A Yeast Mutant Screen Identifies TORC and Lys63 Polyubiquitination Pathway Genes among Determinants of Sensitivity to the Cancer Stem Cell-Specific Drug Salinomycin

Donald Rozario[#], Sammer Zeglam and Wolfram Siede[*]

Department of Cell Biology and Immunology, University of North Texas Health Science Center, Fort Worth, Texas, USA

Abstract: The antibiotic salinomycin (SM) acts as a selective potassium ionophore. In budding yeast (*Saccharomyces cerevisiae*), we describe that the agent inhibits cell growth, elevates reactive oxygen species (ROS) levels and prominently causes mitochondrial damage, as revealed by the emergence of perpetually respiration-defective cells. The collection of systematic gene deletions in haploid yeast was screened to characterize genes whose deletion confers SM sensitivity or resistance if glycerol is provided as the only carbon source, thus requiring active respiration for growth. Mutants conferring the highest SM resistance were those of the Mms2-Ubi13 E2-ubiquitin conjugating enzyme (Lys63 polyubiquitination) and the TORC pathway, such as Sch9. Sch9 phosphorylation is reduced after SM treatment and, whereas initial SM-enhanced ROS levels are not diminished in the mutant, we suggest that a protective response is mounted in the absence of Sch9 that promotes mitochondrial stability under conditions of potassium ion loss. As indicated by other isolated mutants with altered SM sensitivity, levels and modifications of ribosomal proteins may also play a role in these responses. SM has attracted considerable attention due to its cancer stem-cell specific mode of action. Even if not all of its cancer stem cell targets may have an equivalent in yeast, these studies may suggest strategies for mitigating its side effects during treatment of cancer patients.

Keywords: Salinomycin, cancer stem cells, mitochondria, oxidative stress, TORC pathway, polyubiquitination.

INTRODUCTION

A tumor does not consist of identical cells but typically comprises an assembly of subclones that may represent various stages of differentiation. In recent years, multiple studies have suggested that often only a minority of cells, termed cancer stem cells, are endowed with the capability to self-renew and thus with tumorigenic potential [1, 2]. Consequently, as the only long-lived cells within the tumor, these cells may have been the primary targets for transforming mutations. Such cancer stem cells have been reproducibly isolated from various types of tumors, using unique combinations of surface markers. Examples of cancers where the stem cell concept has been successfully applied to are human myeloid leukemia, brain, prostate and breast cancer.

This concept of cancer stem cells has consequences for chemo- or radiation therapy where, typically, a reduction in overall tumor mass is considered therapy success. The stem cell concept, however, implies that, since only a small fraction of cells is responsible for self-renewal and productive metastasis, their selective elimination should be the ultimate goal of therapy.

In 2009, a screen for compounds with selective toxicity for breast cancer stem cell-like cells was successfully conducted [3]. Out of 16,000 screened compounds, salinomycin (SM) exhibited the best selective toxicity for the stem cell model line. If tested directly in breast cancer cell lines, the fraction of presumed stem cells was dramatically lowered following SM treatment whereas it was actually increased following conventional paclitaxel treatment [3].

SM is a 751 Da monocarboxylic polyether antibiotic isolated from *Streptomyces albus* that had long been known to be active against gram-positive bacteria, in addition to mycobacteria, certain filamentous fungi and eukaryotic parasites. SM has been widely used to prevent coccidiosis in poultry. The newly discovered cancer stem cell-specific properties prompted a variety of studies [4-6]. Anti-cancer activity through stem cell inactivation was confirmed for breast and ovarian cancer, human promyeloblastic leukemia, murine gastrointestinal stromal tumors, human lung adenocarcinoma, osteosarcoma, and gastric cancer. Because of considerable side effects already known from veterinary medicine [7], the fate of SM as a clinically useful drug is still unclear. Nevertheless, individual case studies have been quite promising [5].

Further studies revealed additional anti-cancer properties of SM that appear to go beyond its stem-cell specific activity. For example, the agent induces apoptosis in various human cancer cells irrespective of

*Address correspondence to this author at the Santa Fe BioLabs LLC, 3400 Camp Bowie Blvd., CBH214, Fort Worth, Texas 76107, USA; Tel: +1 817 808 7747; E-mail: wolfram@santafebiolabs.com

[#]Present address: Bio-Rad Laboratories, 1418 W Pipeline Road STE 220, Hurst, Texas 76180, USA.

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p53 status, even if considered resistant, but less so in normal cells [8]. SM was found to inhibit p-glycoprotein and may overcome multiple-drug resistance [9, 10]. SM can penetrate the blood brain barrier [11] – a fact that may be relevant for promising approaches to brain cancer therapy with SM since glioblastoma cells have largely stem-cell like properties [12-15].

The mechanistic basis for these remarkable activities of SM is not entirely understood. SM has long been known to act as an ionophore targeting cytoplasmic and mitochondrial membranes [16, 17], showing selectivity for alkali ions and especially potassium. SM efficiently blocks the retention of potassium ions in mitochondria and perturbs mitochondrial function [18]. In general, such efflux of potassium ions is correlated with activation of apoptosis [19], and apoptosis in response to mitochondrial membrane depolarization is likely to be a relevant mechanism of SM toxicity. The resulting oxidative stress has been found to be associated with SM-induced apoptosis and autophagy [20-23].

Other important targets of SM in human cancer cells have been identified [4]. Suppression of Wnt/beta-catenin signaling through binding of LRP6 by SM has been reported [24-27]. In addition to the Wnt signaling pathway as a target, the influence of SM on the Hedgehog pathway may also account for its selective cytotoxicity [28]. Another active area of research concerns the use of SM as a sensitizer in chemotherapy [29, 30].

Availability of a systematic collection of deletions of non-essential genes makes budding yeast (*Saccharomyces cerevisiae*) an attractive model organism for toxicogenomic studies [31]. We carried out a screen for yeast mutants that are SM sensitive or resistant in order to provide information on potential cellular SM targets and, as a consequence, suggest mechanisms for its various activities. Among numerous other proteins, we found that inactivation of certain proteins of the TORC and Lys63 polyubiquitination pathways confers SM resistance under conditions that require functional mitochondria.

