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A Small Molecule Suppressor of FK506 that Targets the Mitochondria and Modulates Ionic Balance in *Saccharomyces cerevisiae*

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Summary

FK506 inhibits the evolutionarily conserved, Ca^{2+}-dependent phosphatase calcineurin, which in yeast is essential for growth during sodium stress. We undertook a chemical genetic modifier screen to identify small molecules that suppress the ability of FK506 to inhibit yeast growth in high NaCl. One of these small molecule suppressors, SFK1 (suppressor of *FK506 1*), causes a mitochondrially induced death in low salt, concomitant with the release of reactive oxygen species. Biochemically, SFK1 interacts with Por1p, a channel protein in the outer mitochondrial membrane, suggesting that SFK1 interacts with the mitochondria directly. A genome-wide screen of yeast deletion strains for hypersensitivity to SFK1 yielded several strains with impaired mitochondrial function, as well as several with reduced sodium tolerance. Our data link ionic balance to mitochondrial function and suggest a role for calcineurin in mediating this signaling network.

Introduction

Small molecules offer a means to alter conditionally protein function across cell types and organisms. The immunosuppressant FK506 binds FKBP12 (for FK506 binding protein, 12 kDa) and specifically inhibits the highly conserved Ca^{2+}-dependent protein phosphatase calcineurin [1]. Although originally identified for its ability to suppress the proliferation of T lymphocytes, FK506 has been used as a tool to study the role of calcineurin in a wide variety of organisms from yeast to humans. In *S. cerevisiae*, calcineurin is only required under certain stress conditions, such as high concentrations of Na^+, Li^+, Mn^{2+}, and OH^-, as well as prolonged exposure to mating factor [2–5]. Hence, FK506 potently inhibits the growth of yeast only under such stress conditions. In order to develop a set of small molecule tools to investigate the role of calcineurin in yeast, we screened for small molecules that would suppress FK506's ability to inhibit yeast growth in high concentrations of NaCl. This method of probing the function of the target of a known small molecule with a collection of diverse small molecules is referred to as a chemical genetic modifier screen, by analogy to the suppressor/enhancer screen of classical genetics. As a genetically tractable and well-studied organism, yeast provides an ideal model system for performing and, especially, for following up such a screen.

In yeast, calcineurin plays an important role in sodium tolerance, simultaneously inhibiting Na^+ uptake and stimulating Na^+ extrusion. Calcineurin induces the transcription of a number of genes via the transcription factor, Tcn1 [6, 7]. During sodium stress, calcineurin stimulates the transcription of Ena1, a P-type ion pump in the plasma membrane that is critical for sodium extrusion [7]. In addition to the calcineurin pathway, a number of other pathways, such as the Hog1 mitogen-activated protein kinase [8], the Hal3-Ppz1 [9–11], and the Ure2-Gln3 pathways [12], regulate Ena1 expression. In strains lacking functional calcineurin, several mutations have been identified that stimulate these parallel pathways, increasing Ena1 expression, thereby promoting sodium extrusion and cell survival [11, 12].

In addition to stimulating Na^+ extrusion, calcineurin inhibits Na^+ uptake. One of the main routes for Na^+ entry is thought to be via the K^+ transporters, Trk1 and Trk2. Under conditions of sodium stress, calcineurin is thought to increase the K^+/Na^+ discrimination of Trk1, thereby limiting Na^+ entry [3]. A major determinant of sodium uptake during sodium stress is the membrane potential of the plasma membrane, and a number of mutations are thought to improve growth in high Na^+ by depolarizing the plasma membrane, thereby reducing the driving force for cation uptake [13–17]. The proton-pumping ATPase Pma1 is the major generator of plasma membrane potential in yeast, and several *pma1* mutants with reduced electrogenic activity have been identified that are resistant to Na^+ and other toxic cations [12, 13].

We screened a large collection of chemically diverse small molecules for the ability to promote the growth of calcineurin-deficient yeast in high-NaCl media. Here, we report on one molecule that scored in this screen, which we call SFK1 for *s*uppressor of *FK506 1*. While SFK1 improves the growth of yeast under high NaCl in the presence of FK506, it causes death under low-salt conditions, at similar concentrations. Yeast cells lacking mitochondrial DNA (ρ^0) are resistant to SFK1-mediated death, suggesting that this death requires respiration-competent mitochondria. Biochemically, SFK1 interacts with Por1p (YVDAC1), a voltage dependent anion-selective channel in the outer mitochondrial membrane (OMM), suggesting that SFK1 may be localized to the mitochondria. To explore the mechanism of SFK1-mediated death, we screened the set of viable, haploid yeast deletion strains for mutants that selectively show sensitivity to SFK1. Our data suggest that the mitochondria act as a sensor for the ionic balance of the cell and that SFK1 targets protein(s) critical to this sensing mechanism.

