Sulforaphane Alters the Acidification of the Vacuole to Trigger Cell Death

Alexander Wilcox[1]*, Michael Murphy[1]*, Douglass Tucker[1]*, David Laprade[1], Breton Roussel[1], Victoria Hallisey[1], Christopher Chin[2], Abraham Brass[2], and Nicanor Austriaco[1]

[1]Department of Biology, Providence College, 1 Cunningham Square, Providence, RI 02918, USA.

[2]Department of Microbiology and Physiological Systems, University of Massachusetts School of Medicine, 368 Plantation St., ASC 1001, Worcester, MA 01605, USA.

*These authors contributed equally to the manuscript.

Abstract

Sulforaphane (SFN) is a compound [1-isothiocyanato-4-(methylsulfinyl)-butane] found in broccoli and other cruciferous vegetables that is currently of interest because of its potential as a chemopreventive and a chemotherapeutic drug. Recent studies in a diverse range of cellular and animal models have shown that SFN is involved in multiple intracellular signaling pathways that regulate cell death, cell cycle progression, and cell invasion. In order to better understand the mechanisms of action behind SFN-induced cell death, we undertook an unbiased genome wide screen with the yeast knockout (YKO) library to identify SFN sensitive (SFN^S) mutants. Our mutants were enriched with knockouts in genes linked to vacuolar function suggesting a link between this organelle and SFN's mechanism of action in yeast. Our subsequent work revealed that SFN increases the vacuolar pH of yeast cells and that varying the vacuolar pH can alter the sensitivity of yeast cells to the drug. In fact, several mutations that lower the vacuolar pH in yeast actually made the cells resistant to SFN (SFN^R). Finally, we show that human lung cancer cells with more acidic compartments are also SFN^R suggesting that SFN's mechanism of action identified in yeast may carry over to higher eukaryotic cells.

Introduction

The consumption of broccoli and other cruciferous vegetables belonging to the *Brassica* family has been shown to have protective effects against several types of cancer, including prostate, breast, colon, and lung cancer. [1, 2] Though these plants contain a diverse range of metabolites and antioxidants, the chemical agents believed to be responsible for these effects are the naturally occurring organosulfur compounds called isothiocyanates (ITCs; R-N=C=S). [3, 4]. These molecules are the products of the reaction of plant glucosinolates with myrosinase, an enzyme released by the disruption of plant tissues.

Studies undertaken during the past two decades have reported that the isothiocyanate in cruciferous vegetables primarily responsible for their chemopreventive effects is the isothiocyanate called sulforaphane (SFN; 1-isothiocyanato-4-(methylsulfinyl)butane). [5] Numerous experiments have shown that SFN can defend healthy cells against chemical and radiation induced carcinogenesis and can inhibit the proliferation, migration, and survival of tumor cells. [6, 7] There is also extensive evidence that reveals that SFN is a chemoprevention agent against cardiovascular diseases, neurodegenerative diseases, autism, and diabetes. [8-10]

Sulforaphane affects many molecular targets in cellular and animal models. However, its cytoprotective function has been attributed primarily to its diverse abilities. These include SFN's abilities to inhibit phase 1 metabolizing enzymes (mostly cytochrome P450); to alter the localization of the transcription factor Nrf2 so that it can enter the nucleus to regulate the basal and inducible expression of a

multitude of antioxidant proteins, detoxification enzymes, and xenobiotic transporters; and to suppress pro-inflammatory responses within the cell. [4] SFN is also known to inhibit histone deacetylase, which could explain its ability to induce cell cycle arrest and apoptosis, and to regulate different microRNAs. [11-13] Finally, there is data that suggests that SFN can trigger cell death by upregulating caspases and downregulating anti-apoptotic factors. [14-16]

In order to better understand the mechanisms of action of SFN in eukaryotes and to possibly uncover novel ones, we undertook an unbiased genome wide screen with the *Saccharomyces cerevisiae* knockout (YKO) library, a collection of 4,775 individual yeast strains, each of which contains a deletion of a single non-essential yeast ORF, to identify mutations that affect the cell's sensitivity to SFN. The YKO collection has been used extensively over the past decade to identify the mechanisms of actions of a wide range of small molecules and drugs. [17, 18] Our screen uncovered numerous SFN^S mutants. Notably, they were enriched with knockouts in genes linked to vacuolar function suggesting a link between this organelle and SFN's mechanism of action in yeast. Our subsequent work revealed that SFN increases the vacuolar pH of yeast cells and that varying the vacuolar pH can alter the sensitivity of yeast cells to SFN. In fact, several mutations that lower the vacuolar pH in yeast actually made the cells resistant to SFN. Finally, we show that human lung cancer cells with decreased endosomal pH are also SFN^R suggesting that SFN's mechanism of action identified in yeast may carry over to higher eukaryotic cells.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions. All experiments were done with isogenic *Saccharomyces cerevisiae* strains in either the BY4742 (MATα *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*) or the PSY316AR (MATα RDN1::ADE2 his3- 200 leu2-3,112 lys2 ura3-52) backgrounds. For all the experiments described in this paper, cells were cultured and treated using standard yeast protocols. [19] Unless noted otherwise, all drugs and reagents were purchased from SIGMA-Aldrich. Isothiocyanates were resuspended in acetonitrile as a solvent.

Spot Assay. Seed cultures of the BY4742 and PSY316AR yeast strains were grown overnight in YPD. Each strain was diluted to an OD_{600} of 0.1 in fresh YPD and grown for at least 2 doublings (~5 hours). After the yeast strains entered log phase (OD_{600} ~0.4-0.8), sulforaphane (LKT Laboratories), BITC, or PEITC was added to the cultures at the indicated concentrations with the solvent, acetonitrile alone, as the no-drug control. Following the indicated incubation times, cells were removed, spun, washed, and diluted. For each strain, a series of 10-fold dilutions was then prepared in water over a range of concentrations from 10^{-1} to 10^{-5} relative to the initial culture. Spots of 5µl from each dilution series were then plated on the indicated media and cultured at 30˚C for 2 days. All spot assays were repeated at least three times and a representative experiment is shown.

Liquid Viability Assay. Seed cultures of each yeast strain were grown overnight in YPD. Each strain was diluted to an OD_{600} of 0.1 in fresh YPD and grown for at least 2

doublings (~5 hours). After the yeast strains entered log phase (OD_{600} ~0.4-0.8), SFN was added at the indicated concentrations. Cell viability was measured at the indicated time points following drug addition using a Nexcelom Vision Cell Analyzer with propidium iodide as a vital stain (1µg/ml). Statistical significance was determined with the Student's t-test, using Graph Pad Prism 6. By default, one asterisk is $p<0.05$; two asterisks is $p<0.01$; three asterisks is $p<0.001$; and four asterisks is $p<0.0001$.

Genetic Screen for SFN^S Mutants. Seed cultures of individual yeast strains from the BY4742 knockout library (Dharmacon Yeast Knock Out MATalpha Collection) were grown overnight at 30°C in 96-well plates in complete synthetic defined (SD) media. A 10µl aliquot of each culture was then transferred to a well of two different sets of 96-well plates, each of which contained 150µl fresh complete SD media. Cells were allowed to reach exponential phase (OD_{600} ~0.4-0.8). SFN was then added to one of the sets of 96-well plates to a final concentration of 200µg/ml. Relative growth for SFN^S mutants was determined by visual inspection of the wells, comparing wells with drug with wells without drug, after they had been cultured at 30˚C for 2 days.

Functional Gene Ontology Annotation. The Cytoscape 2.8.3 plugin BiNGO (v2.44) was used to identify enriched biological processes in the SFN^S mutant pool after Benjamini & Hochberg false discovery correction for multiple hypothesis testing.