MATERIAL AND METHODS

Yeast Strains and Plasmids

Unless indicated, all strains were derived from *S. cerevisiae* BY4741 (*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). The screen using SM was carried out with the commercially available yeast knock-out deletion collection in BY4741, purchased from OpenBiosystems. Strain Y100 is MATa *ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 can1-100* (from S. Elledge). Plasmid pRS416 containing 3HA-tagged Sch9 was obtained from Y. Jiang, originally from R. Loewith [32].

Screen for Yeast Deletion Mutants Showing SM Sensitivity or Resistance

From 96-well plates, the array of haploid deletion mutants was replicated onto YPD (1% yeast extract, 2% peptone, 2% dextrose) plates and grown for two days at 30°C, then replicated onto YPG (1% yeast extract, 2% peptone, 3% glycerol) plates. After another two days, cells samples of each patch were picked up with the replicator tool and resuspended in 30 µl water each, using a 96 well dish. Drops were placed onto YPG plates containing salinomycin sodium salt (Cayman Chemical Corp.; dissolved in ethanol), at 8 µg/ml for selection of sensitive mutants, at 12 µg/ml for selection of resistant mutants. Putative mutants with altered sensitivity were initially identified after 2 days of incubation while putative sensitive clones were monitored for up to 1 week. SM sensitive and resistant candidate clones were first verified by growing cells to late-logarithmic phase in YPG and streaking on SM gradient plates, containing 0–5 µg/ml SM for sensitive mutants (for YPG), 0–25 µg/ml SM for resistant mutants (YPD) or 0–12 µg/ml SM for resistant mutants (YPG). Gradient plates were generated by pouring a SM containing agar layer in slanted rectangular petri dishes and then overlaying when solidified with the same amount of SM-free medium. Overnight cultures pre-grown in YPG to late log phase were streaked on gradient plates.

We tested the same clones also for sensitivity or resistance to Narasin (Sigma) in YPG gradient plates of 0–5 or 0–7 µg/ml and to Nigericin (Calbiochem) in YPG gradient plates of 0–2 or 0–4.5 µg/ml. All results were verified by reisolating the identified mutants from a different version of the used deletion collection (OpenBiosystems v. 1.0) and repeating the described analysis.

Growth Curves

To establish the influence of SM on growth parameters of liquid cultures, we precultured the strains at 30°C with shaking in YPG liquid medium until a titer of $3x10^7$ cells/ml was reached. The culture was then diluted into fresh YPD or YPG at $2.5x10^4$ cells/ml or $1x10^5$ cells/ml, respectively. SM or the same volume of

ethanol (solvent control) was added and growth was monitored during incubation at 30°C by photometrically measuring the optical density of the cultures at 600 nm.

Detection of Sch9 Phosphorylation

Cultures were grown in YP media with either dextrose (YPD) or glycerol (YPG) as a carbon source to OD_{600} = 0.5 or 0.1, respectively. Following incubation with 200 ng/ml rapamycin for 30 minutes and 10 µg/ml SM for 5 hours, respectively, samples of $2x10^7$ cells were prepared using a modification of a published protocol [32]. The harvested samples were washed with water, resuspended in 0.5 ml 20% TCA, pelleted and frozen at – 80°C. For lysis, cells were washed twice with acetone, resuspended in urea buffer (50 mM Tris [pH 7.5], 5 mM EDTA, 6 mM urea, 1% SDS, 1 mM PMSF and 1ul PPi 1 and 2), vortexed with zirconium beads (12 min at 4°C) and subsequently heated for 10 min at 65°C. For chemical fragmentation by 2-nitro-5-thiocyanobenzoic acid (NTCB), 30 µl of 0.5 M N-cyclohexyl-2-aminoethanesulfonic acid (CHES, pH 10.5) and 7.5 mM NTCB were added and incubated overnight at room temperature. Two volumes of 20 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were added together with 1x SDS loading buffer, proteins were separated on a 7% SDS-PAGE gel and transferred to nitrocellulose. Sch9-HA was detected using mouse monoclonal anti-HA antibody 12CA5 (1:1000) (Covance). Secondary antibodies used were HRP-conjugated bovine anti-mouse antibodies (Santa Cruz Biotechnology).

Flow-Cytometric Analysis of Cellular ROS Content

Cells were grown overnight to saturation, diluted to a titer of $1x10^6$ cell/ml in fresh media (YPD or YPG) and incubated at 30°C for 5 hours. 10 µl of 10 µg/ml SM was added and incubation continued for additional 5 hours. Samples of $1x10^6$ cells were then washed twice with PBS and resuspended in 1 ml PBS. Cells were stained with 1 µM of 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein (CM-H_2DCFDA, Invitrogen) in dimethyl sulfoxide (DMSO), incubated for 20 min at room temperature and analyzed for fluorescence on a FC500 Flow Cytometer (Beckman Coulter).

RESULTS

Salinomycin is Active in Budding Yeast where it Prominently Targets Mitochondria

We intended to use budding yeast as a convenient eukaryotic model to analyze cellular responses to SM. First, we determined that wild-type yeast is susceptible to the drug. SM is known as an ionophore similar to nigericin that is known to cause mitochondrial damage in yeast [33]. When logarithmic-phase haploid cells were continuously treated with SM, significantly slower growth as compared to untreated cells (Figure **1**) and dose-dependent inactivation of colony formation (data not shown) was demonstrated.