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Results

Screening for Small Molecule Suppressors of FK506 in Yeast

Approximately 100,000 compounds from either commercial or in-house small molecule libraries were screened for their ability to allow growth of calcineurin-deficient yeast in media containing 0.7 M NaCl. Small molecules were robotically pinned into 384-well plates containing high-NaCl media. Then, either calcineurin-deleted (Δ*cna1* Δ*cna2*) or FK506-treated wild-type yeast were added. The plates were allowed to incubate at room temperature for 3 days, at which time compounds that allowed growth were identified as potential positives. Only a very small percentage of compounds (~0.02%) scored and retested as positives.

We focused on one positive from the screen, SFK1 (Figure 1A). SFK1 improves the growth of FK506-treated yeast in high-NaCl media with an IC_{50} of 1.5–2.5 μM (Figure 1B). While SFK1 improves growth in high-NaCl media in the presence of FK506, it inhibits growth in YPD (Figure 1C). Because these two phenotypes occur at similar concentrations, we hypothesized that they might be related. That is, SFK1 might improve growth in high-NaCl plus FK506 and inhibit growth in low NaCl by interacting with the same protein target.

To characterize SFK1 further, we tested the ability of SFK1 to affect growth under a variety of conditions (Figure 1D). High concentrations of NaCl prevent SFK1-mediated growth inhibition. However, SFK1 does not improve growth in high NaCl in the absence of FK506. Hence, calcineurin must be inactivated in order for SFK1 to improve growth in high NaCl. Like high concentrations of NaCl, high concentrations of the nontoxic salt, KCl, prevent growth inhibition by SFK1. High concentrations of the osmotic stabilizer sorbitol, however, do not prevent this growth inhibition, suggesting that SFK1 is not inhibiting growth by causing cell lysis. Growth inhibition by SFK1 is also pH dependent, since lowering the pH of the media improves growth in the presence of SFK1.

Structure-Activity Analysis of SFK1

In order to explore the structural requirements for the activity of SFK1, we synthesized several derivatives of the molecule. The activity of SFK1 is highly sensitive to structural modifications. Shortening of the alkyl chain of the molecule results in a gradual loss in the ability to improve growth of calcineurin-deficient (Δ*cna1* Δ*cna2*) yeast in high NaCl (Figure 2A, upper graph). Similarly, shortening of the alkyl chain results in a gradual loss in the ability of the molecule to inhibit growth in low NaCl (Figure 2A, lower graph). The correspondence between the ability of a given derivative to improve growth in high NaCl and to inhibit growth in low NaCl provides further evidence that the two effects are related.

Analogs of SFK1 with a benzyl ester instead of a cyclohexylmethyl ester show reduced activity, suggesting that modifications to that end of the molecule are also detrimental to activity, increasing the IC_{50} and decreasing the magnitude of activity (Figure 2B). This pattern is most clearly evident when comparing $SFK1_7$ and $SFK1_{bz,7}$. $SFK1_{bz,7}$ is 2-fold less active in terms of its ability to improve growth in high NaCl, and it is 2-fold less active in terms of its ability to inhibit growth in low NaCl. Again, the ability of a given derivative to improve growth in high NaCl and the ability to inhibit growth in low NaCl roughly correspond. The remarkable sensitivity of SFK1's activity to small changes in its structure was also shown using transcriptional profiling (see below).

SFK1 Interacts Biochemically with Por1p, a Pore Protein in the OMM

In order to isolate proteins that interact with SFK1, an amino derivative of SFK1 was synthesized and immobilized on an NHS-ester Hi-Trap sepharose resin (Figure 3A). The Boc-protected amino derivative of SFK1 (**2**) inhibited growth in low salt at 40 μM and improved growth slightly in the presence of NaCl and FK506 at a similar concentration. Yeast lysate was first incubated with a blank resin loaded with ethanolamine in order to clear it of proteins that nonspecifically interact with the blank resin. This cleared lysate was then incubated with the SFK1 resin (**3**). The SFK1 resin was washed, and then proteins that specifically interact with the resin were eluted with $SFK1_4$, a more water-soluble version of SFK1 (Figure 2). A 30 kDa band reproducibly eluted from the column. This band could also be eluted with more "active" versions of SFK1, such as $SFK1_6$ (data not shown). Microsequencing of this band identified it as Por1p, a pore protein in the OMM. Because Por1p is a highly expressed protein used as a marker for the OMM [18], this result suggested that SFK1 might be localized to the mitochondria. Por1p and the related protein, Por2p, do not appear to be unique targets of SFK1, however, since SFK1 still has activity in a Δ*por1* strain and in a Δ*por1* Δ*por2* strain (data not shown).