129 *Confocal Imaging of Yeast Cells.* BCECF-AM (Molecular Probes, Eugene, OR) staining
130 was performed as described [20] with the following modifications: Seed cultures
131 were grown overnight in YPD. Each culture was then diluted to an OD_{600} of 0.1 in
132 fresh YPD and grown for at least 2 doublings (~5 hours). Once the cells were in log
133 phase, sulforaphane, BITC, or PEITC were added to the cultures at the indicated
134 concentrations with the solvent, acetonitrile alone, as the no-drug control. After they
135 were allowed to grow at 30°C for an additional 18 hours, cells were harvested,
136 washed, and resuspended in an equivalent amount of APG (a synthetic minimal
137 medium containing 10mM arginine, 8mM phosphoric acid, 2% glucose, 2mM
138 MgSO4, 1mM KCl, 0.2mM CaCl2, and trace minerals and vitamins titrated to pH 7.0
139 with KOH and 10mM MES). Two 200µl aliquots of each yeast culture were then
140 transferred to a 96-well plate. They were incubated with 50 µM BCECF-AM at 30°C
141 for 30 min, washed, and resuspended in APG medium to be imaged. Images were
142 captured with a Zeiss LSM 700 Laser Confocal Microscope (Zeiss, Thornwood, NY),
143 and processed using the Zen 2009 software package.
144
145 *Assay for the Measurement of Yeast Vacuolar pH.* Seed cultures of each yeast strain
146 were grown overnight in YPD. Each strain was diluted to an OD_{600} of 0.1 in fresh
147 YPD and grown for at least 2 doublings (~5 hours). After the yeast strains entered
148 log phase (OD_{600} ~0.4-0.8), cells were spun down and resuspended in APG media
149 titrated to pH 3, 5, 7, 9, or 11. After an additional hour of growth in this media, the
150 cells were incubated with 50µM BCECF-AM at 30°C for 30 min, washed, and
151 resuspended in APG medium to be imaged. Images were captured with a Zeiss LSM

700 Laser Confocal Microscope (Zeiss, Thornwood, NY), and processed using the Zen 2009 software package. The vacuolar pH was estimated from a calibration curve that plotted the vacuolar pH of cells grown in APG media titrated to different pH values against the fluorescence intensities measured by the LSM700. Results and statistics were plotted using Graph Pad Prism 6.

Cell Lines. The pQCXIP and IFITM3 plasmids and A549 cell lines were characterized previously. [21, 22] Briefly, A549 cells were grown in complete DMEM (Invitrogen #11965) with 10% FBS (Invitrogen). A549 cells were made by gamma-retroviral transducion with either an empty vector control or a vector containing the full-length human *IFITM3* cDNA. The cells were then selected with 2μg/mL puromycin in complete DMEM. Expression of IFITM3 was confirmed by Western blotting using an SDS-PAGE gel and an anti-IFITM3 antibody against the n-terminus of IFITM3 (Abgent #AP1153a).

Lysotracker Red Staining. Lysotracker Red staining of A549 cells was done as described previously.[22] Briefly, A549 cells transduced with the empty vector or overexpressing IFITM3 were plated on coverslips and cultured for 4 hours in complete DMEM with either 20μM DMSO or SFN at 37°C. [16] For the last hour, Lysotracker Red DND-99 (Invitrogen) was added in the corresponding media to the cells. Cells were fixed with 4% PFA and stained with DAPI (blue). The coverslips were then imaged by a Leica SP-5 confocal microscope.

175 *SFN Survival Assay for Mammalian Cell Lines.* Cells were plated in a 96-well plate at
176 8000 cells per well. They were then cultured with either 20μM DMSO or SFN in
177 complete DMEM for 24 hours. [16] Cells were then fixed and stained with Hoechst
178 and imaged by an IXM microscope. Meta-express software was used to count the
179 number of cells indicated by DAPI staining.

Results and Discussion

Sulforaphane Inhibits the Growth of Wild Type Yeast Cells.

Isothiocyanates have been used as antimicrobials, mainly for food preservation and plant pathogen control. [23] However, since sulforaphane (SFN), to the best of our knowledge at the time, had never been tested on yeast cells, we began by investigating whether the drug was able to inhibit the growth of wild type *Saccharomyces cerevisiae* cells. We plated ten-fold serial dilutions of wildtype cells from the PSY316 strain background on synthetic defined (SD) media with increasing concentrations of SFN (0-200 μg/ml). After two days of growth at 30°C, it was clear that SFN inhibited the growth of the strain (Figure 1A).

Similar results were obtained when we measured the viability of the cells grown in liquid cultures containing 100 μg/ml SFN using propidium iodide as a vital stain, suggesting that the drug actually kills rather than merely inhibits yeast cell growth (Figure 1B). A parallel experiment with wildtype cells from the BY4742 strain background showed that the ability of SFN to trigger yeast cell death is not specific to a particular strain background (Figure 1B).

Given our laboratory's wider interest in apoptotic-like cell death in yeast [24], we checked to see if SFN induces the characteristic hallmarks of this kind of cell death. We discovered that SFN-induced cell death neither generated reactive oxygen species (ROS), as determined by dihydroxyrhodamine 123 staining, nor was inhibited by the absence of oxygen (data not shown). Both are hallmark characteristics of apoptosis in yeast [25] suggesting that SFN-induced cell death is nonapoptotic in nature.

A Genome-wide Screen Links Vacuolar Acidification to SFN's Mechanism of Action.

In order to better understand the mechanisms of action behind SFN-induced cell death, we undertook an unbiased genome wide screen to identify mutations that alter the cell's sensitivity to SFN using the *Saccharomyces cerevisiae* knockout (YKO) library, a collection of 4,775 individual yeast strains in the BY4742 background, each of which contains a deletion of a single non-essential yeast ORF. Our initial experiments had revealed that 200µg/ml SFN significantly inhibits the growth of wildtype BY4742 yeast cells grown in 96-well liquid SD cultures for 48 hours, so we screened the YKO library for mutant BY4742 strains that were unable to grow under these conditions.

Each mutant strain was isolated by visually comparing 96-well plates with SFN to control plates without SFN, to identify wells that had little or no turbidity after 48 hours (Figure 2A). After screening the entire YKO library twice, we identified 311 mutant strains that consistently were unable to grow in liquid SD cultures containing 200µg/ml SFN after two days (Supplementary Table S1). Functional annotation utilizing gene ontology (GO) terms revealed that our screen had preferentially isolated mutants in genes involved in cellular metabolism, in the cell's response to stress, and in the regulation of cell metabolism (Figure 2B). However, a cursory search through the *Saccharomyces* Genome Database (SGD) revealed that many, if not most, of these loss-of-function mutants are also sensitive to a wide range of other cellular insults and stresses suggesting that they may not be SFN-specific.

Intriguingly, however, we noticed that our SFN^S mutants were significantly enriched for genes involved in vacuolar function, especially in vacuolar acidification and/or pH regulation (Figure 2B). The vacuole has been implicated in the mechanism of action of numerous other drugs in yeast. [26-28] Our SFN^S vacuolar function deletion mutants included knockouts of *VMA1, VMA2,* and *VMA4,* which encode three of the subunits of the vacuolar H(+)-ATPase (V-ATPase) that is required for vacuolar acidification [29, 30]; knockouts of genes encoding the vacuolar fusion proteins, Vps41p, Vam3p, Vam6p, and Vam7p [31, 32]; and knockouts of the ergosterol biosynthesis proteins, Erg2p, Erg6p, and Erg24p. Notably, a previous study had linked genes involved in V-ATPase function, vacuolar fusion, and ergosterol biosynthesis to the vacuolar pH-stat of *Saccharomyces cerevisiae* [33], suggesting to us that the vacuole and especially the acidification of the vacuole may be linked to SFN function in yeast. A preliminary microarray and gene ontology (GO) analysis comparing ORFs upregulated in yeast cells grown in SFN as compared to cells grown in media containing other drugs such as benomyl, fluconazole, and paraquat also revealed an enrichment of vacuolar pH genes (data not shown).