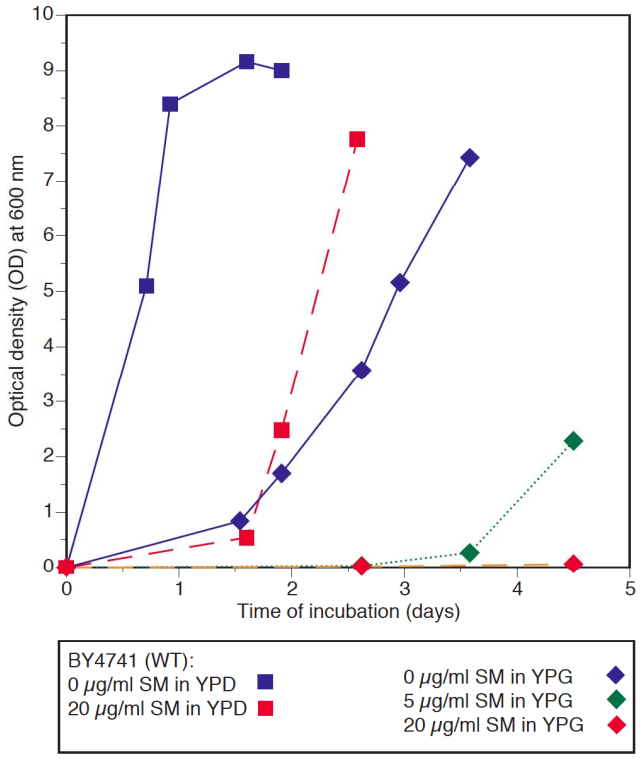

Figure 1: Growth of haploid wild-type yeast in the presence of SM. Cells of the wild-type strain BY4741 were pregrown to logarithmic phase in YPG and then diluted into fresh YPD or YPG with or without SM. Growth was monitored photometrically by measuring optical culture density (OD600) during incubation at 30°C. A representative single experiment is shown. Use of symbols is indicated in the figure.

To ensure that yeast engages mitochondrial oxidative metabolism, we used glycerol as a non-fermentable sole carbon source (YPG medium) for preculturing before diluting into fresh YPG or YPD. The latter medium contains dextrose as a carbon source and thus enables growth by glycolysis. The effect of SM on yeast growth is clearly more severe in YPG (Figure **1**).

In order to probe more directly if SM causes damage to mitochondrial DNA, the formation of respiration-deficient yeast mutants in YPD was also monitored. Dysfunctional mitochondria lead to smaller-sized colonies ('petites'). Red colony pigmentation indicates respiration proficiency if an *ade1* or *ade2*

mutation is present in the strain. Then, 'petites' are easily recognizable by the absence of red pigmentation. In such a strain (Y100), massive induction of petite colonies by SM can be observed (Figure **2A**) providing more evidence that SM targets mitochondria in yeast.

Additionally, we measured by flow cytometry if intracellular ROS are increased as a result of compromised mitochondrial integrity after SM exposure. After several hours of SM treatment, the proportion of cells with ROS levels above background had indeed noticeably increased (Figure **2B**).

When plating on SM-containing YPD plates, SM-resistant mutant clones were readily detectable among the background of poor growing cells after mutagen treatment, i.e. irradiation with 254 nm ultraviolet light