SFK1 Causes a Mitochondrially Induced Death

We next asked whether yeast cells could recover from SFK1 treatment in low-salt media. In order to answer this question, we treated yeast with 10 μM SFK1 for a given amount of time and then washed away the compound and plated the cells in order to count the number of surviving cells. SFK1 does, in fact, cause lethality. However, SFK1-induced death is a relatively slow process, with roughly one-third of cells dead after 1 hr and roughly two-thirds of cells dead after 6 hrs (Figure 4A).

In experiments in which either a prolonged treatment or a high concentration of SFK1 was used, the surviving population of cells was highly enriched in petites (data not shown). Petites are yeast cells that have acquired either nuclear or mitochondrial mutation(s) that make them unable to respire and hence unable to grow on nonfermentable carbon sources. The fact that the petites were resistant to SFK1-mediated death suggested that this death might require respiring mitochondria. In order to test this hypothesis more rigorously, we independently generated a ρ^0 yeast strain through extensive growth on ethidium bromide. Because ρ^0 yeast lack mitochondrial DNA and thus do not express the proteins that are encoded by that DNA, they are not able to respire and are entirely dependent on fermentation for energy generation. Treatment of ρ^0 yeast with 10 μM SFK1 revealed that they are almost completely resistant to SFK1-mediated death, with approximately 93% surviving after 6 hr of treatment