Lowering the Vacuolar pH Makes Yeast Cells Resistant to SFN-induced Cell Death

Because of the enrichment in our SFN^S screen of mutants linked to vacuolar acidification, we determined whether sulforaphane altered the vacuolar pH of the cell. Staining cells grown in SFN with the vacuole specific, pH-sensitive dye, 2,7'-bis (2-carboxyethyl)-5,6-carboxyfluorescein-acetoxymethylester (BCECF-AM), revealed

that SFN significantly increases the vacuolar pH of two wildtype strains of different genetic backgrounds, making them more alkaline (Figure 3A and 3B).

From this observation, we hypothesized that SFN may trigger cell death by increasing the vacuolar pH of the yeast cell. To interrogate this possible mechanism of action, we sought to manipulate the vacuolar pH of the yeast cell to determine if this would alter the cell's sensitivity to SFN. If SFN kills by increasing the pH of the yeast vacuole, we predicted that cells with more alkaline vacuoles than wildtype cells would be more sensitive to SFN because lower concentrations of the drug would more readily push cells beyond the threshold of alkalinity that triggers death. In contrast, we anticipated that cells with more acidic vacuoles would be more resistant to SFN than wildtype because it would take higher concentrations of the drug to push cells beyond a similar threshold.

The regulation of vacuolar pH in yeast is complex. [20] However, we took advantage of a battery of yeast vacuole acidification mutants, first identified by Brett et al. in a screen for genes involved in the vacuolar pH-stat in yeast, to see if we could discern a relationship between the pH of the yeast vacuole and the cell's ability to grow on SFN plates. In this earlier screen, of the 107 mutants that displayed an aberrant vacuolar pH under more than one external pH condition, functional categories of transporters, membrane biogenesis, and trafficking machinery were significantly enriched.

Of the forty-six hyper-alkaline deletion strains determined by Brett et al. [20] to have more alkaline vacuoles than wildtype, eighteen (39%) were identified in our screen as SFN^S mutants. A Fisher exact test revealed that there was a statistically

significant association between the two phenotypes of hyper-alkaline vacuoles and SFN^S ($p<0.0001$). On the other hand, of the seventy-seven hyper-acidic deletion strains known to have more acidic vacuoles than their wildtype counterparts, eleven (14%) were resistant to SFN (Figure 4; Table 1). These included deletions in genes involved in transcriptional and translational regulation (*RPL21B, RPS23B, RTF1, HAT1*) and sterol/lipid biogenesis (*SUR1*). A third of the SFN^R vacuolar hyper-acidic mutants were in genes of unknown function.

It is not clear why only a subset of the vacuolar hyper-acidic mutants were SFN^R, and we could not identify a common molecular explanation that would link them all to reveal SFN's precise mechanism of action. However, given the complexity of the vacuolar pH-stat in yeast and the involvement of many of the hyper-acidic vacuolar genes in other physiological and metabolic pathways in the yeast cell, this should not be surprising. Nonetheless, this finding supports our claim that the mechanism of action of SFN-induced cell death involves the drug's ability to increase the cell's vacuolar pH. We still do not understand how SFN makes yeast vacuoles more alkaline and how an increase in vacuolar pH could trigger cell death. Though there is data that suggests that the V-ATPase promotes vacuolar membrane permeabilization (VMP) and nonapoptotic death in stressed yeast [34], we have discovered that SFN does not trigger this death-inducing mechanism: yeast cells grown in SFN and stained with FM4-64 to visualize their vacuolar membranes do not appear to undergo increased vacuolar fragmentation (data not shown).

295 Sulforaphane's Ability to Increase Vacuolar pH in Yeast is Drug Specific

296 As we have noted, the vacuole has been linked to the mechanisms of actions
297 of a diversity of drugs and small molecules in yeast. [26-28] This raises the real
298 possibility that an increase in the vacuolar pH is a generic response to drug insult in
299 yeast. Recent studies suggest that the isothiocyanates phenethyl isothiocyanate
300 (PEITC) and benzyl isothiocyanate (BITC), like SFN, can inhibit metastatic cell
301 activity and migration. [35, 36] Therefore, to determine if SFN's ability to increase
302 the vacuolar pH is drug-specific, we checked to see if PEITC and BITC could similarly
303 trigger an increase in vacuolar pH. If so, it would suggest that isothiocyanates in
304 general and not SFN specifically kill cells via this mechanism.

305 As with SFN, we began by determining if PEITC and BITC could kill yeast cells
306 in liquid culture. We found that the levels of cell death induced by 0.094μg/ml PEITC
307 and 0.746μg/ml BITC were comparable to that triggered by 400μg/ml SFN (Figure
308 5A). However, in contrast with cells grown in SFN, yeast cells grown in PEITC and
309 BITC did not increase their vacuolar pH as determined by BCEC-F staining (Figure
310 5B). This suggests that SFN's mechanism of action in triggering cell death in yeast is
311 distinct from the mechanisms used by two related isothiocynates, PEITC and BITC,
312 to kill this simple eukaryote.

313

314 Sulforaphane Increases the pH of Endosomes of Human A549 Cells

315 Given SFN's well-studied ability to alter the physiology of mammalian cells,
316 we visually examined A549 cells, a human alveolar adenocarcinoma cell line,
317 cultured with SFN to determine if SFN's mechanism of action in yeast cells is

generally applicable. We discovered that A549 cells grown in media containing 40 μM SFN and the pH-sensitive dye, Lysotracker Red, show a decreased fluorescence as compared to cells grown in the absence of drug suggesting that they have more alkaline endosomes (Figure 6A). This suggests that SFN is able to increase the pH of both yeast vacuoles and mammalian lysosomes.

Finally, in light of our findings that hyper-acidic yeast mutants are also resistant to SFN, we sought to make the lysosomes of mammalian cells more acidic to see if this too would in turn make them resistant to SFN. To do this, we overexpressed the Interferon-inducible Transmembrane Protein 3 (IFITM3) protein that is known to enlarge the late endosomes and lysosomal compartments as well as increase their acidity in A549 cells (Figure 6A) [22], and cultured the cells in SFN. We discovered that A549 cells overexpressing IFITM3 are relatively more resistant to SFN suggesting that lowering endosomal pH levels is also protective in higher eukaryotes (Figure 6B). It is a novel mechanism of action that should help us advance our understanding of sulforaphane's chemopreventive and chemotherapeutic functions. Interestingly, it is a mechanism not unlike the mechanism of chloroquine which is known to increase the pH of the food vacuole of the plasmodium parasite, preventing hemoglobin degradation into a nontoxic digestible form. [37]

REFERENCES

1. Steinmetz KA, Potter JD. Vegetables, fruit, and cancer prevention: a review. J Am Diet Assoc. 1996;96(10):1027-39. Epub 1996/10/01. doi: 10.1016/s0002-8223(96)00273-8. PubMed PMID: 8841165.

2. Becker TM, Juvik JA. The Role of Glucosinolate Hydrolysis Products from Brassica Vegetable Consumption in Inducing Antioxidant Activity and Reducing Cancer Incidence. Diseases. 2016;4(2). Epub 2016/06/17. doi: 10.3390/diseases4020022. PubMed PMID: 28933402; PubMed Central PMCID: PMCPMC5456278.

3. van Poppel G, Verhoeven DT, Verhagen H, Goldbohm RA. Brassica vegetables and cancer prevention. Epidemiology and mechanisms. Adv Exp Med Biol. 1999;472:159-68. Epub 2000/03/29. PubMed PMID: 10736624.

4. Sturm C, Wagner AE. Brassica-Derived Plant Bioactives as Modulators of Chemopreventive and Inflammatory Signaling Pathways. Int J Mol Sci. 2017;18(9). Epub 2017/09/02. doi: 10.3390/ijms18091890. PubMed PMID: 28862664; PubMed Central PMCID: PMCPMC5618539.

5. Zhang Y, Talalay P, Cho CG, Posner GH. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. Proc Natl Acad Sci U S A. 1992;89(6):2399-403. Epub 1992/03/15. PubMed PMID: 1549603; PubMed Central PMCID: PMCPMC48665.