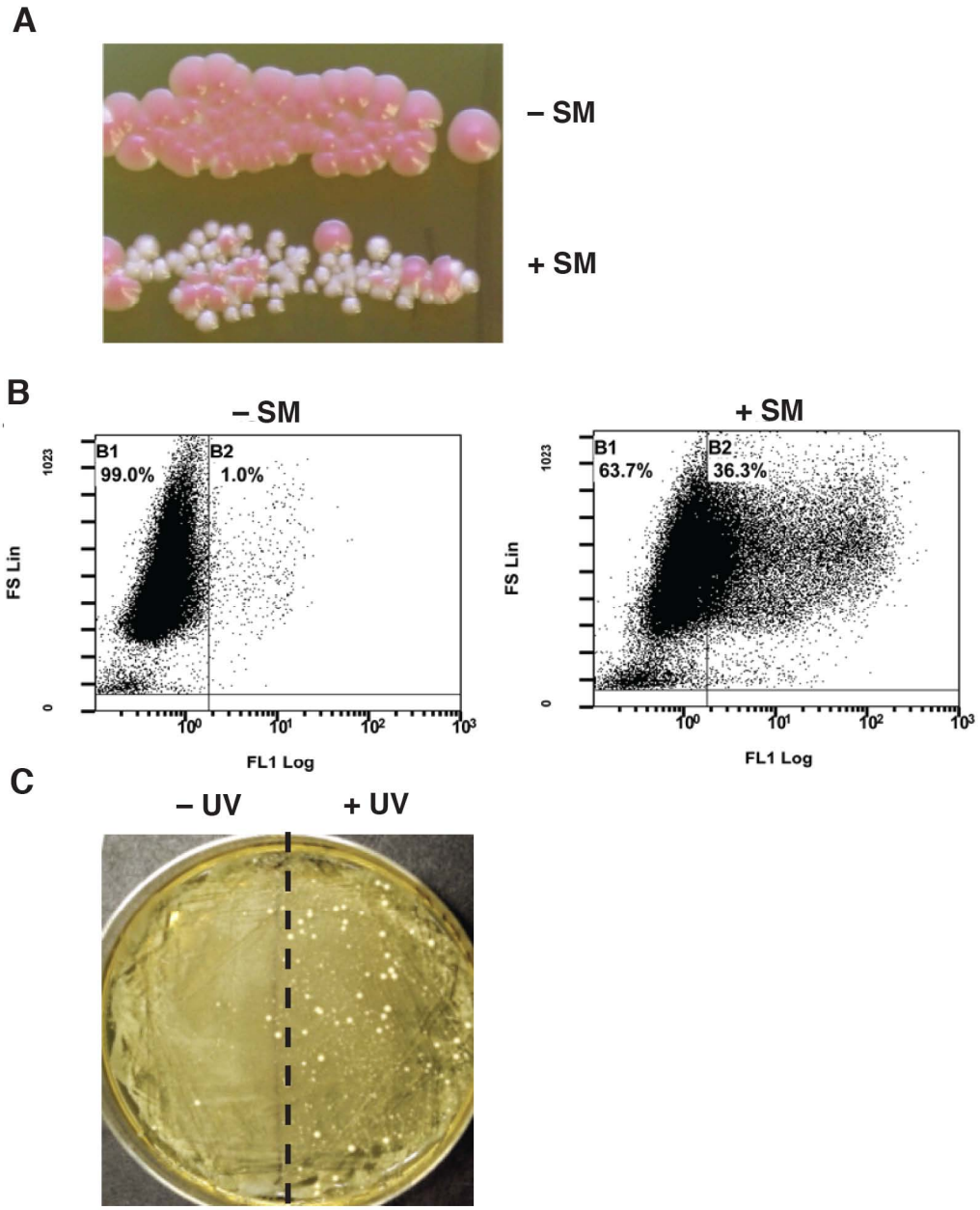

Figure 2: Cellular effects of SM in yeast. **A**) A high fraction of white respiration-deficient colonies ("petites") was observed when a culture sample of a yeast wild type which accumulates a red pigment (strain Y100) was plated after treatment with SM (30 µg/ml) for 20 hrs. (on YPD, 30°C). **B**) Increase in intracellular ROS measured by CM-H_2DCFDA. Wild-type yeast (BY4741) was pre-grown in YPG, then transferred to fresh YPG media with or without SM (10 µg/ml) and stained with CM-H_2DCFDA after 5 hrs. The fluorescence analysis dot blot shows forward scatter (linear Y axis) versus fluorescence (X axis). **C**) Detection of mutagen-induced SM-resistant mutants. 10^6 yeast wild-type cells were spread on a YPD plate containing SM (10 µg/ml), half of the plate was irradiated with 30 J/m^2 UV (254 nm) as indicated.

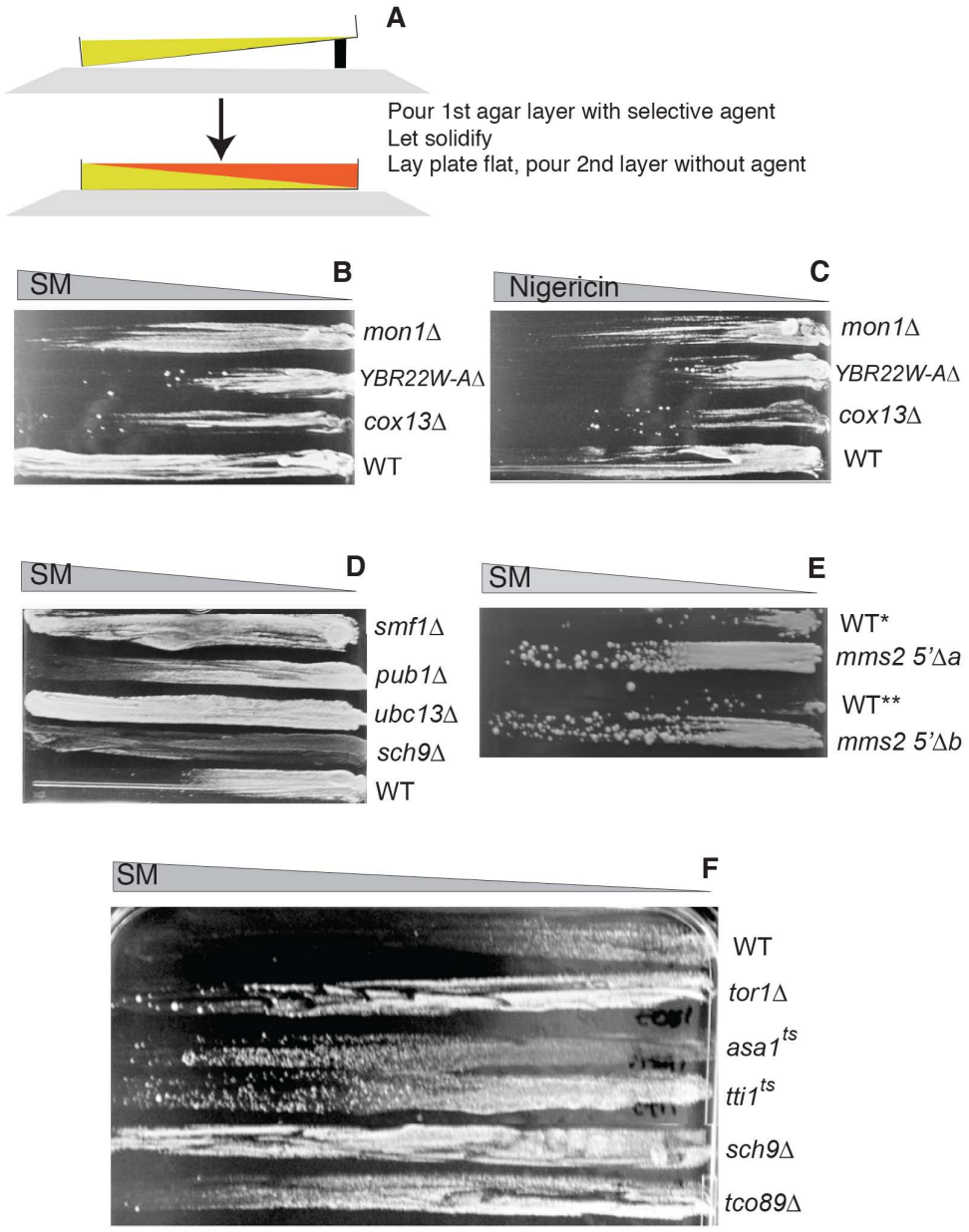

Figure 3: SM-sensitive and -resistant mutants characterized with gradient plates. **A**) General outline of the method. **B**) An example of a low concentration SM (0–5 µg/ml) gradient plate used to characterize SM sensitive mutants. **C**) The same strains were characterized on a nigericin gradient plate. **D**) An example of high concentration SM (0–10 µg/ml) gradient plate used to characterize SM resistant clones. **E**) Results for *mms2*Δ were confirmed by testing 5' truncation mutants of MMS2 from a different genetic background on a high concentration SM plate. Two different congenic strains are shown, WT** is also congenic, whereas WT* is from an unrelated genetic background. **F**) Relative SM resistance of TORC1 related mutants is shown on a high concentration SM plate.

(UV) (Figure **2C**). The estimated high frequency of SM resistant mutants indicates multiple target genes that confer resistance if mutated.

