash, mass death occurred within 8–14 days of the initial eruption and more than 60% of the grazing livestock died from HF poisoning in less than a year [17]. In the summer of 1783 the fallout created a haze in parts of France that increased the human death rate by 38% [16]. Currently, in the US over 200,000 children are in areas with fluoride levels in drinking water higher than 4 ppm [18] and the incidence of dental fluorosis has risen by 9% within the last 15–20 years [14]. Therefore, fluoride at permissible concentrations protects against tooth decay, but at high concentrations fluoride is a toxin.

Enamel development occurs in stages [19]. The cells of the enamel organ responsible for enamel formation are the ameloblasts. They occur as a single layer of cells located directly adjacent to the forming enamel. During the secretory stage the tall columnar ameloblasts secrete proteins into the enamel matrix. During the maturation stage the ameloblasts shorten and reabsorb the secreted proteins. It is during the maturation stage when most of the mineral precipitates and this process generates abundant hydrogen ions causing the ameloblasts to be exposed to an acid environment (pH<6.0) [20]. Acid promotes the conversion of fluoride into highly toxic HF that can easily penetrate the cell membrane. We Have shown that acid increases fluoride toxicity and that the acid environment of maturation stage ameloblasts makes these cells more susceptible to the toxic effects of fluoride exposure. Specifically, we demonstrated previously that mRNA expressed during the maturation stage (*Klk4* and *Amtn*) displayed reduced expression *in vivo* after fluoride exposure whereas fluoride had no effect on the expression levels of mRNA generated during the secretory stage (*Ambn*, *Amel*, *Enam* and *Mmp20*) [21]. Kalikrein-4 (KLK4) is important because it cleaves enamel matrix proteins during the maturation stage to facilitate their export out of the hardening enamel. Since fluorosed enamel has a greater than normal protein content, a lack of KLK4 activity may be responsible for the elevated protein levels. We and others have shown that high doses of fluoride cause cell stress [22–24] and that cells of dental tissues such as ameloblasts [25–27] and odontblasts [28] are affected. However, little is known about the molecular mechanisms that cause or protect cells and tissues from fluoride toxicity.

Sirtuins are nicotinamide adenine dinucleotide (NAD^+)-dependent class III deacetylases and are the mammalian homologues of yeast silent information regulator-2 (Sir2) [29–31]. Sirtuins remove acetyl groups from protein substrates ranging from histones to transcriptional regulators thereby regulating the biological functions of the substrates post-translationally [32]. In mammals the sirtuin family comprises seven proteins (SIRT1 to SIRT7) [33]. SIRT1 is most closely related to the *Sir2* gene of *Saccharomyces cerevisiae* [34] and is itself regulated post-transcriptionally via phosphorylation [35–37]. Residues Thr530 and Ser540 are phosphorylated by cyclinB/Cdk1 [36], and Ser27, Ser47 and Thr530 are phosphorylated by c-Jun N-terminal kinase 1 (JNK1) [37]. Phosphorlyated SIRT1 (p-SIRT) is an active deacetylase compared to its non-phosphorylated form [36]. By

deacetylating target substrates, including FOXOs, PGC-1α and p53, SIRT1 assists in resisting stress caused by caloric restriction (CR), oxidative stress and endoplasmic reticulum (ER) stress [38–41]. Thus, SIRT1 promotes cell survival by modulating cellular processes involved in the maintenance of homeostasis and stress adaptation.

SIRT1 regulates autophagy during cell stress [42, 43]. Macroautophagy, generally referred to as autophagy, is a phylogenetically conserved intracellular catabolic process that allows for the degradation of cytoplasmic components, such as damaged proteins and organelles [44–46]. Autophagic activities are mediated by a multi-step process, involving the formation of double-membrane vesicles known as autophagosomes. Autophagic activities are mediated by a complex molecular machinery including approximately 50 lysosomal hydrolases and more than 30 autophagy related genes (*Atg*) that are conserved from yeast to mammals [47, 48]. Autophagy is induced by a variety of stress stimuli, including CR [49], hypoxia [50], oxidative stress [51], ER-stress [52], and DNA damage [53]. Autophagy is mostly cytoprotective and ameliorates cell stress. Pharmacological induction of autophagy improves cell survival while autophagy inhibition promotes cell death [54–56]. SIRT1 deacetylates the major regulators of autophagy [57] including ATG5, ATG7 and ATG8/LC3 [43, 58–60].

Resveratrol (RES) is a natural herbal compound found in grapes, peanuts and other plants. RES activates SIRT1 [61], induces autophagy [62] and increases adenosine 5´-monophosphate-activated protein kinase (AMPK) phosphorylation [63]. To date, many studies have assessed the biology and pharmacology of RES, including the molecular mechanisms of resveratrol´s cytoprotection [64] which led to the identification of multiple molecular RES targets [65]. RES is not only a SIRT1 activator, but also functions as a phytoalexin, antioxidant, cyclooxygenase (COX) inhibitor, peroxisome proliferator-activated receptor-alpha (PPAR-α) activator, and an endothelial nitric oxide synthase (eNOS) inducer. Recent results show several potential beneficial effects of RES administration. RES prevents or slows the progression of a wide variety of pathologies, including neuronal disease [66–69], obesity [70], cardiovascular disease [71], and cancer [72]. Therefore, RES may someday be of therapeutic value.

At low doses as recommended by the CDC, fluoride protects against dental caries. However at high doses, fluoride is toxic and its toxicity increases in an acidic environment. Herein, we examine how cells adapt to the toxic effects of high dose fluoride exposure by demonstrating *in vitro* and *in vivo* that SIRT1 and autophagy are key components in the adaptive response to fluoride toxicity.

2. Materials and methods

2.1. Animals

Sprague-Dawley rats (6-week-old) were purchased from Charles River Laboratories (Wilmington, MA) and were provided water containing 0, 50, 100 or 125 ppm fluoride as sodium fluoride *ad libitum*. After 6 weeks, animals were euthanized and incisor enamel organs, separated into the secretory and maturation stages of enamel development, were extracted for qPCR assays and complete incisors were used for immunohistochemical analysis. All animals were treated humanely and all handling procedures were approved by the Institutional Animal Care Use Committee (IACUC) at The Forsyth Institute. The Forsyth Institute is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and follows the *Guide for the Care and Use of Laboratory Animals* (NRC1996).

2.2. Cell culture

The mouse ameloblast-derived cell line (LS8) was maintained in alpha minimal essential medium with GlutaMAX (Life Technologies, Grand Island, NY) supplemented with fetal bovine serum (10%) and sodium pyruvate (1 mM). Sodium fluoride: NaF (Cat. S299-100, Fisher Scientific, Pittsburgh, PA), Resveratrol (Cat. R5010-100MG, Sigma, St. Louis, MO), and Inauhzin (Cat. 566332, Calbiochem, San Diego, CA) were included as indicated.

2.3. Real-time PCR analysis (*qPCR*)

Total RNA was extracted from LS8 cells or rat enamel organs using Direct-zol™ RNA MiniPrep (Zymo Research Corp., Irvine, CA) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Minneapolis, MN). The cDNA was subjected to real-time PCR amplification on a Light Cycler 480 Real-Time PCR System (Roche Diagnostics). The internal reference control gene was *Eef1a1*. We have performed several analyses to identify an internal reference control gene with a consistent expression level among various fluoride treatment concentrations and *Eef1a1* was the gene of choice (unpublished data). The relative expression of the target gene was determined by the $2^{-\Delta\Delta CT}$ method [73]. The following primers were synthesized by Invitrogen (Grand Island, NY). For murine LS8 cells: *Sirt1*, forward: 5'-GCTGGGGTTTCTGTCTCCTG-3', reverse:5'-GACACAGAGACGGCTGGAAC-3'; *Atg5*, forward: 5'-GTGCTTCGAGATGTGTGGTTTGGA-3', reverse: 5'-CGTCAAATAGCTGACTCTTGGCAA-3'; *Atg7*, forward: 5'-GCTAATGGACACCAGGGAGA-3', reverse: 5'-AAAAAGTGAGGAGCCCAGGT-3'; *Atg8/LC3*, forward: 5'-ATCATCGAGCGCTACAAGGGTGA-3', reverse: 5'-GGATGATCTTGACCAACTCGCTCAT-3'; *Eef1a1* forward: 5'-GTCGCAGGGGCTTGTCAGTT-3', reverse:5'-ACCCGCAAAGATGGCAGTG-3'. For rat enamel organ: *Sirt1*, forward: 5'-GGTATTTATGCTCGCCTTGCTG-3', reverse: 5'-GTGACACAGAGATGGCTGGAACT-3'; *Atg5*, forward: 5'-GCTTCGAGACGTGTGGTTTGGA-3', reverse:5'-GCGTCAAATAGCTGACTCTTGGCA-3'; *Atg7*, forward: 5'-GCTGGTCTCCTTGCTCAAAC-3', reverse: 5'-GGGTGCTGGGTTAGGTTACA-3'; *Atg8/LC3*, forward: 5'-ATCATCGAGCGCTACAAGGGTGA-3', reverse: 5'-GGATGATCTTGACCAACTCGCTCAT-3'; *Eef1a1*, forward: 5'-TGATGCCCCAGGACACAGAGACT-3', reverse: 5'-GATACCAGCTTCAAATTCCCCAACAC-3'.

2.4. Western blot analysis

LS8 cells were treated with NaF for the indicated concentrations and times and proteins were extracted with RIPA buffer (25mM Tris·HCl pH7.4, 150mM NaCl, 1% NP-40, 1mM EDTA, 5% glycerol) containing Halt protease inhibitor cocktail (Cat.78442, Thermo Scientific, Rockford, IL). Proteins (10–30 μg) were loaded onto Mini-Potean® TGX™ gels (Biorad, Hercules, CA), transferred to Trans-Blot Turbo Transfer nitrocellulose membranes (Biorad) and probed with primary antibodies. Primary antibodies included: rabbit anti-pSIRT1 [pSer47] (Bioss, Inc., Woburn, MA); rabbit anti-SIRT1, rabbit anti-β-actin, rabbit anti-ATG5, rabbit anti-ATGg7, rabbit anti-LC3, rabbit anti-Caspase-3 and rabbit anti-PARP (Cell Signaling, Danvers, MA). The secondary antibody was HRP-conjugated goat anti-rabbit IgG (Biorad).

2.5. Immunocytochemistry and immunohistochemistry

LS8 cells were cultured on micro cover glasses (VWR, Radnor, PA) in 24 well plates and treated with NaF (3.0 mM) or RES (100 μM) for 2 h. Cells were fixed with 4%

formaldehyde and stained with rabbit anti-pSIRT [pSer47] (Bioss). The secondary antibody was Alexa Fluor 488-conjugated goat anti-rabbit IgG (Cell signaling). DRAQ5 (Cell signaling) was used for nuclear staining and subsequent analysis by confocal fluorescence microscopy (Leica Microsystems, Mannheim, Germany). Immunohistochemistry was performed as described previously [27]. Briefly, rat incisors were extracted, fixed and embedded in paraffin. Sections were incubated with rabbit anti-pSIRT [pSer47] (Bioss), rabbit anti-ATG5 or rabbit anti- LC3A/B (Abcam, Cambridge, MA), followed by incubation with a peroxidase-conjugated antibody, Vectastain Elite ABC Regent, (Vector Labs, Burlingame, CA) and ImmPACT™ DAB kit (Vector Labs). Sections were counterstained with 0.1% Fast Green in PBS and examined by light microscopy.