6. Sestili P, Fimognari C. Cytotoxic and Antitumor Activity of Sulforaphane: The Role of Reactive Oxygen Species. Biomed Res Int. 2015;2015:402386. Epub

2015/07/18. doi: 10.1155/2015/402386. PubMed PMID: 26185755; PubMed Central PMCID: PMCPMC4491563.

7. Lenzi M, Fimognari C, Hrelia P. Sulforaphane as a promising molecule for fighting cancer. Cancer Treat Res. 2014;159:207-23. Epub 2013/10/12. doi: 10.1007/978-3-642-38007-5_12. PubMed PMID: 24114482.

8. Bai Y, Wang X, Zhao S, Ma C, Cui J, Zheng Y. Sulforaphane Protects against Cardiovascular Disease via Nrf2 Activation. Oxid Med Cell Longev. 2015;2015:407580. Epub 2015/11/20. doi: 10.1155/2015/407580. PubMed PMID: 26583056; PubMed Central PMCID: PMCPMC4637098.

9. Yamagishi S, Matsui T. Protective role of sulphoraphane against vascular complications in diabetes. Pharm Biol. 2016;54(10):2329-39. Epub 2016/02/04. doi: 10.3109/13880209.2016.1138314. PubMed PMID: 26841240.

10. Singh K, Zimmerman AW. Sulforaphane Treatment of Young Men with Autism Spectrum Disorder. CNS Neurol Disord Drug Targets. 2016;15(5):597-601. Epub 2016/04/14. PubMed PMID: 27071786.

11. Kaufman-Szymczyk A, Majewski G, Lubecka-Pietruszewska K, Fabianowska-Majewska K. The Role of Sulforaphane in Epigenetic Mechanisms, Including Interdependence between Histone Modification and DNA Methylation. Int J Mol Sci. 2015;16(12):29732-43. Epub 2015/12/26. doi: 10.3390/ijms161226195. PubMed PMID: 26703571; PubMed Central PMCID: PMCPMC4691138.

12. Tortorella SM, Royce SG, Licciardi PV, Karagiannis TC. Dietary Sulforaphane in Cancer Chemoprevention: The Role of Epigenetic Regulation and HDAC Inhibition. Antioxid Redox Signal. 2015;22(16):1382-424. Epub 2014/11/05. doi:

383 10.1089/ars.2014.6097. PubMed PMID: 25364882; PubMed Central PMCID:
384 PMCPMC4432495.
385 13. Dacosta C, Bao Y. The Role of MicroRNAs in the Chemopreventive Activity of
386 Sulforaphane from Cruciferous Vegetables. Nutrients. 2017;9(8). Epub 2017/08/22.
387 doi: 10.3390/nu9080902. PubMed PMID: 28825609; PubMed Central PMCID:
388 PMCPMC5579695.
389 14. Choi S, Singh SV. Bax and Bak are required for apoptosis induction by
390 sulforaphane, a cruciferous vegetable-derived cancer chemopreventive agent.
391 Cancer Res. 2005;65(5):2035-43. Epub 2005/03/09. doi: 10.1158/0008-5472.can-
392 04-3616. PubMed PMID: 15753404.
393 15. Choi S, Lew KL, Xiao H, Herman-Antosiewicz A, Xiao D, Brown CK, et al. D,L-
394 Sulforaphane-induced cell death in human prostate cancer cells is regulated by
395 inhibitor of apoptosis family proteins and Apaf-1. Carcinogenesis. 2007;28(1):151-
396 62. Epub 2006/08/22. doi: 10.1093/carcin/bgl144. PubMed PMID: 16920735.
397 16. Sakao K, Singh SV. D,L-sulforaphane-induced apoptosis in human breast
398 cancer cells is regulated by the adapter protein p66Shc. J Cell Biochem.
399 2012;113(2):599-610. Epub 2011/10/01. doi: 10.1002/jcb.23386. PubMed PMID:
400 21956685; PubMed Central PMCID: PMCPMC3253217.
401 17. Smith AM, Ammar R, Nislow C, Giaever G. A survey of yeast genomic assays
402 for drug and target discovery. Pharmacol Ther. 2010;127(2):156-64. Epub
403 2010/06/16. doi: 10.1016/j.pharmthera.2010.04.012. PubMed PMID: 20546776;
404 PubMed Central PMCID: PMCPMC2923554.

405 18. Giaever G, Nislow C. The yeast deletion collection: a decade of functional
406 genomics. Genetics. 2014;197(2):451-65. Epub 2014/06/19. doi:
407 10.1534/genetics.114.161620. PubMed PMID: 24939991; PubMed Central PMCID:
408 PMCPMC4063906.
409 19. Dunham MJ, Gartenberg MR, Brown GW, editors. Methods in Yeast Genetics
410 and Genomics: A Cold Spring Harbor Laboratry Course Material. 1 ed. Cold Spring
411 Harbor, NY: Cold Spring Harbor Laboratory Press; 2015.
412 20. Brett CL, Kallay L, Hua Z, Green R, Chyou A, Zhang Y, et al. Genome-wide
413 analysis reveals the vacuolar pH-stat of Saccharomyces cerevisiae. PLoS One.
414 2011;6(3):e17619. Epub 2011/03/23. doi: 10.1371/journal.pone.0017619. PubMed
415 PMID: 21423800; PubMed Central PMCID: PMCPMC3056714.
416 21. Brass AL, Huang IC, Benita Y, John SP, Krishnan MN, Feeley EM, et al. The
417 IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile
418 virus, and dengue virus. Cell. 2009;139(7):1243-54. Epub 2010/01/13. doi:
419 10.1016/j.cell.2009.12.017. PubMed PMID: 20064371; PubMed Central PMCID:
420 PMCPMC2824905.
421 22. Feeley EM, Sims JS, John SP, Chin CR, Pertel T, Chen LM, et al. IFITM3 inhibits
422 influenza A virus infection by preventing cytosolic entry. PLoS Pathog.
423 2011;7(10):e1002337. Epub 2011/11/03. doi: 10.1371/journal.ppat.1002337.
424 PubMed PMID: 22046135; PubMed Central PMCID: PMCPMC3203188.
425 23. Dufour V, Stahl M, Baysse C. The antibacterial properties of isothiocyanates.
426 Microbiology (Reading, England). 2015;161(Pt 2):229-43. Epub 2014/11/08. doi:
427 10.1099/mic.0.082362-0. PubMed PMID: 25378563.

24. Carmona-Gutierrez D, Bauer MA, Zimmermann A, Aguilera A, Austriaco N, Ayscough K, et al. Guidelines and recommendations on yeast cell death nomenclature. Microbial cell (Graz, Austria). 2018;5(1):4-31. Epub 2018/01/23. doi: 10.15698/mic2018.01.607. PubMed PMID: 29354647; PubMed Central PMCID: PMCPMC5772036.

25. Carraro M, Bernardi P. Calcium and reactive oxygen species in regulation of the mitochondrial permeability transition and of programmed cell death in yeast. Cell Calcium. 2016;60(2):102-7. Epub 2016/03/21. doi: 10.1016/j.ceca.2016.03.005. PubMed PMID: 26995056; PubMed Central PMCID: PMCPMC4996716.

26. Krasowska A, Chmielewska L, Adamski R, Luczynski J, Witek S, Sigler K. The sensitivity of yeast and yeast-like cells to new lysosomotropic agents. Cell Mol Biol Lett. 2004;9(4a):675-83. Epub 2005/01/14. PubMed PMID: 15647790.

27. Liao C, Hu B, Arno MJ, Panaretou B. Genomic screening in vivo reveals the role played by vacuolar H+ ATPase and cytosolic acidification in sensitivity to DNA-damaging agents such as cisplatin. Mol Pharmacol. 2007;71(2):416-25. Epub 2006/11/10. doi: 10.1124/mol.106.030494. PubMed PMID: 17093137.