Screen of the Yeast Deletion Mutant Collection Identifies Various Pathways Determining Cellular SM Sensitivity

To identify cellular pathways that modulate cellular responses to SM we performed a screen for differential sensitivity using the commercially available Yeast Knockout Collection (YKO), made in haploid strain BY4741. Screens for SM-sensitive and resistant mutants were performed by replicating mutant colonies onto SM-containing plates, and candidate clones were retested on SM-gradient plates (Figure **3A**). Since our previous results showed that SM prominently causes mitochondrial damage, these screens were performed on YPG medium to enforce aerobic respiration and inhibit colony formation if respiration is deficient.

Table 1: List of Identified Yeast Genes that Alter SM-Sensitivity when Deleted

A. Genes conferring SM-sensitivity when deleted:	
VPS5, *VPS15*, *VPS16*, *VPS20*, *VPS25*, *VPS30*, *VPS34*, *VPS52*, *VPS53*, *VPS66* *SNF7*, *SNF8*, *YPT7* *CCZ2*, *MON1* *VAM3*, *VAM10* *MNN11* *CDC50*, *SLA1*	Vacuolar/Golgi/endosomal function
VPS30, *MON1*, *CCZ1*	Autophagy
ASP5	Cytosolic aspartate aminotransferase
ATX1	Cytosolic copper chaperone
TAF14	Chromatin modification, transcriptional complexes
CAJ1	Heat shock protein of DnaJ family
LAT1	Dihydrolipoamide acetyltransferase component (E2) of pyruvate dehydrogenase complex
RTK1	Kinase involved in ribosome biosynthesis
COX13	Subunit of cytochrome C oxidase
RPL42B	60S ribosomal subunit, redundant with *RPL42A*
YBR221W-A	4 kD protein of unknown function
B. Genes conferring SM-resistance when deleted:	
SCH9, *TCO89*	mTORC1 pathway
MMS2, *UBC13*	E2 ubiquitin ligation (Lys63 linkage)
RPL8A, *RPL12B*	60S ribosomal subunit
MEP3, *SMF1*, *YMC2*	Transport proteins
PUB1	polyA binding
VAC8	Vacuolar membrane protein

First, deletion mutants were isolated that show *enhanced sensitivity* to a low SM concentration. The majority of these mutants involves vacuolar functions (Table **1**). This is not an unexpected finding, since the vacuole is well established as the location for drug accumulation and detoxification in fungal and plant cells. Several additional findings are noteworthy. SM sensitivity results also from the deletions of *VPS30*, *MON1* or *CCZ1* which all play a role in autophagy. Remarkably, we also found a deletion of an extremely small *ORF*, *YBR221W*-A, encoding a (hypothetical) 4 KD protein of unknown function, to confer a high degree of SM sensitivity. A representative SM gradient plate with deletion mutants of *MON1*, *COX13* and *YBR221W-A* is shown in Figure **3B** to illustrate the method of characterization by gradient test. Other gene deletions that confer sensitivity belong to various pathways such as protein folding, stress responses, ribosomal biogenesis and metabolic oxidation (Table **1**).

Relative mutant sensitivities to the SM-derivative narasin and the similar-acting ionophore nigericin were characterized in parallel. In comparison to SM, responses were identical to narasin (not shown) and largely similar to nigericin (Figure **3C**) with only minor quantitative differences.

Second, in order to identify mutants showing *enhanced resistance* to SM, its concentration in the initial selection plates was increased so that the wild type was no longer able to grow. SM concentrations in gradient plates were also adjusted accordingly. As predicted by the UV experiment (Figure **2C**), various gene deletions were indeed found to confer SM resistance (Table **1**). These included ribosomal and vacuolar proteins, certain transporters and poly-A binding protein. Representative examples are shown in Figure **3D**. In the following, we will focus on two pathways of major interest where gene deletions conferred the highest levels of SM resistance observed – an E2-ubiquitin-conjugating enzyme (Mms2-Ubc13) and the target of rapamycin complex (TORC).

Deficiencies in the E2-Ubiquitin-Conjugating Enzyme Ubc13-Mms2 Confer Protection Against SM

Among deletion mutants showing a high level of SM resistance were those of *UBC13* and *MMS2* forming an

Table 2: SM Sensitivity of Deletion Mutants of Either One of Two Ribosomal Protein Paralogs (A or B)

Locus designation	Phenotype of ΔA	Phenotype of ΔB
RPL6	–	+
RPL12	+	+
RPL22	+	0
RPL35	+	+
RPL37	+	–
RPL40	+	+
RPL41	+	+
RPP1	0	+
RPP2	0	+
RPS1	+	0
RPS6	+	+
RPS9	+	0
RPS10	+	0
RPS11	+	+
RPS18	+	+
RPS22	+	+
RPS23	0	+
RPS27	0	+
RPS29	+	+

– = more sensitive to SM than WT.
+ = more resistant to SM than WT.
0 = ~ WT response.

E2-ubiquitin-conjugating enzyme for Lys63 polyubiquitination. To confirm this result (Figure **3D**, Table **1**), we also tested 5' terminal truncations of *MMS2* of different genetic background [34] which behaved identically (Figure **3E**). We also verified that gradient assay data predict the results of liquid assays of shorter duration and showed faster culture growth of *mms2*Δ as compared to wild type in both YPD and YPG media following preculture in YPG (Figure **4A**, **B**). Mms2-Ubc13 mediated polyubiquitination plays a known role in the Rad5-dependent DNA damage tolerance pathway [35]; a *RAD5* deletion, however, did not confer SM resistance (data not shown).

TORC1 Pathway Deficiencies Confer Protection from SM

We found that deficiency in certain TORC signaling components (*SCH9, TCO89*) resulted in resistance to SM (Figure **3D**, **F**). *SCH9* is a downstream effector of TOR complex 1 (TORC1) whereas *TCO89* is part of TORC1 itself [36, 37]. In yeast, TORC1 is comprised of Tor1/Tor2 along with accessory proteins; Tor1 is a major kinase of TORC1. In a survey of other viable TORC mutants, we found that *tor1*Δ conferred SM resistance similar to *sch9*Δ and *tco89*Δ (Figure **3F**). Tti1 is part of the triple T (TTT) complex and assists along with Asa1 in PIKK folding. $Tti1^{ts}$ and $asa1^{ts}$ are temperature sensitive alleles; however, restrictive temperature was not required to detect a SM resistance phenotype (Figure **3F**).