2.6. (NAD^+)-dependent deacetylase activity assay (SIRT1–7)

LS8 cells were cultured in 96 well plates and treated with NaF (0.0, 0.5, 1.0, 3.0 and 5.0 mM) for 6 h. The Cyclex SIRT1/Sir2 Deacetylsase Fluorometric Assay Kit (CycLex Co., Lid.,Nagano, Japan) was used according to the manufacturer's protocol. In brief, cell proteins were extracted with lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate) and added to reaction mixture containing 50 mM Tris–HCl pH 8.8, 4 mM $MgCl_2$, 0.5 mM DTT, 0.25 mAU/ml lysyl endopeptidase, 1 mM trichostatin A, 20 mM fluoro-substrate peptide, and 200 mM (NAD^+). The samples were mixed and incubated for 30 min at RT and the fluorescence intensity (ex. 355 nm, em. 460 nm) was recorded every 10 min over the course of 1 h and results were normalized to the protein concentration. The control (0.0 mM NaF) was assigned as 1, and the effects of NaF treatment were expressed as ratios relative to this value.

2.7. Cell proliferation assay

To assess cell proliferation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed. LS8 cells were cultured overnight in 96-well plates and then the indicated concentrations of NaF were added in the presence or absence of RES (10 and 100 μM). After 24 h, the MTT assay (Sigma) was performed as described previously [27].

2.8. Statistical analysis

For quantitative real-time PCR (qPCR) results of *Sirt1* and *Atgs in vitro*, differences among the two groups were analyzed by Student's *t*-test. *Sirt1* qPCR results were also assessed by regression analysis. All data were presented as the mean ± standard deviation (SD). For Sirtuin deacetylase activity and qPCR results of *Atgs in vivo*, regression analyses were performed. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Fluoride induces Sirt1 expression in dose dependent manner

Since *Sirt1* expression is induced by ER-stress and since we have previously demonstrated that fluoride causes ER-stress in cell lines and ameloblasts [25, 27], we asked if fluoride also induces *Sirt1* expression. The ameloblast-derived cell line (LS8) was treated with 0.0, 0.5, 1.0 or 3.0 mM fluoride as sodium fluoride for 4 h and the expression of *Sirt1* mRNA was quantified by qPCR with *Eef1a1* as the reference control gene. Fluoride significantly increased *Sirt1* gene expression ($p < 0.01$) and this expression increased with increasing fluoride concentrations (Fig. 1A). A regression analysis (Fig. 1B) revealed a highly significant value ($p < 0.0001$) demonstrating that fluoride strongly induces *Sirt1* in a dose-dependent manner.

3.2. Fluoride induces SIRT1 phosphorylation

The phosphorylated form of SIRT1 has increased enzymatic activity [36, 37]. So, we asked if in addition to increasing *Sirt1* gene expression, does fluoride also induce SIRT1 phosphorylation. LS8 cells were treated with 5.0 mM fluoride for 0–6 h and phosphorylation of Ser47 was evaluated. Western blots demonstrated that fluoride did enhance SIRT1 phosphorylation (p-SIRT1) in a time dependent manner (Fig. 2A)The ratio of p-SIRT1 to total Sirt1 (t-SIRT1) was increased time dependently (approximately 2 fold at 1h and 9 fold at 6h compared to the 0h time point). Enhancement of p-SIRT1 by lower concentrations of NaF (2.0 mM) was also observed after 24 h and 48 h of fluoride treatment (data not shown). RES activates SIRT1 by stimulating its phosphorylation [74] and immunocytochemical experiments showed that both fluoride (3.0 mM; 2 h) and RES (100 μM; 2 h) enhanced SIRT1 phosphorylation in LS8 cells (Fig. 2B).

3.3. Fluoride induces SIRT1–7(NAD^+)-dependant deacetylase activity

Since fluoride induced SIRT1 phosphorylation and SIRT1 deacetylase activity is typically enhanced by phosphorylation, we sought to confirm that fluoride stimulates NAD(+)-dependent deacetylase activity. LS8 cells were treated with 0.0–5.0 mM fluoride at 1.0 mM increments for 6 h and deacetylase activity was quantified by use of a florometric assay. Fluoride did significantly increase (NAD^+)-dependent deacetylase activity ($p < 0.0001$) in a dose dependent manner (Fig. 3) confirming that the fluoride-mediated increase in SIRT1 phosphorylation is associated with a corresponding increase in deacetylase activity.

3.4. RES activates SIRT1 and ameliorates the anti-proliferative effects observed in fluoride treated LS8 cells

Here we test whether SIRT1 acts to mitigate the toxic effects of fluoride exposure. Previously, we showed that fluoride inhibits proliferation of LS8 cells [27] and now we ask if enhancing SIRT1 activity with RES will help recover LS8 proliferation. In addition to our immunocytochemical results (Fig. 2B), we performed western blots to confirm RES induces SIRT1 phosphorylation and to determine if this phosphorylation is time dependent. RES (100 μM) induced SIRT1 phosphorylation within 2 h and this induction remained steady and elevated for the next 3 h (Fig. 4A). LS8 cells were treated with 5.0 mM fluoride for 24 h and were then harvested and counted in the presence of a viability stain. Fluoride treatment significantly inhibited cell proliferation, but did not affect LS8 viability (Fig. 4B). Next we performed MTT assays over a wide range of fluoride concentrations to evaluate the effect of fluoride and RES on LS8 cell proliferation. LS8 cells were treated with 0.0–10.0 mM fluoride at 1.0 mM increments for 24 h in the absence or presence of 10 μM RES. MTT assays showed that RES promoted a higher rate of LS8 proliferation starting with the 2.0 mM fluoride dose that continued for each higher dose measured (Fig. 4C). Inauhzin (INZ) inhibits SIRT1 activity [75, 76]. So we employed MTT assays to evaluate the effect of SIRT1 inhibitors on proliferation of LS8 cells treated with 5.0 mM fluoride for 24 h. RES at 10 or 100 μM significantly ($p < 0.01$) reversed the anti-proliferative effects of fluoride in a dose-dependent manner. In contrast, the SIRT1 inhibitor INZ eliminated the RES-mediated amelioration of LS8 cell proliferation in the presence of fluoride (Fig. 4C). These data suggest that augmentation of SIRT1 plays a protective role against fluoride cytotoxicity.

3.5. Fluoride induces autophagy in LS8 cells

SIRT1 deacetylase activity is induced by cell stress and is an important regulator of autophagy [43, 59, 60]. Since we demonstrated that fluoride augments SIRT1 deacetylase activity, we also asked if fluoride is capable of initiating autophagy via induction of autophagy related gene expression. LS8 cells were treated with 3.0 mM fluoride for 4 h and expression of *Atg5*, *Atg7* and *Atg8/LC3* mRNA were quantified by qPCR. Fluoride treatment

significantly increased ($p < 0.01$) the expression of *Atg5* (2.4 fold), *Atg7* (7.5 fold) and *Atg8/LC3* (3.3 fold) compared to the 0.0 mM treatment control (Fig. 5A). Western blots were performed to determine if fluoride also induced ATG protein levels. LS8 cells were treated with the indicated concentration of fluoride for 24 h prior to protein extraction. Fluoride increased ATG5 and ATG7 expression in a dose dependent manner. Fluoride also augmented the formation of LC3 II which is an indicator of autophagozome formation. (Fig. 5B). However LC3 II levels in cells treated with higher concentrations of fluoride (3 mM) were the same as control levels after 24 h treatment, suggesting that LC3 II formation may be a "switch" that responds to relatively low dose fluoride, but that remains off at higher doses that may lead to a different pathway such as apoptosis. Therefore, these data demonstrate that fluoride exposure induces both gene and protein expression of autophagy-related genes necessary for an autophagic stress response.

3.6. SIRT1 activation by RES promotes autophagy and attenuates fluoride-mediated apoptosis

Previously we showed that fluoride induces caspase-mediated apoptosis in LS8 cells [27]. So, we asked if RES could ameliorate apoptosis caused by fluoride exposure. LS8 cells were treated with 1.0 mM or 5.0 mM fluoride for 24 h in the absence or presence of 1, 10 or 100 μM RES. With the 5 mM but not 1.0 mM fluoride treatment, RES increased the levels of the active ATG protein LC3 II over that of the levels observed by use of fluoride alone (Fig. 6A). This indicates that at the higher dose fluoride treatment, RES enhanced the fluoride-mediated autophagic response. LS8 cells were next treated with 5.0 mM fluoride for 6 h with or without RES to determine if RES inhibited fluoride-mediated apoptosis. Western blots revealed that RES attenuated fluoride-mediated caspase-3 cleavage/activation and PARP cleavage/activation (Fig. 6B), indicating that augmentation of SIRT1 activity and autophagy by RES helped rescue LS8 cells from the toxic effects of 5.0 mM fluoride. This was consistent with results presented in Figure 5 that suggest high dose fluoride elicits a different stress response pathway (apoptosis) than does lower dose fluoride.

3.7. Rats treated with fluoride have increased levels of SIRT1 in their maturation stage enamel organs and ameloblasts

We showed *in vitro* in LS8 cells that fluoride induces *Sirt1* expression (Fig. 1) and phosphorylation (Fig. 2). Next we asked if fluoride induces *Sirt1* expression and phosphorylation *in vivo*. Note that rats given 50 ppm F^- in drinking water have serum fluoride levels similar to humans ingesting 2–5 ppm fluoride [77]. Rodents are more efficient at clearing fluoride from their bodies as compared to humans [78]. Also, development of the rodent incisor takes approximately 35 days to complete whereas in humans, depending on the tooth type, it takes from three to more than ten years to complete [77]. Thus, human ameloblasts have a much longer exposure to fluoride present in drinking water than do rodent ameloblasts and this is likely a factor in the large difference in fluoride sensitivity between humans and rodents.

Sprague-Dawley rats (6-week-old) were provided 50, 100 or 125 ppm fluoride as NaF in drinking water. Rodent incisors erupt continuously so every stage of enamel development persists along the adult rodent incisor. After 6 weeks of fluoride treatment incisors were collected for extraction of enamel organ RNA or were processed for SIRT1 immunohistochemistry. *Sirt1* expression was quantified by qPCR in secretory or maturation stage enamel organ. *Sirt1* expression increased significantly ($p < 0.05$) in the fluoride-treated maturation stage, but not the secretory stage enamel organ (Fig 7A). The difference in *Sirt1* expression between the secretory and maturation stage enamel organs in rats treated with 100 ppm fluoride was highly significant ($p < 0.01$). SIRT1 phosphorylation was assessed by immunohistochemical analysis of rat incisor paraffin sections. A small increase in SIRT1

phosphorylation was observed in ameloblasts of the secretory stage enamel organs from the 100 ppm and 125 ppm fluoride treatment groups. However, a much larger increase in SIRT1 phosphorylation was noted in ameloblasts and papillary layer of the maturation stage enamel organ from these two treatment groups (Fig. 7B). Therefore, fluoride treatment induced *Sirt1* expression and phosphorylation in rat maturation stage, but not secretory stage enamel organ.