28. de Castro PA, Savoldi M, Bonatto D, Malavazi I, Goldman MH, Berretta AA, et al. Transcriptional profiling of Saccharomyces cerevisiae exposed to propolis. BMC Complement Altern Med. 2012;12:194. Epub 2012/10/25. doi: 10.1186/1472-6882-12-194. PubMed PMID: 23092287; PubMed Central PMCID: PMCPMC3598864.

450 29. Parra KJ, Chan CY, Chen J. Saccharomyces cerevisiae vacuolar H+-ATPase
451 regulation by disassembly and reassembly: one structure and multiple signals.
452 Eukaryot Cell. 2014;13(6):706-14. Epub 2014/04/08. doi: 10.1128/ec.00050-14.
453 PubMed PMID: 24706019; PubMed Central PMCID: PMCPMC4054264.
454 30. Kane PM. Proton Transport and pH Control in Fungi. Adv Exp Med Biol.
455 2016;892:33-68. Epub 2016/01/02. doi: 10.1007/978-3-319-25304-6_3. PubMed
456 PMID: 26721270.
457 31. Wickner W. Membrane fusion: five lipids, four SNAREs, three chaperones,
458 two nucleotides, and a Rab, all dancing in a ring on yeast vacuoles. Annu Rev Cell
459 Dev Biol. 2010;26:115-36. Epub 2010/06/05. doi: 10.1146/annurev-cellbio-
460 100109-104131. PubMed PMID: 20521906.
461 32. Orr A, Wickner W, Rusin SF, Kettenbach AN, Zick M. Yeast vacuolar HOPS,
462 regulated by its kinase, exploits affinities for acidic lipids and Rab:GTP for
463 membrane binding and to catalyze tethering and fusion. Mol Biol Cell.
464 2015;26(2):305-15. Epub 2014/11/21. doi: 10.1091/mbc.E14-08-1298. PubMed
465 PMID: 25411340; PubMed Central PMCID: PMCPMC4294677.
466 33. Zhang YQ, Gamarra S, Garcia-Effron G, Park S, Perlin DS, Rao R. Requirement
467 for ergosterol in V-ATPase function underlies antifungal activity of azole drugs. PLoS
468 Pathog. 2010;6(6):e1000939. Epub 2010/06/10. doi:
469 10.1371/journal.ppat.1000939. PubMed PMID: 20532216; PubMed Central PMCID:
470 PMCPMC2880581.
471 34. Kim H, Kim A, Cunningham KW. Vacuolar H+-ATPase (V-ATPase) promotes
472 vacuolar membrane permeabilization and nonapoptotic death in stressed yeast. J

473 Biol Chem. 2012;287(23):19029-39. Epub 2012/04/19. doi:
474 10.1074/jbc.M112.363390. PubMed PMID: 22511765; PubMed Central PMCID:
475 PMCPMC3365936.
476 35. Lai KC, Hsiao YT, Yang JL, Ma YS, Huang YP, Chiang TA, et al. Benzyl
477 isothiocyanate and phenethyl isothiocyanate inhibit murine melanoma B16F10 cell
478 migration and invasion in vitro. International journal of oncology. 2017;51(3):832-
479 40. Epub 2017/08/03. doi: 10.3892/ijo.2017.4084. PubMed PMID: 28766686.
480 36. Ma YS, Hsiao YT, Lin JJ, Liao CL, Lin CC, Chung JG. Phenethyl Isothiocyanate
481 (PEITC) and Benzyl Isothiocyanate (BITC) Inhibit Human Melanoma A375.S2 Cell
482 Migration and Invasion by Affecting MAPK Signaling Pathway In Vitro. Anticancer
483 research. 2017;37(11):6223-34. Epub 2017/10/25. doi:
484 10.21873/anticanres.12073. PubMed PMID: 29061805.
485 37. Yayon A, Cabantchik ZI, Ginsburg H. Identification of the acidic compartment
486 of Plasmodium falciparum-infected human erythrocytes as the target of the
487 antimalarial drug chloroquine. Embo j. 1984;3(11):2695-700. Epub 1984/11/01.
488 PubMed PMID: 6391917; PubMed Central PMCID: PMCPMC557751.
489
490

FIGURE LEGENDS

FIGURE 1: Sulforaphane Inhibits the Growth of Wild Type Yeast Cells. (A) Ten-fold serial dilutions of wildtype yeast cells from the PSY316 strain background were plated on synthetic defined media with increasing concentrations of SFN and allowed to grow at 30°C for two days. (B) Wildtype cells from both the BY4742 and PSY316 strain backgrounds were grown in synthetic defined liquid cultures containing 100µg/mL of SFN. The viability of the cells at the indicated time points was determined using propidium iodide as a vital stain. The difference in viabilities was deemed statistically significant by the Student's t-test ($p<0.05$). Error bars indicate standard deviations for trials with at least three independent cultures.

FIGURE 2: **A Genome-wide Screen Links Vacuolar Acidification to SFN's Mechanism of Action.** (A) Seed cultures of individual yeast strains from the BY4742 knockout library were grown overnight at 30°C in 96-well plates in complete synthetic defined (SD) media and then transferred to media with and without 200µg/ml of SFN. Relative growth for SFN^S mutants was determined by visual inspection of the wells, comparing wells with drug with wells without drug, after they had been cultured at 30˚C for 2 days. A representative pair of 96-well plates is shown. (B) Functional annotation utilizing gene ontology (GO) terms revealed that our screen had preferentially isolated mutants in genes involved in vacuolar function, especially in vacuolar acidification and/or pH regulation.

513 Asterisks indicate statistical significance of the enrichment of ORFs identified in the
514 screen as compared to their representation in the genome.
515
516 **FIGURE 3: Sulforaphane Increases the pH of the Yeast Vacuole.** (A) Wildtype
517 cells from the BY4742 strain background were grown in synthetic defined liquid
518 cultures containing 200μg/mL of SFN and were stained with the vacuole specific,
519 pH-sensitive dye, BCECF-AM. Cells grown in SFN were significantly more fluorescent
520 than their counterparts grown in media without drug. (B) The vacuolar pH of the
521 cells imaged in Figure 3A was estimated from a calibration curve that plotted the
522 vacuolar pH of cells grown in APG media titrated to different pH values against the
523 fluorescence intensities measured by the LSM700. The difference in viabilities was
524 deemed statistically significant by the Student's t-test ($p<0.05$). Error bars indicate
525 standard deviations for trials with at least three independent cultures.
526
527 **FIGURE 4: Mutations That Alter the Vacuolar pH of Yeast Cells Alter their**
528 **Sensitivity to SFN.** Ten-fold serial dilutions of yeast cells from the BY4742 strain
529 background with mutations in genes known to regulate the pH of the yeast vacuole
530 were plated on synthetic defined media with 400μg/mL SFN and allowed to grow at
531 30°C for two days. Deletions in genes known to increase vacuolar pH (*VMA2)*
532 increased the sensitivity of cells to SFN while deletions in genes known to decrease
533 vacuolar pH (*RRG8*, *RPL218*, *SUR1*, and *LCL1*) increased the resistance of cells to the
534 drug.
535

TABLE 1: Eleven Genes Whose Deletions Are Known to Decrease Vacuolar pH Also Increase the Resistance of Yeast Cells to Sulforaphane.