The effect of a *SCH9* deletion was characterized in more detail. First, the gradient assay result was confirmed for liquid cultures (Figure **4A**, **B**). Similarly, the improved growth in the presence of SM correlates with an absence of induction of "petite" (= respiration-deficient, Rho^- or Rho^0) colonies by SM (Figure **5A**, **B**). Since this result suggests that the main protective effect of the *SCH9* deletion affects mitochondrial stability, we asked if Sch9 affects the ROS levels that are elevated as a consequence of SM treatment. The absence of an ROS increase in an isogenic Rho^- wild type (without functional mitochondria) proves their exclusive mitochondrial origin. ROS levels in *sch9*Δ, however, appeared to be quite comparable to wild type (Figure **5C**). We conclude that initial oxidative stress

level by the ionophore action of SM is not reduced in the absence of Sch9.

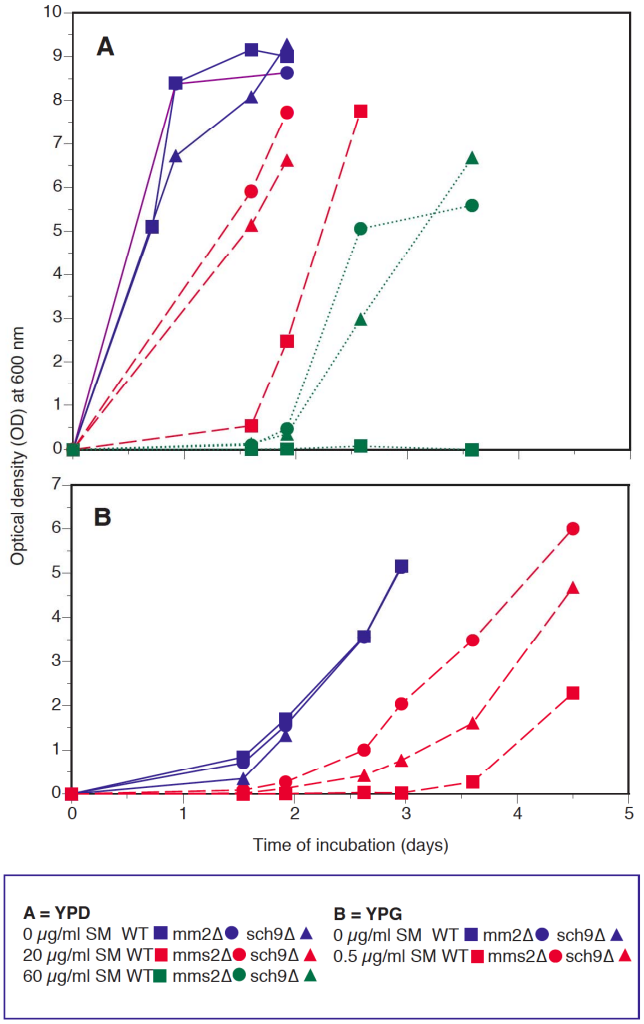

Figure 4: Growth of *sch9*Δ and *mms2*Δ mutants as compared to wild type in the presence of SM in YPD (**A**) and YPG (**B**). Use of symbols and SM concentrations are indicated in the figure.

TORC1 serine-threonine protein kinase phosphorylates Sch9 at its C-terminal on multiple residues [32]. We also wanted to determine if SM downregulates the TORC1 pathway upstream of Sch9. We found that SM like rapamycin results in a reduction in TORC1 kinase activity as indicated by nearly undetectable levels of phosphorylated Sch9 in wild type yeast (Figure **6**).

Ribosome Protein Paralog Deletions Confer Altered SM Sensitivity

The rapamycin-sensitive phosphoproteome indicates that Sch9 might be a central node in TORC1 mediated ribosomal biogenesis [38, 39]. Interestingly, strains with deleted *RPL12B* and *RPL8A* genes also conferred resistance to SM (Table **2**). Because of a large-scale duplication of the budding yeast genome, a number of ribosomal proteins are each encoded by two paralogs. Our initial screen revealed three genes coding for ribosomal proteins, *RPL8A*, *RPL12B* and *RPL42B*, whose deletion rendered cells resistant or sensitive to SM. We surveyed all available deletions of ribosomal genes (if yielding viable cells on non-fermentable media) to detect even minor effects that may have been overlooked in the initial screen, using previously described SM gradient plates (Table **2**). In two instances, deletion of one gene resulted in enhanced SM resistance whereas deletion of the paralogous gene led to enhanced sensitivity. In the remaining cases, deletion of one paralog rendered cells more resistant whereas deletion of the corresponding paralog enhanced resistance as well or had no effect. Response to nigericin was also tested in parallel, with mostly similar results (not shown).

DISCUSSION

SM with its hydrophobic surface appeared to be able to enter yeast cells as indicated by decreasing growth rates (Figure **1**) and cell killing at higher concentrations (not shown). SM treatment resulted in the induction of 'petite' colonies (which may include both rho^{-} or rho^{0}, the latter with undetectable mitochondrial DNA) that are indicative of irreversibly damaged mitochondria (Figure **2A**). We suspect that, like nigericin, SM is incorporated into the mitochondrial membrane, leading to an ion imbalance across the organelle's membrane and depolarization of the membrane potential. Loss in membrane integrity may then increase the amount of ROS in the cell. Our results confirm this notion since we detected increased intracellular ROS after SM treatment (Figure **2B**). This increase is respiration dependent and not found in respiration-deficient cells (Figure **5C**).

Given that its highest affinity is for K^{+} ions, incorporation of SM into the mitochondrial membrane would disrupt K^{+} ion homeostasis, leading to accumulation of K^{+} ions and water in the mitochondrial matrix causing swelling, as demonstrated for nigericin [33, 40]. An ion imbalance caused by excessive K^{+} ion influx would then disrupt Ca^{2+} homeostasis [41]. Since calcium is one of the key regulators of mitochondrial function and acts at several levels within the organelle to stimulate ATP synthesis, a cascade of events would ensue that includes enhanced generation of ROS, triggering of the permeability transition pore and release of cytochrome c that may ultimately result in apoptosis in mammalian cells [40].