3.8. Rats treated with fluoride have increased levels of autophagy in their maturation stage enamel organs and ameloblasts

Next we asked if autophagy gene expression is induced in the enamel organs of fluoride-treated rats. Rat incisor enamel organs at the secretory or maturation stage of development were extracted for RNA and qPCR was performed to determine if treatment with 50 or 100 ppm fluoride induced the expression of *Atg5*, *Atg7* and *Atg8/LC3*. Each of these genes were significantly induced ($p < 0.05$) in the fluoride-treated maturation stage rat enamel organ and the expression of each of these genes was greater in rats treated with 100 ppm fluoride as compared to those treated with 50 ppm fluoride (Fig. 8A). Among treatment groups, no significant differences in gene expression were observed for the secretory stage enamel organ. We performed immunohistochemistry on paraffin-embedded rat incisor sections to identify cells of the enamel organ with increased protein levels of ATG5 and ATG8/LC3. In the 100 ppm fluoride treatment groups the maturation stage ameloblasts stained strongly for ATG5 and ATG8/LC3 while the secretory stage ameloblasts did not show staining with fluoride treatment (Fig. 8B). This result was consistent with the qPCR results. Therefore, fluoride induces *Sirt1* expression, SIRT1 phosphorylation and autophagy both *in vitro* and *in vivo*.

4. Discussion

Here we show *in vitro* that fluoride induces *Sirt1* gene expression in dose dependent manner, induces SIRT1 phosphorylation, and induces SIRT deacetylase activity. We demonstrate that RES activates SIRT1 and ameliorates the anti-proliferative effects observed in fluoride treated LS8 cells. We also found that fluoride induces autophagy in LS8 cells and that SIRT1 activation by RES promotes autophagy and attenuates fluoride-mediated apoptosis. *In vivo*, we demonstrated that rats treated with fluoride have increased levels of *Sirt1* gene expression in their maturation stage enamel organs and have increased p-SIRT protein levels in their ameloblasts. These rats also had increased levels of autophagy related gene expression and autophagy related protein levels in their maturation stage enamel organs and ameloblasts after fluoride ingestion. Previously, fluoride was shown to induce autophagy in the rat exocrine pancreas [24], but the participation of *Atg* genes was not examined and this is the first report implicating SIRT1 in a protective response to fluoride toxicity. Although a relationship among ER-stress, SIRT1 and autophagy was reported in various tissues and organs [60], the relationship between these three responses in enamel development have not been previously demonstrated. Also, the SIRT1/autophagy pathway was not previously shown to play a protective role against fluoride-mediated cell stress. So, we show several novel findings that help illuminate the molecular pathways that are activated as a result of fluoride-mediated toxicity.

Previously we reported that fluoride initiates an ER-stress response in ameloblasts and that the ER-stress response inhibited protein synthesis and secretion during enamel formation. We therefore implicated ER-stress as a possible cause of dental fluorosis [25, 27]. Since ER-stress can induce the expression of SIRT1 and autophagy [60], this is what may occur upon exposure to fluoride in the ameloblasts and surrounding enamel organ to alleviate the ER-stress response.

Interestingly, the fluoride-mediated SIRT1/autophagy induction was observed in the maturation stage but not in the secretory stage of enamel development suggesting that only the maturation stage enamel organ, and its ameloblasts, were undergoing a stress response. Previously, we showed in both mice and rats [21, 25] that fluoride exposure caused the transient attenuation of protein translation via phosphorylation of the translation initiation factor, eukaryotic initiation factor-2, subunit alpha (eIF2α). This fluoride-mediated stress response also occurred during the maturation stage, but not during the secretory stage of enamel development. The stage specific fluoride-mediated SIRT1/autophagy and ER-stress results are consistent with our proposed hypothesis that fluoride is converted at a greater rate to highly toxic HF in the acidic environment of the maturation stage. We proposed that HF flows down a steep pH concentration gradient from the enamel matrix into the ameloblast cytosol. The neutral pH inside the ameloblast reverts HF to fluoride which maintains the concentration gradient and the accumulation of fluoride within the ameloblasts elicits an ER-stress response [21]. Therefore, the fluoride-mediated stage-specific SIRT1/autophagy results are consistent with our previously published results demonstrating that the acid generated by massive hydroxyapatite precipitation makes the ameloblasts of the maturation stage enamel organ more susceptible to fluoride toxicity.

JNK1 is activated by reactive oxygen species (ROS) [79] and by a response to ER-stress termed the unfolded protein response (UPR) [80–82]. Previously we demonstrated that fluoride induces the phosphorylation of JNK1 and c-jun [21] and others have demonstrated that JNK1 phosphorylates SIRT1 at three residues (Ser^{27}, Ser^{47} and Thr^{530}) which promotes SIRT1 enzymatic activity during oxidative stress [37]. Fluoride was also shown to induce oxidative stress [83, 84] and phosphorylate JNK1 [21, 85]. These results suggest that JNK1 may play a role in alleviating fluoride-induced cell stress by phosphorylating SIRT1 so that it becomes a highly active deacetylase.

To identify SIRT1 as playing a protective role in fluoride toxicity, we treated LS8 cells with NaF in the presence or absence of RES. The polyphenol RES artificially activates SIRT1 [61] via a direct “assisted allosteric” mechanism [86] and in our system RES did induce the phosphorylation of SIRT1 (Figs. 2B and 4A). In the presence of fluoride, RES enhanced LS8 cell proliferation and reduced the activation of apoptosis mediators (Figs. 4B, C and 6B). These results show that SIRT1 plays a protective role to mitigate the toxic effects of fluoride exposure. The therapeutic value of SIRT1 activation by RES was reported for several diseases [67–72] suggesting that RES may also be useful for prevention of dental fluorosis. However, how RES activates SIRT1 is still controversial because RES has multiple cellular targets in addition to SIRT1, such as AMP-activated protein kinase (AMPK) [87]. AMPK is well-described inhibitor of mammalian-homolog-target-of-rapamycin (mTOR) [88] and it can therefore induce autophagy via this pathway. Moreover, recent studies suggest that RES does not directly interact with SIRT1 [89–92] suggesting the possibility of participation of other molecular pathways. In addition, numerous studies have utilized a wide range of RES concentrations in various disease models and have demonstrated the effects of RES are not always protective. For example, one study found that RES adversely affected mouse hippocampal neurogenesis and cognitive function by a mechanism involving activation of AMPK and suppression of CREB and brain-derived neurotrophic factor signaling [93]. Therefore, although our data suggest that augmentation of SIRT1/autophagy by RES may ameliorate dental fluorosis, further elucidation of RES molecular mechanisms are required to address potential toxic effects of high-dose RES ingestion.

In conclusion, we show that SIRT1 and autophagy were induced in ameloblasts responsible for enamel formation as adaptive responses against cellular stresses, including ER-stress and oxidative stress, caused by fluoride. Augmentation of SIRT1/autophagy by RES protected

LS8 cells from fluoride-induced cytotoxicity and inhibited the activation of apoptosis mediators *in vitro*. Our results provide significant insight into new molecular mechanisms activated to alleviate fluoride toxicity in ameloblasts. This knowledge may someday enable development of new dental fluorosis therapies by use of chemical compounds that activate SIRT1.

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Abbreviations

AMPK	AMP-activated protein kinase
Atg	autophagy related gene
CR	caloric restriction
eIF2α	eukaryotic initiation factor-2, subunit alpha
ER	endoplasmic reticulum
HF	hydrogen fluoride
JNK1	c-Jun N-terminal kinase-1
LS8	ameloblast-derived cells
mTOR	inhibitor of mammalian-homolog-target-of-rapamycin
MTT	3-(4,5-dimethylthazol-2-yl)-2,5-di-phenyltetrazolium bromide
(NAD^+)	nicotinamide adenine dinucleotide
qPCR	quantitative real-time PCR
RES	resveratrol
ROS	reactive oxygen species
SIRT1	sirtuin1
UPR	unfolded protein response

References

1. Dean HT, Elvove E. Some Epidemiological Aspects of Chronic Endemic Dental Fluorosis. American journal of public health and the nation's health. 1936; 26:567–575.
2. DenBesten PK. Biological mechanisms of dental fluorosis relevant to the use of fluoride supplements. Community Dent.Oral Epidemiol. 1999; 27:41–47. [PubMed: 10086925]
3. Den Besten PK. Effects of fluoride on protein secretion and removal during enamel development in the rat. J Dent Res. 1986; 65:1272–1277. [PubMed: 3463596]
4. DenBesten PK, Heffernan LM. Enamel proteases in secretory and maturation enamel of rats ingesting 0 and 100 PPM fluoride in drinking water. Adv Dent Res. 1989; 3:199–202. [PubMed: 2701159]
5. Shinoda H. Effect of long-term administration of fluoride on physico-chemical properties of the rat incisor enamel. Calcif Tissue Res. 1975; 18:91–100. [PubMed: 1148900]
6. Shinoda H, Ogura H. Scanning electron microscopical study on the fluorosis of enamel in rats. Calcif Tissue Res. 1978; 25:75–83. [PubMed: 647440]