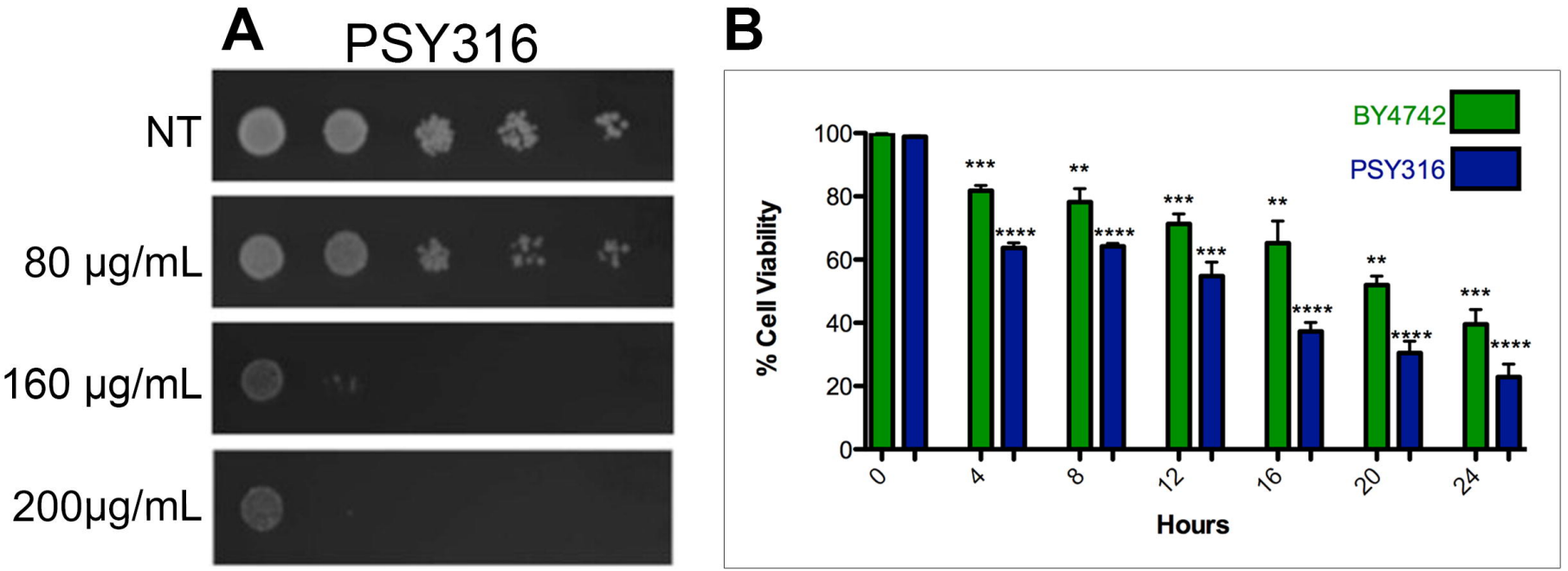
FIGURE 5: Sulforaphane's Ability to Increase Vacuolar pH in Yeast is Drug Specific. (A) Ten-fold serial dilutions of wildtype yeast cells from the BY4742 strain background cultured in synthetic defined liquid cultures containing the indicated drugs, were plated on SD media and allowed to grow at 30°C for two days. (B) Wildtype cells from the BY4742 strain background were grown in synthetic defined liquid cultures containing the indicated drugs and were stained with the vacuole specific, pH-sensitive dye, BCECF-AM. Cells grown in SFN were fluorescent while their counterparts grown in media with the other drugs were not.

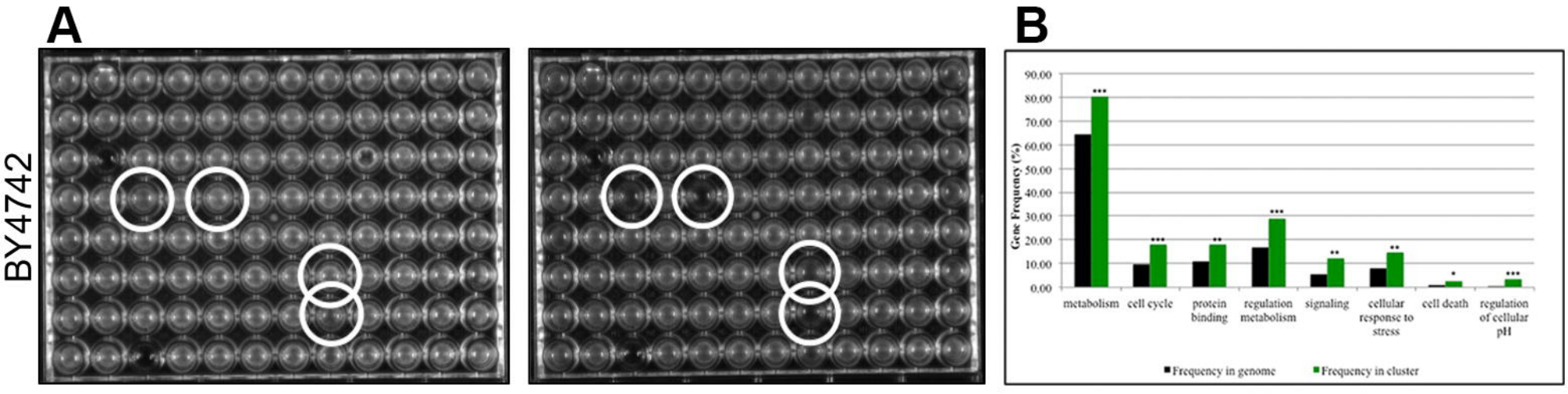
FIGURE 6: Sulforaphane Increases the pH of Endosomes of Human A549 Cells. (A) Cells from the A549 human alveolar adenocarcinoma cell line were cultured in media containing 40μM SFN and the pH-sensitive dye, Lysotracker Red. We also overexpressed the Interferon-inducible Transmembrane Protein 3 (IFITM3) protein that is known to enlarge the late endosomes and lysosomal compartments as well as increase their acidity in A549 cells. Cells grown with SFN are less positive for the dye than cells grown in the absence of the drug suggesting that they have more alkaline endosomes. (B) The viability of A549 cells with or without IFITM3 that had were cultured in media with or without SFN was determined by Hoechst staining. The difference in viabilities was deemed statistically significant by the Student's t-

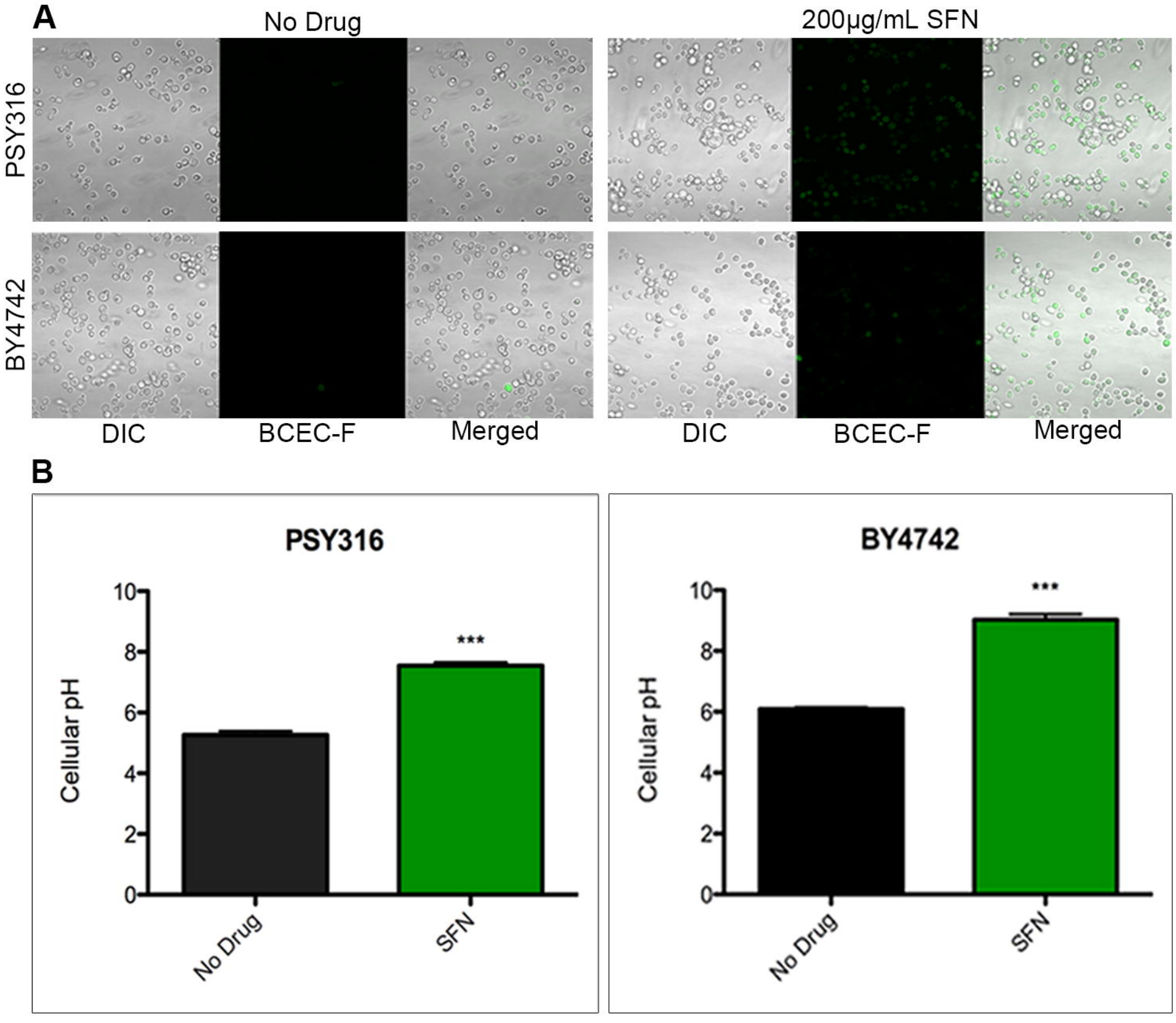
558 test ($p<0.05$). Error bars indicate standard deviations for trials with at least three