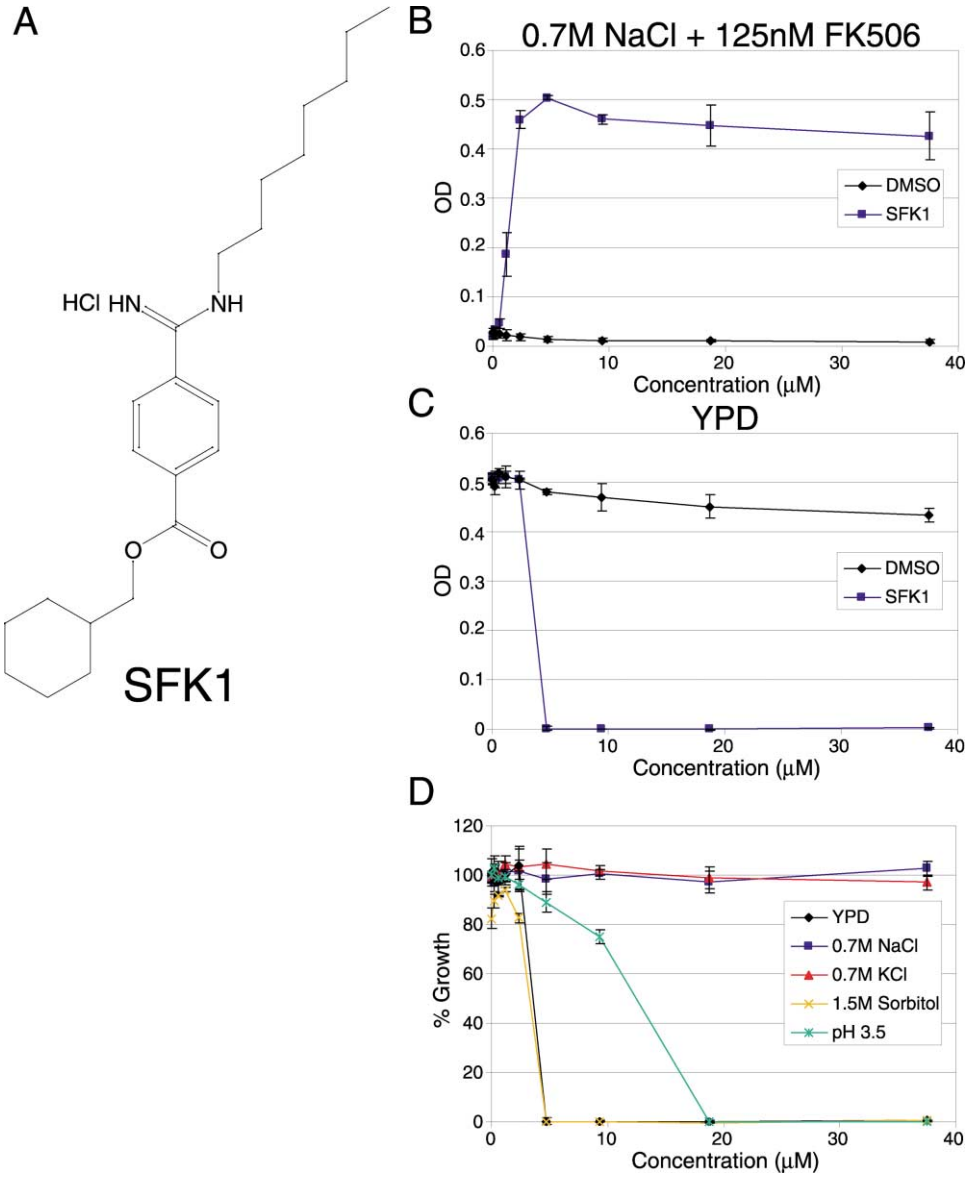


Figure 1. The Effect of SFK1 Treatment on Growth under Various Conditions

(A) The chemical structure of SFK1.

(B) SFK1 improves growth of wild-type yeast in YPD in the presence of 0.7 M NaCl and 125 nM FK506.

(C) SFK1 inhibits growth of wild-type yeast in YPD.

(D) Effect of SFK1 on growth in YPD, YPD with 0.7 M NaCl, YPD with 0.7 M KCl, YPD with 1.5 M sorbitol, and low-pH YPD (pH 3.5). The concentration of SFK1 is listed along the x-axis. Percentage growth in a given media refers to the percentage growth in the presence of SFK1 referenced to growth in the presence of vehicle (DMSO). Low-pH YPD contained 50 mM succinate and was adjusted to pH 3.5 with HCl.

(Figure 4B). Although ρ^0 yeast are resistant to SFK1-mediated death, ρ^0 yeast are only partially resistant to SFK1-mediated growth inhibition. Furthermore, SFK1 is still able to improve the growth of ρ^0 yeast in high NaCl/FK506 (data not shown).

Because mitochondrially induced death in yeast is often associated with increased ROS production, we tested the effect of SFK1 on ROS levels. 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) was used as an indicator of ROS [19]. Because H_2DCF-DA fluoresces green when oxidized by ROS, ROS production could be monitored by immunofluorescence (Figure 4C). After 1 hr of treatment with 10 μM SFK1, 69.7% (±10.4%) of cells were producing ROS, compared to 0% (±0%) of control cells. Because the percentage of ROS-producing cells is higher than the percentage of dead cells following treatment with SFK1 for 1 hr, increased ROS production probably precedes SFK1-induced death and may contribute to that death.

Mitochondrial membrane potential ($\Delta\Psi_m$) is a useful indicator of mitochondrial function. To monitor $\Delta\Psi_m$, we used the cationic, lipophilic dye $DiOC_6$, which accumulates in the mitochondria in accordance with $\Delta\Psi_m$. Treatment with 5 μM SFK1 strongly hyperpolarizes the mitochondria (Figures 4D and 4E). In accordance with their reduced activity,