7. Wright JT, Chen SC, Hall KI, Yamauchi M, Bawden JW. Protein characterization of fluorosed human enamel. J.Dent.Res. 1996; 75:1936–1941. [PubMed: 9033447]
8. Zhou R, Zaki AE, Eisenmann DR. Morphometry and autoradiography of altered rat enamel protein processing due to chronic exposure to fluoride. Arch Oral Biol. 1996; 41:739–747. [PubMed: 9022911]
9. Triller M. Structural and histochemical observations of fluorotic enamel matrix. J Dent Res. 1979; 58:1028–1029. [PubMed: 84005]
10. Boivin G, Chavassieux P, Chapuy MC, Baud CA, Meunier PJ. Skeletal fluorosis: histomorphometric analysis of bone changes and bone fluoride content in 29 patients. Bone. 1989; 10:89–99. [PubMed: 2765315]
11. Zager RA, Iwata M. Inorganic fluoride. Divergent effects on human proximal tubular cell viability. Am.J.Pathol. 1997; 150:735–745. [PubMed: 9033286]
12. Thrane EV, Refsnes M, Thoresen GH, Lag M, Schwarze PE. Fluoride-induced apoptosis in epithelial lung cells involves activation of MAP kinases p38 and possibly JNK. Toxicol.Sci. 2001; 61:83–91. [PubMed: 11294978]
13. Ghosh D, Das SS, Maiti R, Jana D, Das UB. Testicular toxicity in sodium fluoride treated rats: association with oxidative stress. Reprod Toxicol. 2002; 16:385–390. [PubMed: 12220599]
14. Beltran-Aguilar ED, Barker LK, Canto MT, Dye BA, Gooch BF, Griffin SO, Hyman J, Jaramillo F, Kingman A, Nowjack-Raymer R, Selwitz RH, Wu T. Surveillance for dental caries, dental sealants, tooth retention, edentulism, and enamel fluorosis--United States, 1988–1994 and 1999–2002. MMWR Surveill Summ. 2005; 54:1–43. [PubMed: 16121123]
15. Browne D, Whelton H, O'Mullane D. Fluoride metabolism and fluorosis. J Dent. 2005; 33:177–186. [PubMed: 15725518]
16. Gudmundsson G. Respiratory health effects of volcanic ash with special reference to Iceland. A review. Clin Respir J. 2011; 5:2–9. [PubMed: 21159135]
17. Thorvaldur T, Self S. Atmospheric and environmental effects of the 1783–1784 Laki eruption: A review and reassessment. Journal of Geophysical Research. 2003; 108:7–29.
18. Doull, J.; Boekelheide, k; Farishain, BG.; Isaacson, RL.; Koltz, JB.; Kumar, JV.; Limeback, H.; Poole, C.; Puzas, JE.; Reed, NMR.; Thiessen, KM.; Webster, TF. Fluoride in Drinking Water: A Scientific Review of the EPA's Standards. Washington, DC: The National Acedemic Press; 2006.
19. Hu JC, Chun YH, Al Hazzazzi T, Simmer JP. Enamel formation and amelogenesis imperfecta. Cells, tissues, organs. 2007; 186:78–85. [PubMed: 17627121]
20. Smith CE, Issid M, Margolis HC, Moreno EC. Developmental changes in the pH of enamel fluid and its effects on matrix-resident proteinases. Advances in dental research. 1996; 10:159–169. [PubMed: 9206332]
21. Sharma R, Tsuchiya M, Skobe Z, Tannous BA, Bartlett JD. The acid test of fluoride: how pH modulates toxicity. PloS one. 2010; 5:e10895. [PubMed: 20531944]
22. Sun Z, Niu R, Wang B, Jiao Z, Wang J, Zhang J, Wang S, Wang J. Fluoride-induced apoptosis and gene expression profiling in mice sperm in vivo. Archives of toxicology. 2011; 85:1441–1452. [PubMed: 21340527]
23. Ogilvie AL. Histologic findings in the kidney, liver, pancreas, adrenal, and thyroid glands of the rat following sodium fluoride administration. Journal of dental research. 1953; 32:386–397. [PubMed: 13061678]
24. Ito M, Nakagawa H, Okada T, Miyazaki S, Matsuo S. ER-stress caused by accumulated intracistanal granules activates autophagy through a different signal pathway from unfolded protein response in exocrine pancreas cells of rats exposed to fluoride. Archives of toxicology. 2009; 83:151–159. [PubMed: 18696052]
25. Sharma R, Tsuchiya M, Bartlett JD. Fluoride induces endoplasmic reticulum stress and inhibits protein synthesis and secretion. Environmental health perspectives. 2008; 116:1142–1146. [PubMed: 18795154]
26. Wei W, Gao Y, Wang C, Zhao L, Sun D. Excessive fluoride induces endoplasmic reticulum stress and interferes enamel proteinases secretion. Environmental toxicology. 2013; 28:332–341. [PubMed: 21626649]

27. Kubota K, Lee DH, Tsuchiya M, Young CS, Everett ET, Martinez-Mier EA, Snead ML, Nguyen L, Urano F, Bartlett JD. Fluoride induces endoplasmic reticulum stress in ameloblasts responsible for dental enamel formation. The Journal of biological chemistry. 2005; 280:23194–23202. [PubMed: 15849362]
28. Jacinto-Aleman LF, Hernandez-Guerrero JC, Trejo-Solis C, Jimenez-Farfan MD, Fernandez-Presas AM. In vitro effect of sodium fluoride on antioxidative enzymes and apoptosis during murine odontogenesis. Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology. 2010; 39:709–714. [PubMed: 20738751]
29. Landry J, Sutton A, Tafrov ST, Heller RC, Stebbins J, Pillus L, Sternglanz R. The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. Proceedings of the National Academy of Sciences of the United States of America. 2000; 97:5807–5811. [PubMed: 10811920]
30. Imai S, Armstrong CM, Kaeberlein M, Guarente L. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature. 2000; 403:795–800. [PubMed: 10693811]
31. Smith JS, Brachmann CB, Celic I, Kenna MA, Muhammad S, Starai VJ, Avalos JL, Escalante-Semerena JC, Grubmeyer C, Wolberger C, Boeke JD. A phylogenetically conserved NAD+-dependent protein deacetylase activity in the Sir2 protein family. Proceedings of the National Academy of Sciences of the United States of America. 2000; 97:6658–6663. [PubMed: 10841563]
32. Blander G, Guarente L. The Sir2 family of protein deacetylases. Annual review of biochemistry. 2004; 73:417–435.
33. Morris BJ. Seven sirtuins for seven deadly diseases of aging. Free radical biology & medicine. 2013; 56:133–171. [PubMed: 23104101]
34. Frye RA. Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. Biochemical and biophysical research communications. 2000; 273:793–798. [PubMed: 10873683]
35. Beausoleil SA, Jedrychowski M, Schwartz D, Elias JE, Villen J, Li J, Cohn MA, Cantley LC, Gygi SP. Large-scale characterization of HeLa cell nuclear phosphoproteins. Proceedings of the National Academy of Sciences of the United States of America. 2004; 101:12130–12135. [PubMed: 15302935]
36. Sasaki T, Maier B, Koclega KD, Chruszcz M, Gluba W, Stukenberg PT, Minor W, Scrable H. Phosphorylation regulates SIRT1 function. PloS one. 2008; 3:e4020. [PubMed: 19107194]
37. Nasrin N, Kaushik VK, Fortier E, Wall D, Pearson KJ, de Cabo R, Bordone L. JNK1 phosphorylates SIRT1 and promotes its enzymatic activity. PloS one. 2009; 4:e8414. [PubMed: 20027304]
38. Kume S, Uzu T, Horiike K, Chin-Kanasaki M, Isshiki K, Araki S, Sugimoto T, Haneda M, Kashiwagi A, Koya D. Calorie restriction enhances cell adaptation to hypoxia through Sirt1-dependent mitochondrial autophagy in mouse aged kidney. The Journal of clinical investigation. 2010; 120:1043–1055. [PubMed: 20335657]
39. Hasegawa K, Wakino S, Yoshioka K, Tatematsu S, Hara Y, Minakuchi H, Washida N, Tokuyama H, Hayashi K, Itoh H. Sirt1 protects against oxidative stress-induced renal tubular cell apoptosis by the bidirectional regulation of catalase expression. Biochemical and biophysical research communications. 2008; 372:51–56. [PubMed: 18485895]
40. Kume S, Haneda M, Kanasaki K, Sugimoto T, Araki S, Isono M, Isshiki K, Uzu T, Kashiwagi A, Koya D. Silent information regulator 2 (SIRT1) attenuates oxidative stress-induced mesangial cell apoptosis via p53 deacetylation. Free radical biology & medicine. 2006; 40:2175–2182. [PubMed: 16785031]
41. Ghosh HS, Reizis B, Robbins PD. SIRT1 associates with eIF2-alpha and regulates the cellular stress response. Scientific reports. 2011; 1:150. [PubMed: 22355666]
42. Hariharan N, Maejima Y, Nakae J, Paik J, Depinho RA, Sadoshima J. Deacetylation of FoxO by Sirt1 Plays an Essential Role in Mediating Starvation-Induced Autophagy in Cardiac Myocytes. Circulation research. 2010; 107:1470–1482. [PubMed: 20947830]
43. Lee IH, Cao L, Mostoslavsky R, Lombard DB, Liu J, Bruns NE, Tsokos M, Alt FW, Finkel T. A role for the NAD-dependent deacetylase Sirt1 in the regulation of autophagy. Proceedings of the National Academy of Sciences of the United States of America. 2008; 105:3374–3379. [PubMed: 18296641]

44. Ohsumi Y. Molecular dissection of autophagy: two ubiquitin-like systems. Nature reviews. Molecular cell biology. 2001; 2:211–216.
45. Shintani T, Klionsky DJ. Autophagy in health and disease: a double-edged sword. Science. 2004; 306:990–995. [PubMed: 15528435]
46. Lum JJ, DeBerardinis RJ, Thompson CB. Autophagy in metazoans: cell survival in the land of plenty. Nature reviews. Molecular cell biology. 2005; 6:439–448.
47. Chen Y, Klionsky DJ. The regulation of autophagy - unanswered questions. Journal of cell science. 2011; 124:161–170. [PubMed: 21187343]
48. He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. Annual review of genetics. 2009; 43:67–93.
49. Boya P, Gonzalez-Polo RA, Casares N, Perfettini JL, Dessen P, Larochette N, Metivier D, Meley D, Souquere S, Yoshimori T, Pierron G, Codogno P, Kroemer G. Inhibition of macroautophagy triggers apoptosis. Molecular and cellular biology. 2005; 25:1025–1040. [PubMed: 15657430]
50. Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, Mukherjee C, Shi Y, Gelinas C, Fan Y, Nelson DA, Jin S, White E. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer cell. 2006; 10:51–64. [PubMed: 16843265]
51. Sid B, Verrax J, Calderon PB. Role of AMPK activation in oxidative cell damage: implications for alcohol-induced liver disease. Biochemical pharmacology. 2013
52. Kouroku Y, Fujita E, Tanida I, Ueno T, Isoai A, Kumagai H, Ogawa S, Kaufman RJ, Kominami E, Momoi T. ER stress (PERK/eIF2alpha phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. Cell death and differentiation. 2007; 14:230–239. [PubMed: 16794605]
53. Apel A, Herr I, Schwarz H, Rodemann HP, Mayer A. Blocked autophagy sensitizes resistant carcinoma cells to radiation therapy. Cancer research. 2008; 68:1485–1494. [PubMed: 18316613]
54. Kroemer G, Levine B. Autophagic cell death: the story of a misnomer. Nature reviews. Molecular cell biology. 2008; 9:1004–1010.
55. Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR, Hengartner M, Knight RA, Kumar S, Lipton SA, Malorni W, Nunez G, Peter ME, Tschopp J, Yuan J, Piacentini M, Zhivotovsky B, Melino G. D. Nomenclature Committee on Cell, Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell death and differentiation. 2009; 16:3–11. [PubMed: 18846107]
56. Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. Physiological reviews. 2007; 87:99–163. [PubMed: 17237344]
57. Geng J, Klionsky DJ. The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. 'Protein modifications: beyond the usual suspects' review series. EMBO reports. 2008; 9:859–864. [PubMed: 18704115]
58. Salminen A, Kaarniranta K. SIRT1: regulation of longevity via autophagy. Cellular signalling. 2009; 21:1356–1360. [PubMed: 19249351]
59. Rubinsztein DC, Marino G, Kroemer G. Autophagy and aging. Cell. 2011; 146:682–695. [PubMed: 21884931]
60. Kroemer G, Marino G, Levine B. Autophagy and the integrated stress response. Molecular cell. 2010; 40:280–293. [PubMed: 20965422]
61. Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL, Scherer B, Sinclair DA. Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. Nature. 2003; 425:191–196. [PubMed: 12939617]
62. Morselli E, Maiuri MC, Markaki M, Megalou E, Pasparaki A, Palikaras K, Criollo A, Galluzzi L, Malik SA, Vitale I, Michaud M, Madeo F, Tavernarakis N, Kroemer G. Caloric restriction and resveratrol promote longevity through the Sirtuin-1-dependent induction of autophagy. Cell death & disease. 2010; 1:e10. [PubMed: 21364612]
63. Fullerton MD, Steinberg GR. SIRT1 takes a backseat to AMPK in the regulation of insulin sensitivity by resveratrol. Diabetes. 2010; 59:551–553. [PubMed: 20190133]