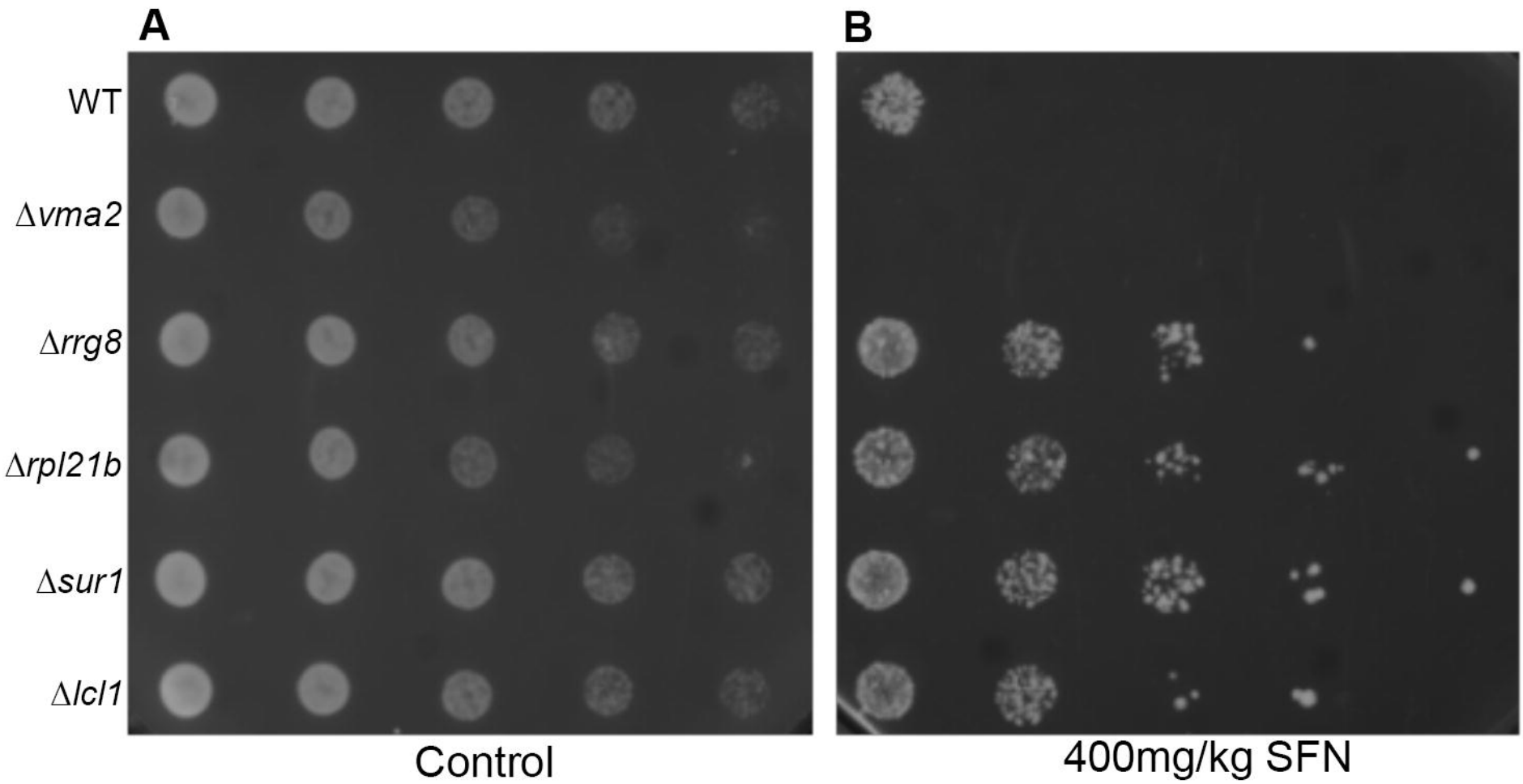
559 independent cultures.