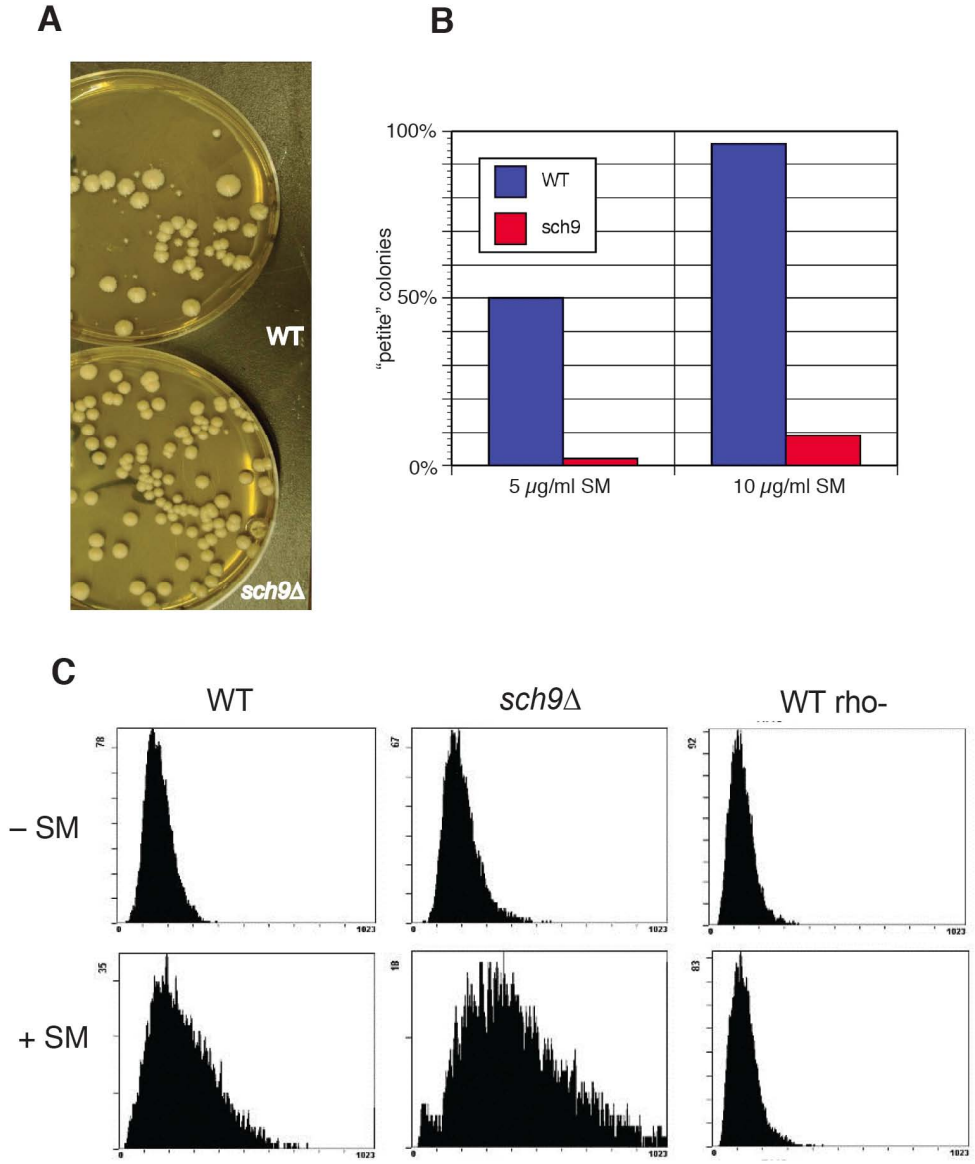

Figure 5: Sch9 deletion prevents induction of respiration deficient cells by SM but not initial ROS formation. **A**, **B**) SM-induced "petite" formation is suppressed by a Sch9 deletion in wild type BY4741. Note the appearance of small, respiration-deficient colonies (A). **C**) Increase in intracellular ROS was measured by CM-H_2DCFDA fluorescence as in Figure **2**. The WT is compared to *sch9*Δ and also to an isogenic rho$^-$ isolate that is incapable of respiration and does not increase ROS following SM treatment. Cell histograms are shown, categorized according to CM-H_2DCFDA fluorescence (X axis).

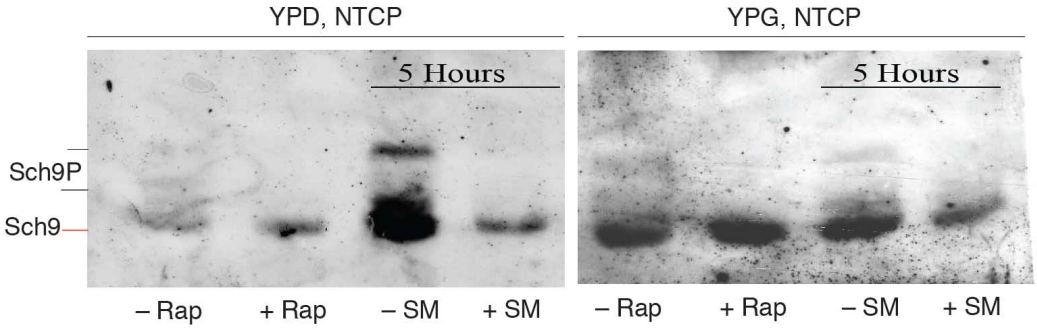

Figure 6: Sch9 kinase assay as a probe for TORC1 activity. Phosphorylation of the C-terminus of Sch9 (Sch9P) was measured as described in materials and methods. SM (after 5 hrs. incubation) causes dephosphorylation of Sch9 that resembles the effect of rapamycin (Rap, 30 min. incubation).