64. Wong YT, Gruber J, Jenner AM, Ng MP, Ruan R, Tay FE. Elevation of oxidative-damage biomarkers during aging in F2 hybrid mice: protection by chronic oral intake of resveratrol. Free radical biology & medicine. 2009; 46:799–809. [PubMed: 19159678]

65. Smoliga JM, Baur JA, Hausenblas HA. Resveratrol and health--a comprehensive review of human clinical trials. Molecular nutrition & food research. 2011; 55:1129–1141. [PubMed: 21688389]

66. Marambaud P, Zhao H, Davies P. Resveratrol promotes clearance of Alzheimer's disease amyloid-beta peptides. The Journal of biological chemistry. 2005; 280:37377–37382. [PubMed: 16162502]

67. Feng X, Liang N, Zhu D, Gao Q, Peng L, Dong H, Yue Q, Liu H, Bao L, Zhang J, Hao J, Gao Y, Yu X, Sun J. Resveratrol inhibits beta-amyloid-induced neuronal apoptosis through regulation of SIRT1-ROCK1 signaling pathway. PloS one. 2013; 8:e59888. [PubMed: 23555824]

68. Araki T, Sasaki Y, Milbrandt J. Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. Science. 2004; 305:1010–1013. [PubMed: 15310905]

69. Parker JA, Arango M, Abderrahmane S, Lambert E, Tourette C, Catoire H, Neri C. Resveratrol rescues mutant polyglutamine cytotoxicity in nematode and mammalian neurons. Nature genetics. 2005; 37:349–350. [PubMed: 15793589]

70. Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, Prabhu VV, Allard JS, Lopez-Lluch G, Lewis K, Pistell PJ, Poosala S, Becker KG, Boss O, Gwinn D, Wang M, Ramaswamy S, Fishbein KW, Spencer RG, Lakatta EG, Le Couteur D, Shaw RJ, Navas P, Puigserver P, Ingram DK, de Cabo R, Sinclair DA. Resveratrol improves health and survival of mice on a high-calorie diet. Nature. 2006; 444:337–342. [PubMed: 17086191]

71. Bradamante S, Barenghi L, Villa A. Cardiovascular protective effects of resveratrol. Cardiovascular drug reviews. 2004; 22:169–188. [PubMed: 15492766]

72. Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC, Pezzuto JM. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science. 1997; 275:218–220. [PubMed: 8985016]

73. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001; 29:e45. [PubMed: 11328886]

74. Nin V, Escande C, Chini CC, Giri S, Camacho-Pereira J, Matalonga J, Lou Z, Chini EN. Role of deleted in breast cancer 1 (DBC1) protein in SIRT1 deacetylase activation induced by protein kinase A and AMP-activated protein kinase. The Journal of biological chemistry. 2012; 287:23489–23501. [PubMed: 22553202]

75. Bitterman KJ, Anderson RM, Cohen HY, Latorre-Esteves M, Sinclair DA. Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1. The Journal of biological chemistry. 2002; 277:45099–45107. [PubMed: 12297502]

76. Zhang Q, Zeng SX, Zhang Y, Zhang Y, Ding D, Ye Q, Meroueh SO, Lu H. A small molecule Inauhzin inhibits SIRT1 activity and suppresses tumour growth through activation of p53. EMBO molecular medicine. 2012; 4:298–312. [PubMed: 22331558]

77. Lyaruu DM, Bronckers AL, Santos F, Mathias R, DenBesten P. The effect of fluoride on enamel and dentin formation in the uremic rat incisor. Pediatric nephrology. 2008; 23:1973–1979. [PubMed: 18563453]

78. Angmar-Mansson B, Whitford GM. Enamel fluorosis related to plasma F levels in the rat. Caries research. 1984; 18:25–32. [PubMed: 6580951]

79. Wong CH, Iskandar KB, Yadav SK, Hirpara JL, Loh T, Pervaiz S. Simultaneous induction of non-canonical autophagy and apoptosis in cancer cells by ROS-dependent ERK and JNK activation. PloS one. 2010; 5:e9996. [PubMed: 20368806]

80. Zhang C, Kawauchi J, Adachi MT, Hashimoto Y, Oshiro S, Aso T, Kitajima S. Activation of JNK and transcriptional repressor ATF3/LRF1 through the IRE1/TRAF2 pathway is implicated in human vascular endothelial cell death by homocysteine. Biochemical and biophysical research communications. 2001; 289:718–724. [PubMed: 11726207]

81. Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, Ron D. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. Science. 2000; 287:664–666. [PubMed: 10650002]

82. Nishitoh H, Matsuzawa A, Tobiume K, Saegusa K, Takeda K, Inoue K, Hori S, Kakizuka A, Ichijo H. ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by

expanded polyglutamine repeats. Genes & development. 2002; 16:1345–1355. [PubMed: 12050113]

83. Hassan HA, Abdel-Aziz AF. Evaluation of free radical-scavenging and anti-oxidant properties of black berry against fluoride toxicity in rats. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 2010; 48:1999–2004. [PubMed: 20472017]
84. Barbier O, Arreola-Mendoza L, Del Razo LM. Molecular mechanisms of fluoride toxicity. Chemico-biological interactions. 2010; 188:319–333. [PubMed: 20650267]
85. Refsnes M, Skuland T, Schwarze PE, Ovrevik J, Lag M. Fluoride-induced IL-8 release in human epithelial lung cells: relationship to EGF-receptor-, SRC- and MAP-kinase activation. Toxicology and applied pharmacology. 2008; 227:56–67. [PubMed: 17996917]
86. Gut P, Verdin E. Rejuvenating SIRT1 activators. Cell metabolism. 2013; 17:635–637. [PubMed: 23663735]
87. Price NL, Gomes AP, Ling AJ, Duarte FV, Martin-Montalvo A, North BJ, Agarwal B, Ye L, Ramadori G, Teodoro JS, Hubbard BP, Varela AT, Davis JG, Varamini B, Hafner A, Moaddel R, Rolo AP, Coppari R, Palmeira CM, de Cabo R, Baur JA, Sinclair DA. SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function. Cell metabolism. 2012; 15:675–690. [PubMed: 22560220]
88. Sarbassov DD, Ali SM, Sabatini DM. Growing roles for the mTOR pathway. Current opinion in cell biology. 2005; 17:596–603. [PubMed: 16226444]
89. Blander G, Olejnik J, Krzymanska-Olejnik E, McDonagh T, Haigis M, Yaffe MB, Guarente L. SIRT1 shows no substrate specificity in vitro. The Journal of biological chemistry. 2005; 280:9780–9785. [PubMed: 15640142]
90. Borra MT, Smith BC, Denu JM. Mechanism of human SIRT1 activation by resveratrol. The Journal of biological chemistry. 2005; 280:17187–17195. [PubMed: 15749705]
91. Pacholec M, Bleasdale JE, Chrunyk B, Cunningham D, Flynn D, Garofalo RS, Griffith D, Griffor M, Loulakis P, Pabst B, Qiu X, Stockman B, Thanabal V, Varghese A, Ward J, Withka J, Ahn K. SRT1720, SRT2183, SRT1460, and resveratrol are not direct activators of SIRT1. The Journal of biological chemistry. 2010; 285:8340–8351. [PubMed: 20061378]
92. Kaeberlein M, McDonagh T, Heltweg B, Hixon J, Westman EA, Caldwell SD, Napper A, Curtis R, DiStefano PS, Fields S, Bedalov A, Kennedy BK. Substrate-specific activation of sirtuins by resveratrol. The Journal of biological chemistry. 2005; 280:17038–17045. [PubMed: 15684413]
93. Park HR, Kong KH, Yu BP, Mattson MP, Lee J. Resveratrol inhibits the proliferation of neural progenitor cells and hippocampal neurogenesis. The Journal of biological chemistry. 2012; 287:42588–42600. [PubMed: 23105098]

HIGHLIGHTS

- Fluoride exposure activates SIRT1 and autophagy
- SIRT1 and autophagy protect cells from fluoride-induced cytotoxcity
- Chemical induction of SIRT1 enhances cytotoxicity protection from fluoride exposure
- Fluoride induces SIRT1 *in vivo* in cells responsible for enamel formation
- SIRT1 and autophagy may play protective roles in dental fluorosis.

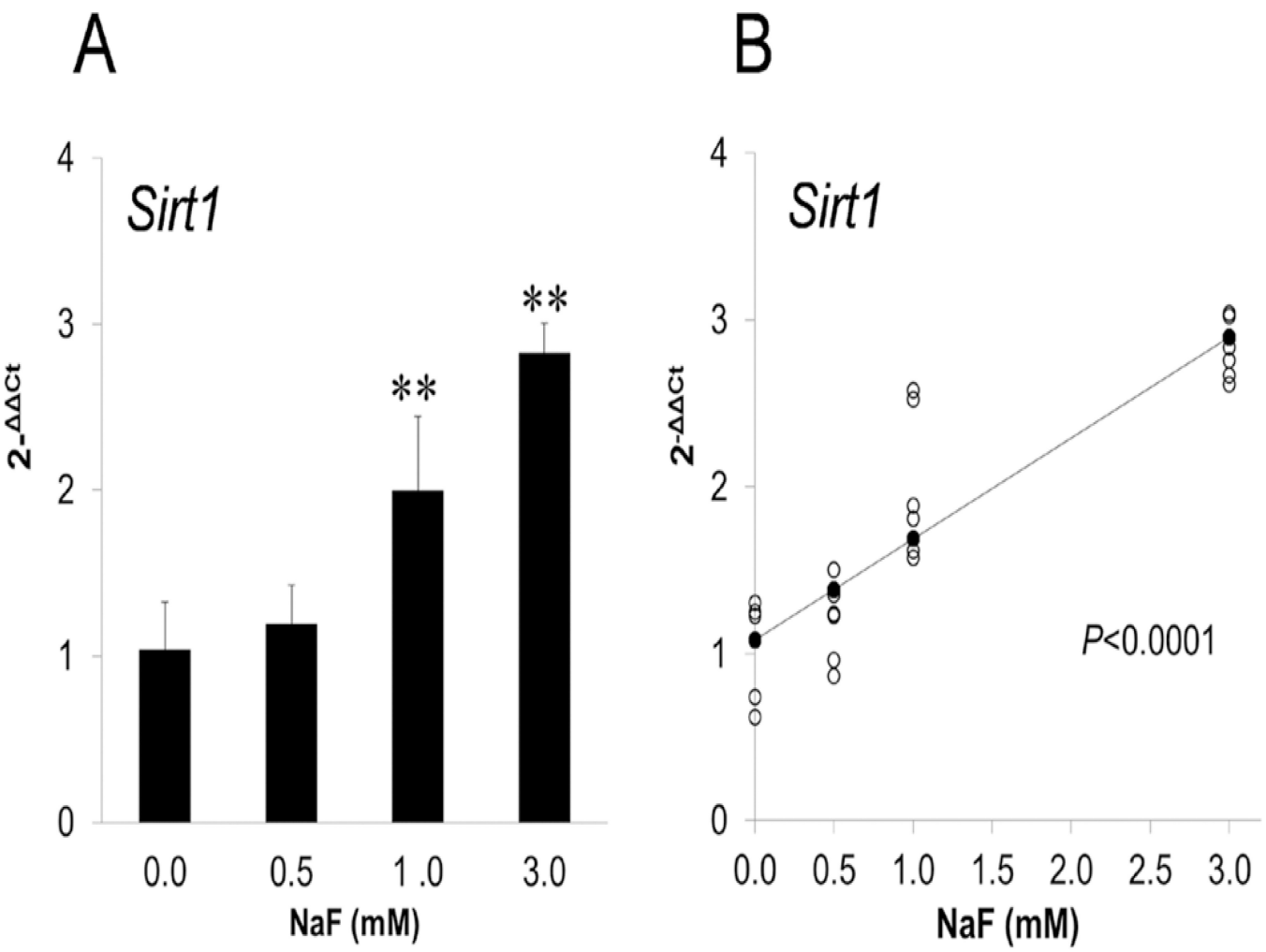

Fig.1.