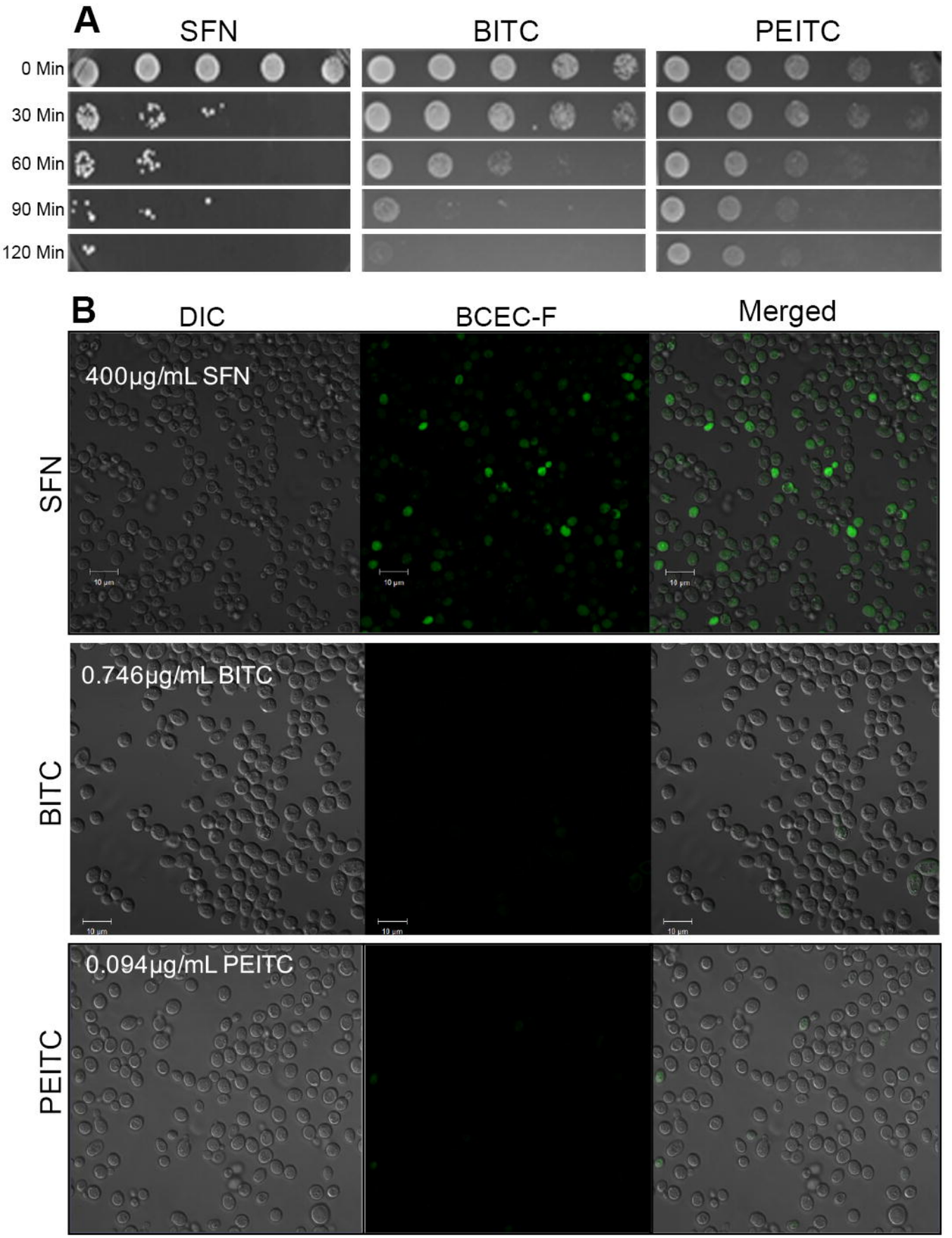
560

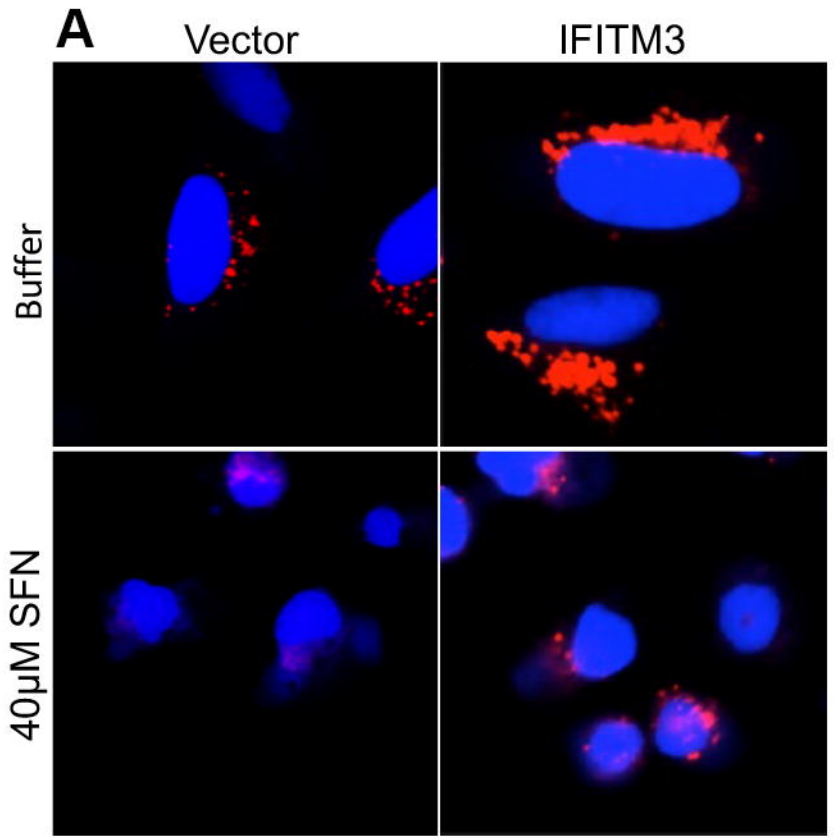

A
PSY316
NT
80 µg/mL
160 µg/mL
200µg/mL
B
BY4742
PSY316
% Cell Viability
100
80
60
40
20
0
0
4
8
12
16
20
24
Hours
***
****
**


A
BY4742
DEFINED MEDIA No Drug
(2 days)
DEFINED MEDIA SFN
(2 days)
B
Gene Frequency (%)
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
***
***
**
***
**
**
*
***
metabolism
cell cycle
protein binding
regulation metabolism
signaling
cellular response to stress
cell death
regulation of cellular pH
Frequency in genome
Frequency in cluster


A
No Drug
200µg/mL SFN
PSY316
BY4742
DIC
BCEC-F
Merged
DIC
BCEC-F
Merged
B
PSY316
Cellular pH
10
8
6
4
2
0
***
No Drug
SFN
BY4742
Cellular pH
10
8
6
4
2
0
***
No Drug
SFN


A
B
WT
Δvma2
Δrrg8
Δrpl21b
Δsur1
Δlcl1
Control
400mg/kg SFN


A
SFN
BITC
PEITC
0 Min
30 Min
60 Min
90 Min
120 Min
B
DIC
BCEC-F
Merged
SFN
400μg/mL SFN
BITC
0.746μg/mL BITC
PEITC
0.094μg/mL PEITC


A
Vector
IFITM3
Buffer
40μM SFN

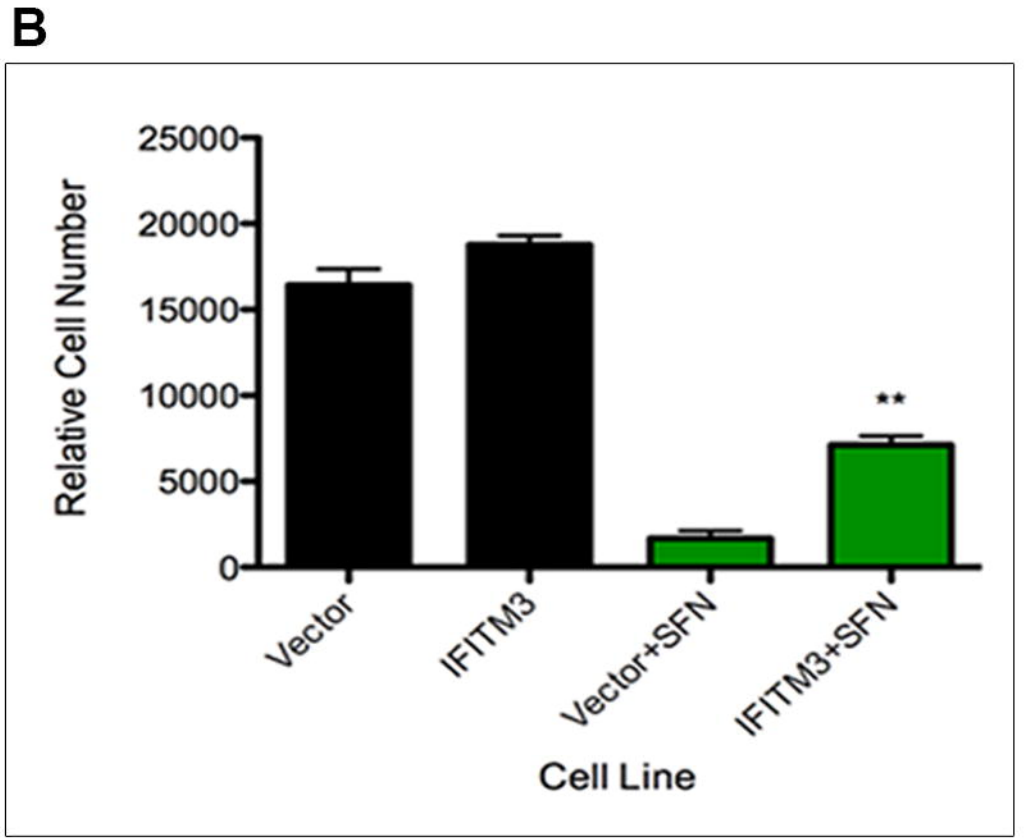

B
Relative Cell Number
25000
20000
15000
10000
5000
0
**
Vector
IFITM3
Vector+SFN
IFITM3+SFN
Cell Line

TABLE 1

Gene Names (SFN^R)	
COS12	*RRG8*
ECM23	*RTF1*
HAT1	*SUR1*
LCL1	*TRM44*
RPL21B	*ULA1*
RPS23B	