Yeast has already been used as a genetic tool to uncover various pathways for other inonophores such as monensin and nigericin [33, 42]. Using budding yeast in a toxicogenomic approach as a model to identify cancer stem cell-specific targets is reasonably justified, since, for example, yeast like most tumor cells prefer glycolysis over respiration even in the presence of abundant oxygen (Warburg effect) [43]. Our screen to find mutants that had altered sensitivities to SM uncovered various pathways. For this study, we focused on mutants of the Mms2-Ubc13 complex and TORC1 related mutants that confer the highest observed degree of relative SM resistance.

Mms2 and Ubc13 are subunits of an E2-ubiquitin conjugating enzyme which is conserved in higher eukaryotes and catalyzes the polyubiquitination of substrates (e.g. PCNA) through Lys63 linkage [35]. This modification typically does not initiate proteasomal degradation but changes the activity and/or interactions of the protein substrate. Interestingly, Lys63-linked polyubiquitination of a yeast membrane permease can signal endocytosis of the protein and vacuolar degradation [44]. The Mms2-Ubc13 complex, however, has known roles in nuclear DNA damage tolerance pathways [35]. Such processes have not been characterized in mitochondria and we have no evidence that similar processes play a role in SM toxicity – no additional related gene was picked up in our screen. Thus, the relevant target remains to be elucidated. It may be found among the other proteins whose inactivation causes altered SM sensitivity.

A number of loss-of-function TORC1 related mutants (*tor1, tel2, asa1, tti1, sch9* and *tco89*) conferred protection from SM (Figure **3D**, **F**). *SCH9* (the homolog of S6K1 in mammals) is a downstream effector of TOR complex 1 (TORC1) whereas *TCO89* is part of TORC1 itself [36, 37]. TORC1 is conserved among all eukaryotes and represents important serine/threonine kinase activities. In response to growth factors and oncogenes, the lipid kinase (PI3K) is able to activate Akt kinase as an upstream effector of TORC1 [45]. The PI3K/AKT/TOR signaling pathway is a significant contributor to disease and various human cancers, including hematologic malignancies. Also, in many cancers there is a frequent hyperactivation mTOR that is a clinically validated target for drug development [46]. mTOR has been implicated in stem cell homeostasis and lifespan determination [47].

We predict that SM is primarily active in yeast due to ROS induced mitochondrial damage (which translates into “petite” induction on fermentable media or cell inactivation on non-fermentable media). TORC1 phosphorylates Sch9 on multiple C-terminal residues. We observed that dephosphorylation of Sch9 occurs after SM treatment (Figure **6**) which suggests reduced kinase activity of TORC1 as a response to mitochondrial dysfunction. This is substantiated by a previous report in which Sch9 was severely dephosphorylated upon exposure to a protonophore [36]. SM was also characterized as a strong antagonist of mTORC1 in breast and prostate cancer cells [27].

The initial burst of intracellular ROS is not abolished by the Sch9 deletion (Figure **5C**) and thus, the complete absence of Sch9 activity must affect protective downstream processes. TORC1 inhibition leads to upregulation of stress response genes such as SOD2 and increased mitochondrial respiration via enhanced translation of mtDNA-encoded oxidative phosphorylation complex subunits [48]. Similar mechanisms may confer SM resistance.

Our data suggest that SM resistance conferred by inactivation of the TORC pathway may at least in part be mediated by modification of translation. Data mined from various sources suggest that TORC1 regulates the translational machinery and ribosome biogenesis mediated by Sch9 as a central node [49]. In *S. cerevisiae*, the majority of ribosomal proteins are encoded by two genes, stemming from an ancient duplication event of its genome [50]. In yeast these paralogs have a complex relationship, as these copies are often not functionally equivalent [51]. Because of a genome duplication, deletion of individual paralogs of yeast ribosomal genes can be studied and it was found that the response to certain genotoxic or cytotoxic agents is altered [50]. These differences have been explained by the existence of ribosomes with differential translation preferences, depending on the individual paralog incorporated, suggesting the presence of a ‘ribosomal code’ [50, 52]. For altered SM sensitivity, only two gene pairs fall in this category (Table **2**), in all other cases, where deletion of paralogs does not result in an opposing effect, reduced protein levels appear more likely as explanations.

Although not a focus of this study, it should be noted that deletion of certain autophagy genes such as *VPS30*, *MON1*, *CCZ1* in addition to a number of vacuolar genes caused SM sensitivity (Table **1**) suggesting that SM may be scavenged by intracellular mechanisms. Autophagy has been suggested as a central anticancer mechanism of SM [53]. It should also

be noted that the lysosomal sequestration of iron by SM has recently been implicated in breast cancer stem cell killing [54].

CONCLUSION

In summary, our analysis of the yeast gene deletions should assist in outlining cellular targets and factors that cause SM sensitivity or resistance. This has also implications for understanding the molecular basis of intrinsic or acquired drug resistance in tumors which is important in developing more beneficial therapies.

The relevance of the yeast model to find cancer-stem cell specific targets remains undecided. For example, nigericin was not identified as very effective in the initial screen for selective killing of cancer stem cells [3]. Consequently, the fact that our analysis characterizes the pharmacogenomics of SM and nigericin as virtually indistinguishable in yeast suggests that relevant cancer stem cell-specific target(s) may not be present in yeast – perhaps another confirmation of the importance of the WNT pathway. At the very least the findings of this study may be useful in devising strategies that minimize damaging side effects of the drug in the patient. Heart and neural toxicity of SM implies mitochondrial damage also as a major toxic consequence in mammals. Our analysis would suggest that an mTOR pathway antagonizing drug like wortmannin could be a useful countermeasure.

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CONFLICT OF INTEREST STATEMENT

Authors have declared no conflicts of interest.

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ABBREVIATIONS

CM-H_2DCFDA = 5-(and-6)-chloromethyl-2',7'-dihydrofluorescein

mtDNA = mitochondrial DNA

NTCB = 2-nitro-5-thiocyanobenzoic acid

PCNA = proliferating cell nuclear antigen

PIKK = phosphatidylinositol 3-kinase-related kinase

ROS = reactive oxygen species

SM = salinomycin

TOR = target of rapamycin

TORC = target of rapamycin complex

WT = wild type

YPD = yeast extract/peptone/dextrose

YPG = yeast extract/peptone/glycerol

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