Fluoride induces expression of *Sirt1* transcripts in a dose-dependent manner. Murine LS8 cells were treated with 0.0, 0.5, 1.0 or 3.0 mM NaF for 4 h and expression of *Sirt1* mRNA was evaluated by qPCR. *Eef1a1* was the reference control gene. (A) Data are expressed as mean ± SD (n = 6). (B) Regression analysis revealed a highly significant positive correlation between fluoride dose and *Sirt1* expression ($p < 0.0001$).

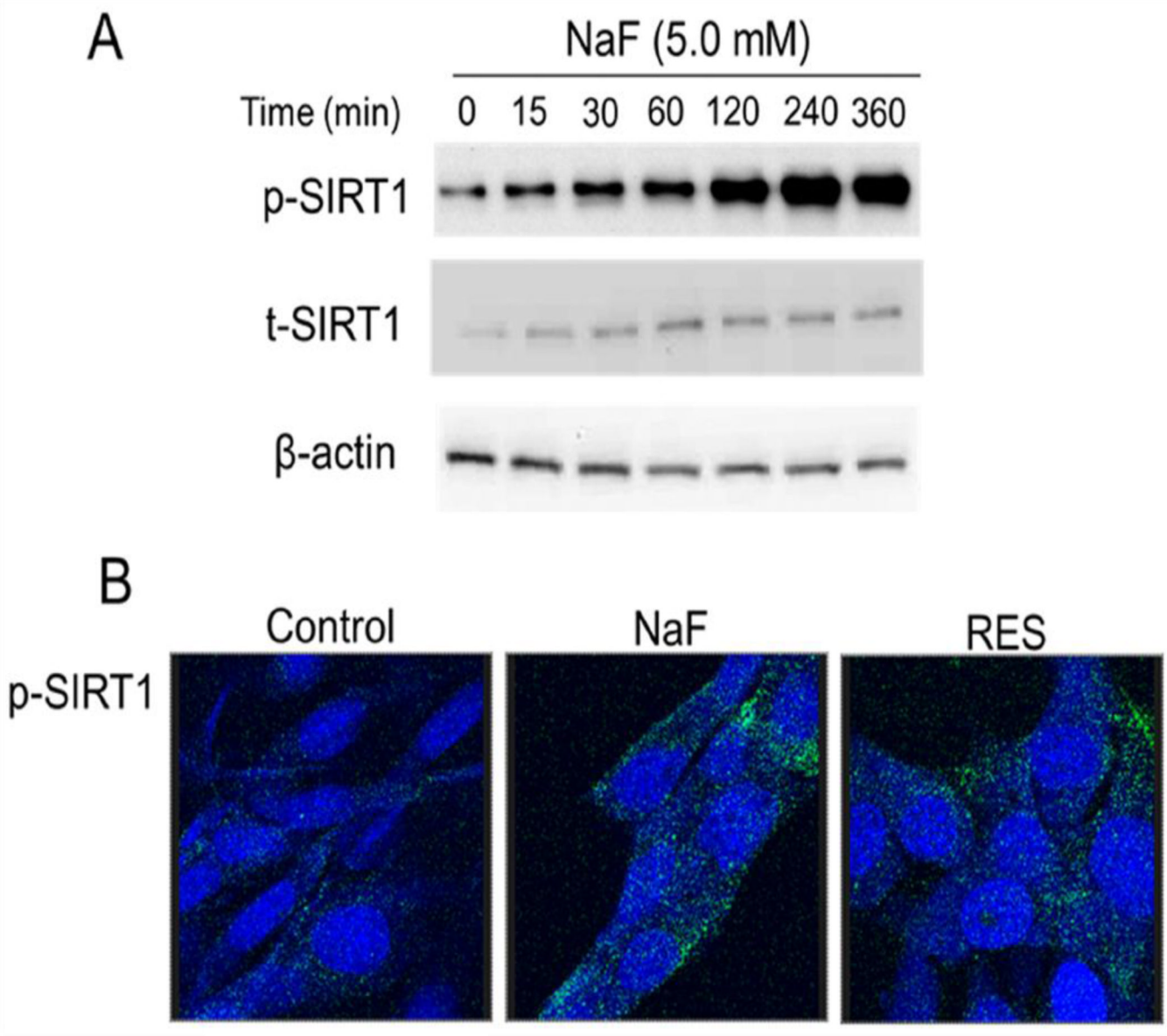

Fig. 2.

Fluoride induces Phosphorylation of SIRT1. (A) LS8 cells were treated with 5.0 mM NaF for the indicated time span and phosphorylation of SIRT1 (p-SIRT1) was analyzed by western blot by use of antibodies for p-SIRT1, total SIRT1 (t-SIRT1) and β-actin which served as the loading control. The ratio of p-SIRT1/t-SIRT1 was analyzed by densitometry. (B) LS8 cells were treated with 3.0 mM NaF for 2 h or 100 μM resveratrol (RES) for 2 h and were stained with anti-p-SIRT1 antibody. Representative confocal microphotographs are shown. Note that both fluoride and RES induced the phosphorylation of SIRT1.

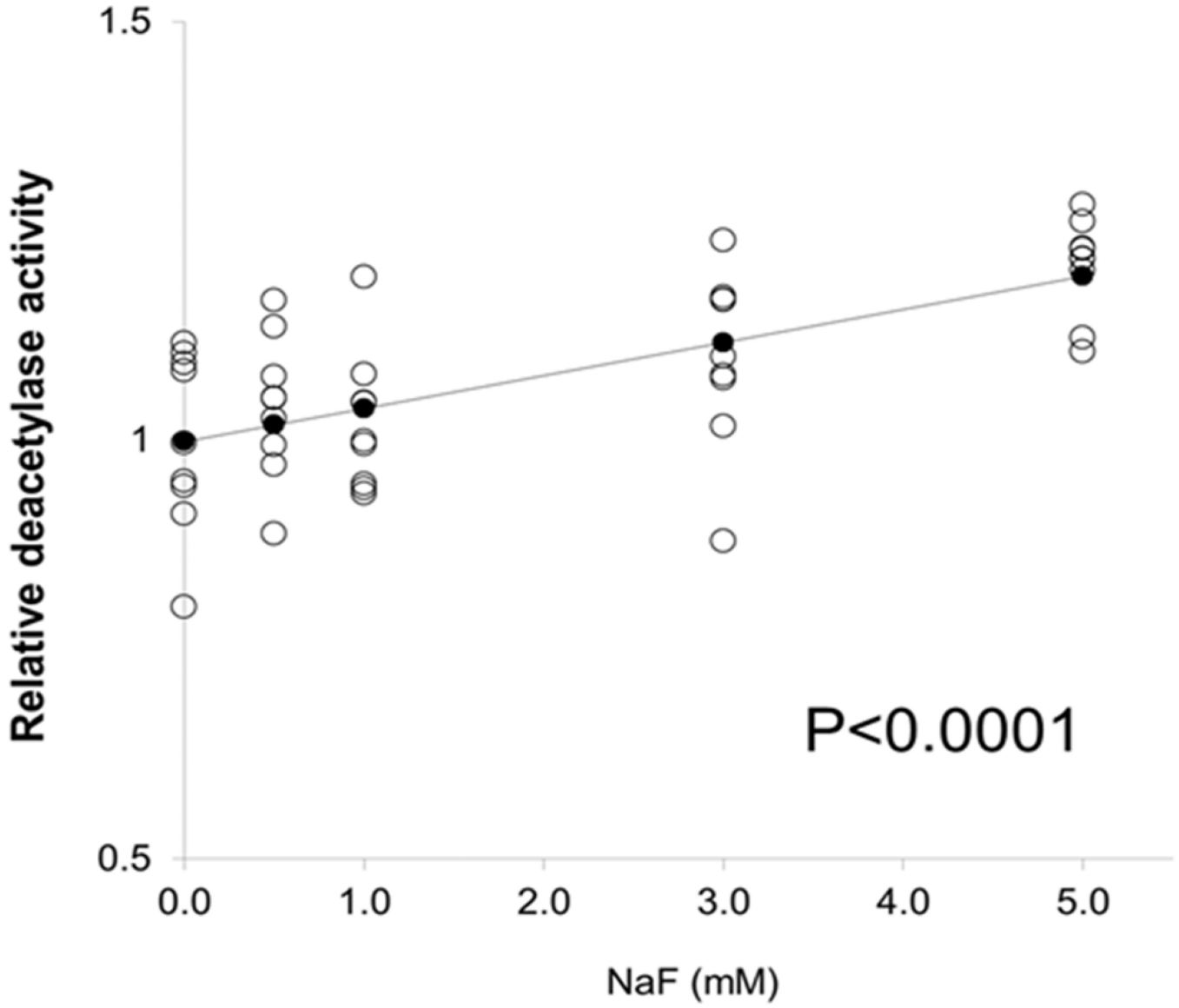

Fig. 3.

Fluoride enhances SIRT1–7 (NAD^+)-dependent deacetylase activity. LS8 cells were treated with 0.0, 0.5, 1.0, 3.0 or 5.0 mM NaF for 6 h. Sirtuin deacetylase activity was quantified by use of a fluorometric assay.. Data show relative (NAD^+)-dependent deacetylase activity from three independent experiments performed in triplicate. A regression analysis revealed that the dose dependent fluoride-mediated increase in deacetylase activity was highly significant ($p < 0.0001$).

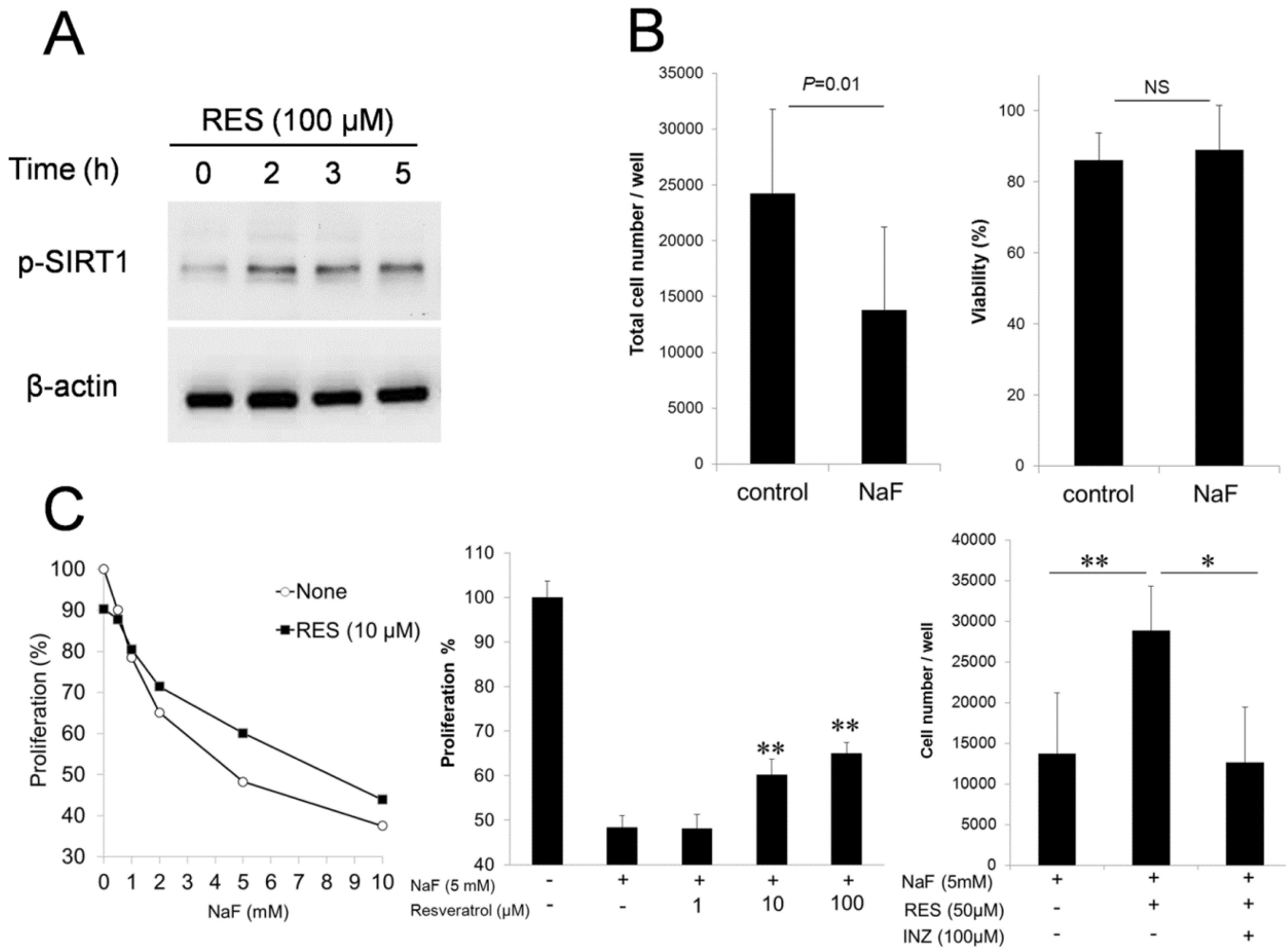

Fig. 4.

RES induces SIRT1 phosphorylation and ameliorates LS8 cell proliferation in the presence of fluoride. (A) LS8 cells were treated with 100 μM RES for 0, 2, 3, or 5 h and SIRT1 phosphorylation was evaluated by western blot analysis. (B) LS8 cells were treated with 5.0 mM NaF for 24 h and then harvested and counted in the presence of a viability stain. Fluoride significantly reduced LS8 cell number ($p < 0.01$), but no difference in cell viability was observed. (C, left) LS8 cells were seeded into 96-well plates and treated for 24 h with the indicated concentrations of NaF with (■) or without (○) 10 μM RES. Cell proliferation percentage was measured by results of the MTT assay (treated A550/untreated A550× 100). (C, middle and right) LS8 cells were seeded into 96-well plates and treated for 24 h with 5.0 mM NaF in the presence of RES (0, 1, 10, 50 and 100 μM), with or without 50 μM inauhzin (INZ). Cell proliferation percentage was measured by MTT assay or by cell number. Four wells were assayed for each experimental treatment, and three separate experiments were performed. Data are expressed as mean ± SD (*$p < 0.05$; **$p < 0.01$).

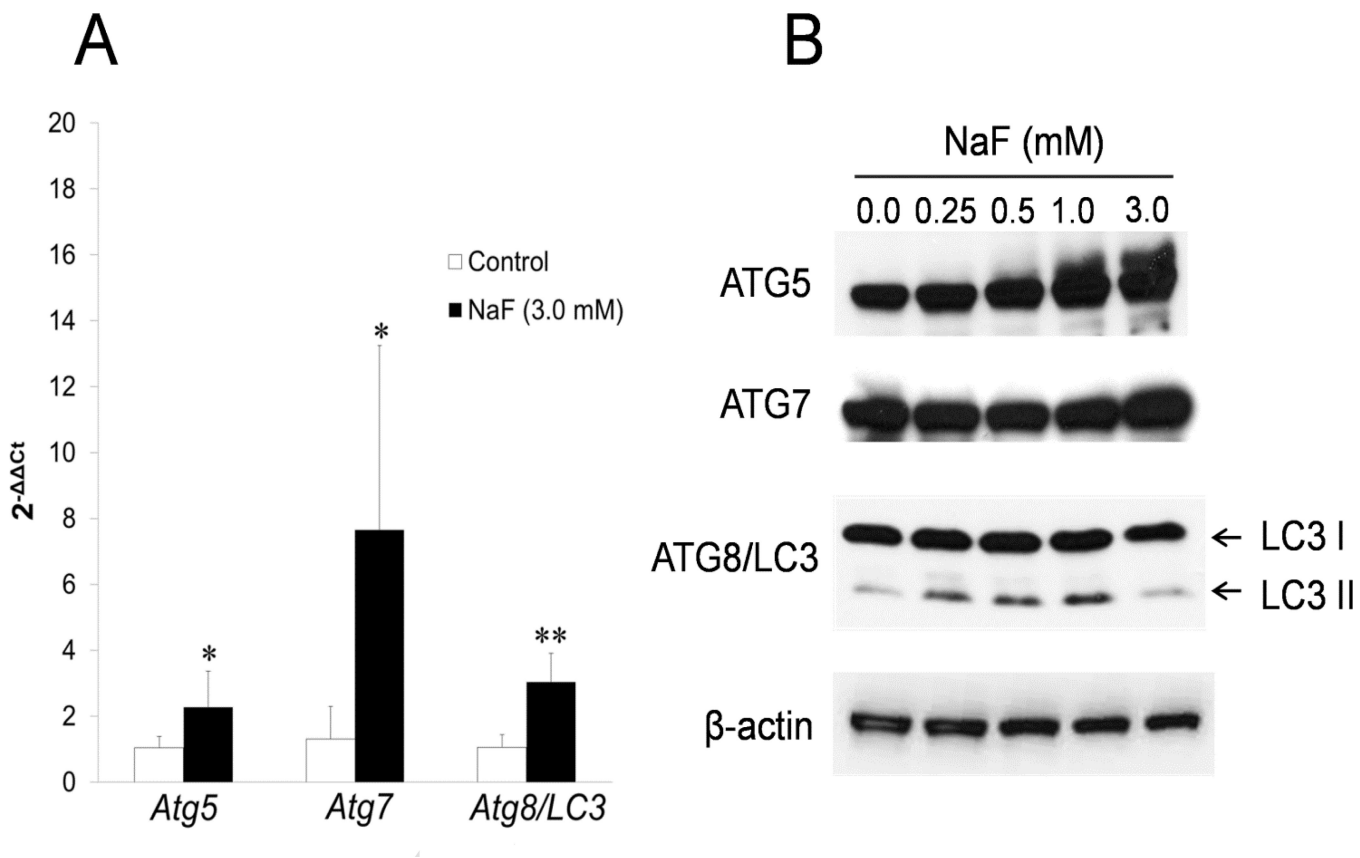

Fig.5.

Fluoride increases mRNA and protein levels of autophagy related genes (*Atg*). (A) LS8 cells were treated with 3.0 mM NaF for 4 h and RNA was extracted for quantification of *Atg5*, *Atg7* and *Atg8/LC3* gene expression by real-time PCR (qPCR). Expression was evaluated by the ΔΔCT method and *Eef1a1* served as reference control gene. Data are expressed as mean ± SD. (**p* < 0.05; ***p* < 0.01). (B) LS8 cells were treated with the indicated doses of NaF for 24 h and analyzed by western blot stained with antibodies against ATG5 (55 kDa), ATG7 (78 kDa), LC3; LC3-I (16 kDa), LC3-II (14 kDa) and β-actin (44 kDa). β-actin was used as the loading control.

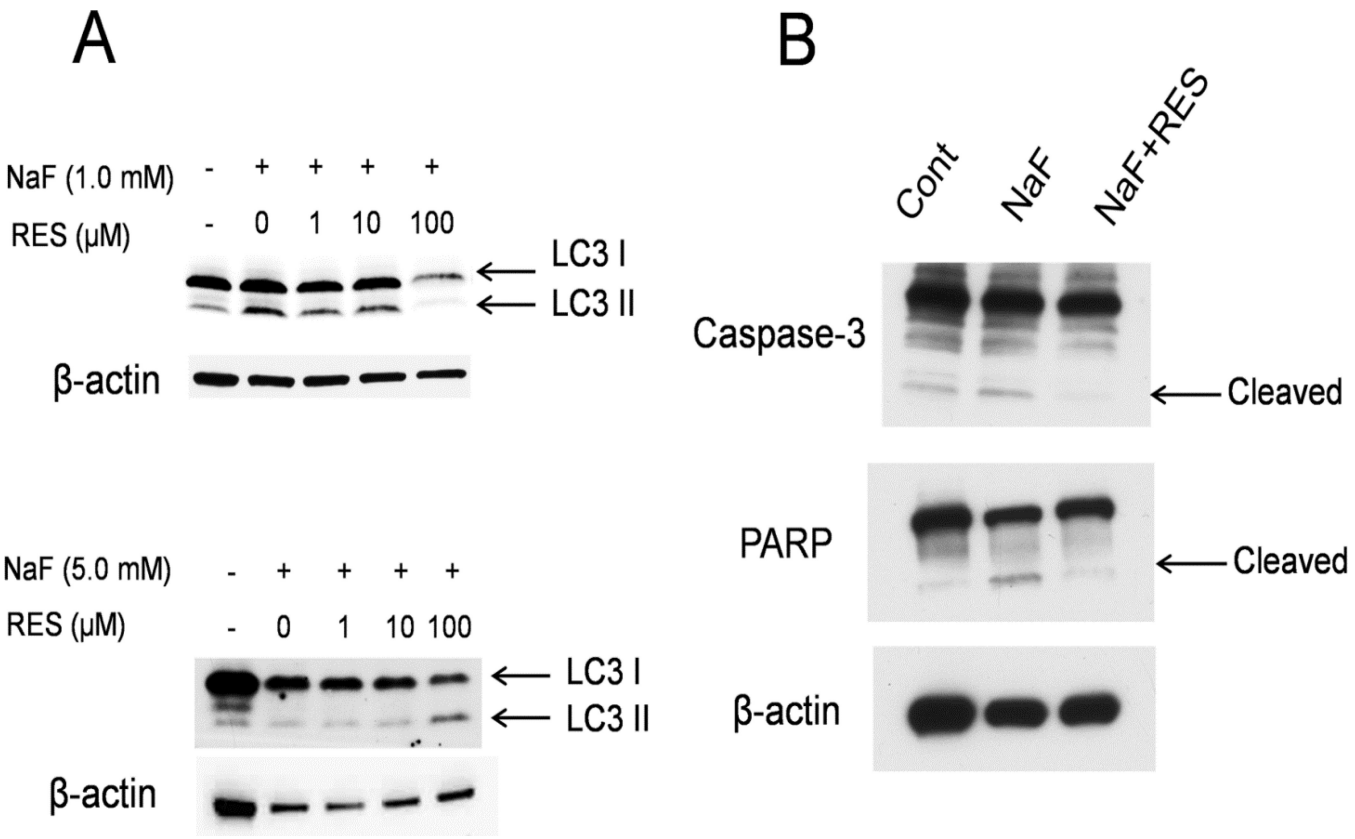

Fig. 6.

RES promoted autophagy and attenuated fluoride-induced activation of apoptosis mediators. Effects of RES on autophagy (A) and apoptosis (B) in LS8 cells treated with NaF were analyzed by western blot. (A) LS8 cells were treated with 1.0 mM or 5.0 mM NaF with or without the indicated dose of RES for 24 h. Fluoride treatment mediated the conversion of the inactive LC3-I (16 kDa) to the active LC3-II (14 kDa) autophagy-related protein. (B) LS8 cells were treated with 5 mM NaF with or without 100 μM RES for 6 h and were immunoblotted by use of anti-caspase-3, PARP and β-actin antibodies. Arrows identify active caspase-3 (17 kDa) and PARP (89 kDa) cleavage forms. β-actin (44 kDa) was used as the loading control. Note that RES attenuated fluoride-mediated PARP and caspase-3 activation.

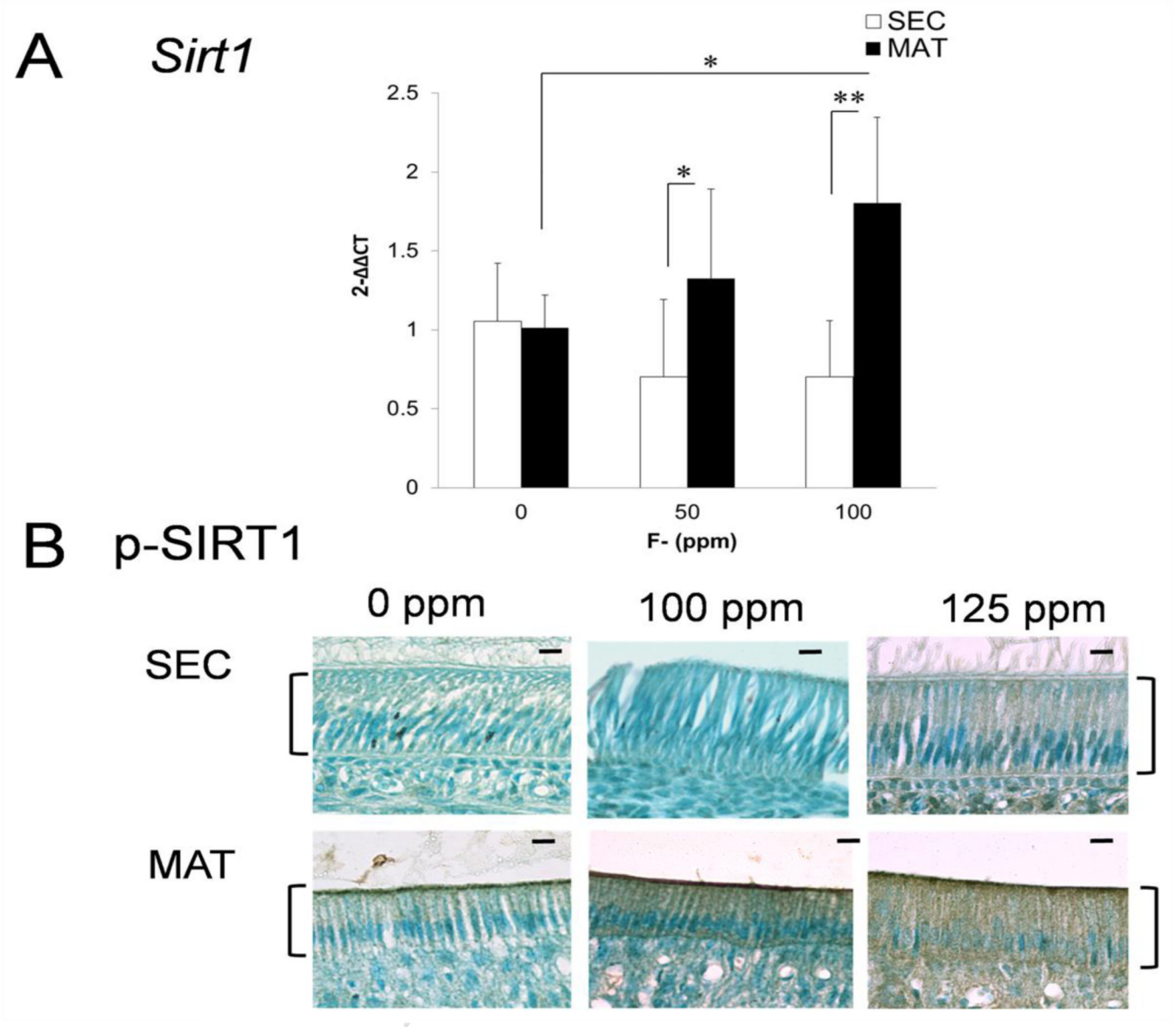

Fig. 7.

Fluoride-mediated *Sirt1* expression occurred in the rat enamel organ at the maturation stage, but not the secretory stage of enamel development. (A) Rats were supplied *ad libitum* with 0, 50 or 100 ppm fluoride in their drinking water for 6 weeks. Incisor enamel organs were separated by developmental stage and qPCR was performed on tissue from the secretory or maturation stages. *Sirt1* expression was quantified by the ΔΔCT method. cDNA from four different rats in each group were assayed in duplicate. Data are expressed as mean ± SD. (**p* < 0.05, ***p* < 0.01). (B) Immunohistochemistry was performed on rat incisor sections treated with 0, 100, or 125 ppm fluoride in drinking water for 6 weeks. Sections were stained with anti-phosphorylated SIRT1 antibody. Scale bar represents 10 μm. Brackets denote ameloblasts.

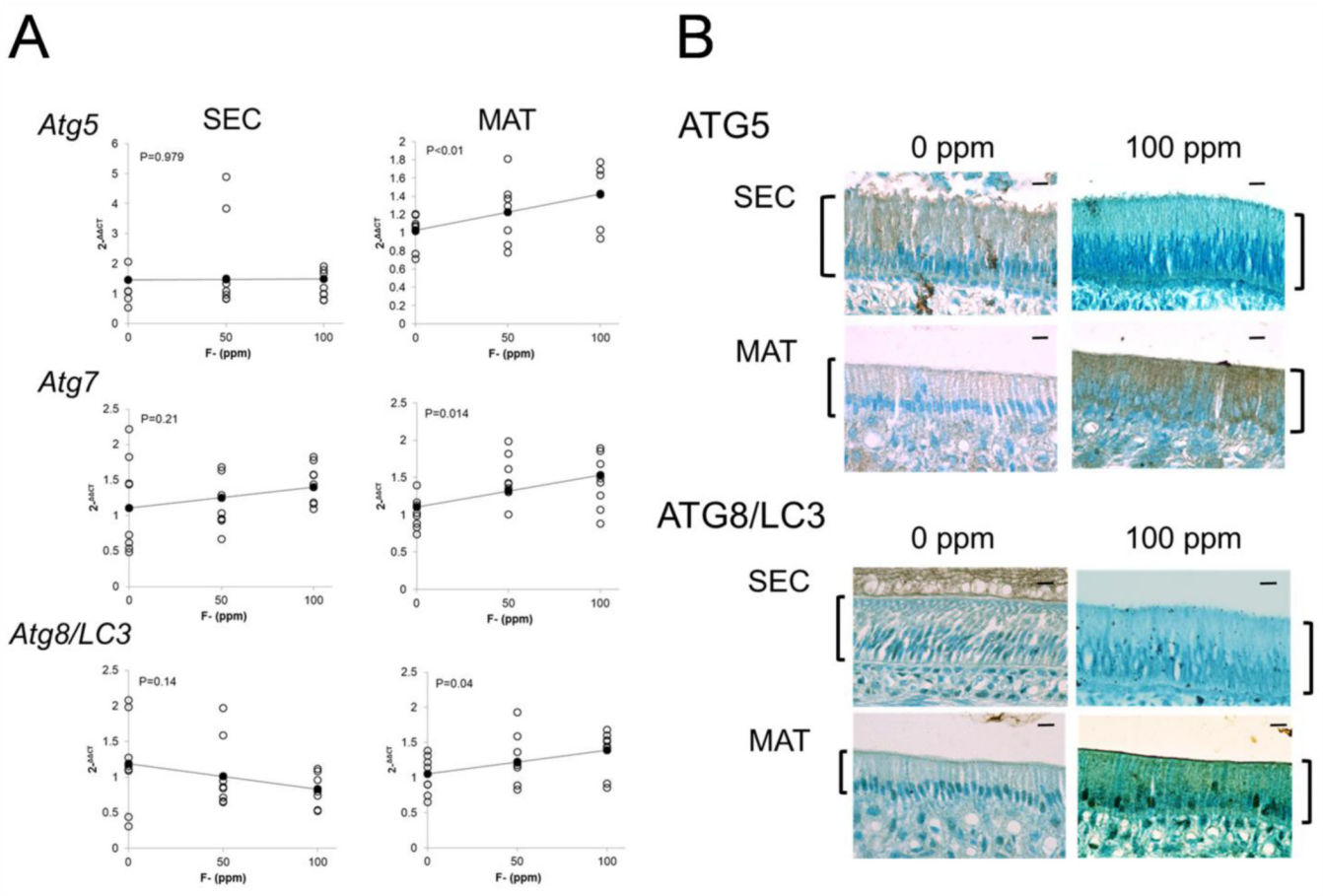

Fig. 8.

Fluoride-mediated *Atg* expression was prevalent in rat maturation stage enamel organ, but not in the secretory stage enamel organ. (A) qPCR was performed on secretory or maturation stage enamel organs from rats treated with 0, 50 or 100 ppm fluoride in drinking water for 6 weeks. Expression of *Atg5*, *Atg7* and *Atg8/LC3* from secretory stage enamel organs (SEC; left panel) and from maturation stage enamel organs (MAT; right panel) were quantified by the ΔΔCT method. Data represent four animal samples from each group performed in duplicate. A regression analysis was performed and the results demonstrated that fluoride dose dependently induced the expression of each *Atg* gene in the maturation stage ($p < 0.05$), but not the secretory stage of enamel development. (B) Rats were treated with 0 or 100 ppm fluoride in their drinking water for 6 weeks. Immunohistochemistry was performed on incisor sections at the secretory (SEC; upper panel) or maturation (MAT; lower panel) stages of enamel development. Both ATG5 and ATG8/LC3 were more highly expressed in the ameloblasts from the maturation stage as compared to ameloblasts from the secretory stage of enamel development. Scale bar represents 10 μm. Brackets denote ameloblasts.