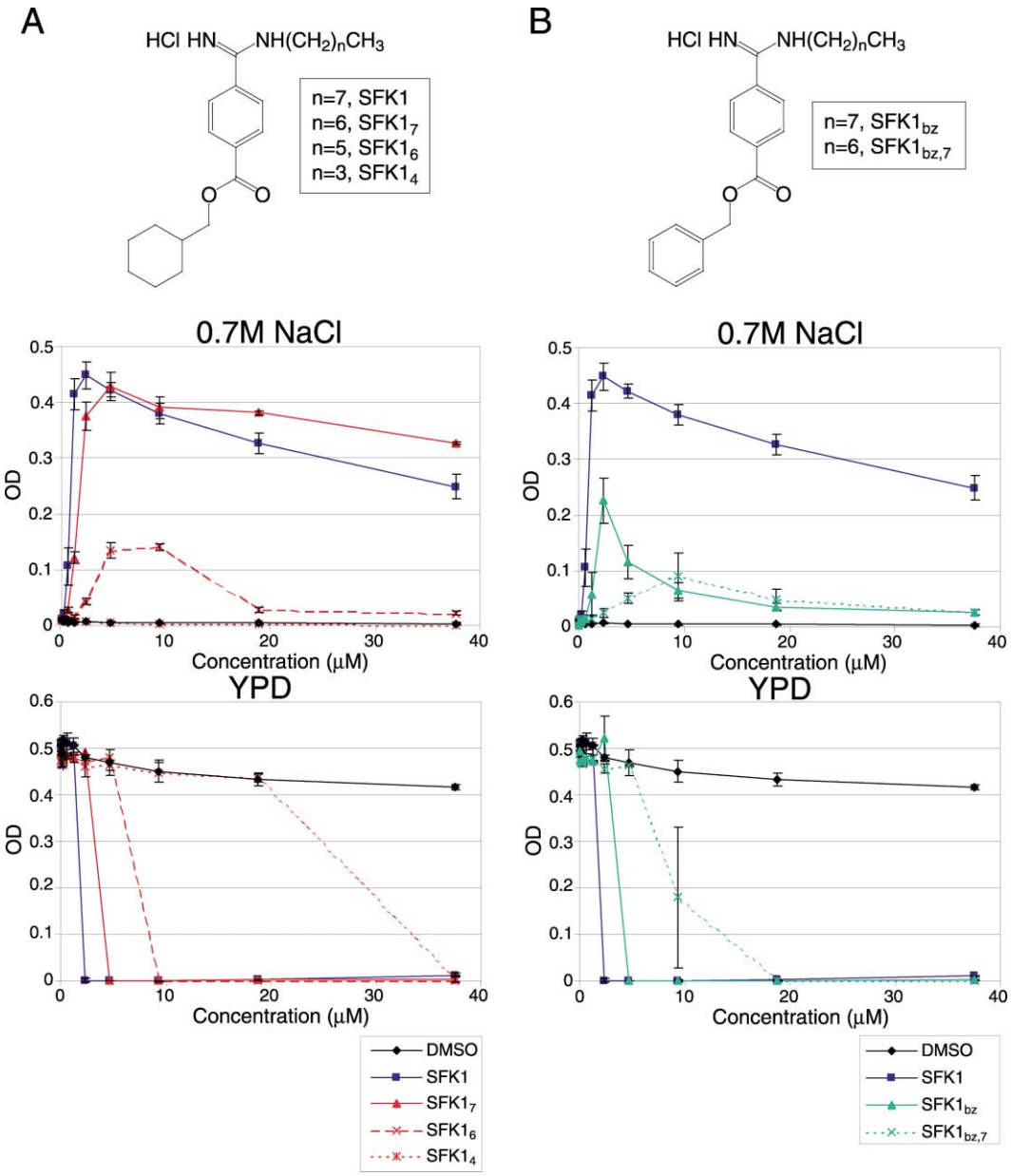


Figure 2. Structure-Activity Relationships of SFK1 Derivatives

(A) The structure of the various derivatives along with the convention used to name the derivatives. Growth of Δ*cna1* Δ*cna2* yeast in 0.7 M NaCl in the presence of various derivatives (top graph). Growth of wild-type yeast in YPD in the presence of various derivatives (bottom graph).

(B) The structure of the various benzyl derivatives along with the convention used to name the derivatives. Growth of Δ*cna1* Δ*cna2* yeast in 0.7 M NaCl in the presence of various benzyl derivatives (top graph). Growth of wild-type yeast in YPD in the presence of various benzyl derivatives (bottom graph).

$SFK1_{bz}$ and $SFK1_{bz,7}$ only weakly hyperpolarize the mitochondria at 5 μM (data not shown). Treatment with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a protonophore, dissipates $\Delta\Psi_m$ (Figure 4D, left plot). Furthermore, CCCP is able to block the ability of SFK1 to hyperpolarize the mitochondria (Figure 4D, right plot). Similarly, sodium azide (NaN_3), an inhibitor of the F_1-ATPase and complex IV, is able to block the ability of SFK1 to hyperpolarize $\Delta\Psi_m$ (Figure 4E). SFK1 is able to hyperpolarize the mitochondria in the presence of either 0.7 M NaCl or 0.7 M KCl, suggesting that SFK1 can still reach its target in high salt (data not shown). Interestingly, SFK1 is able to hyperpolarize $\Delta\Psi_m$ even in ρ^0 cells (data not shown). ρ^0 cells are thought to maintain a $\Delta\Psi_m$, possibly through the rapid electrogenic exchange of ATP^{4-} for ADP^{3-} using the nuclearly encoded F_1-ATPase and adenine nucleotide translocator [20–22]. Thus, although the mitochondrial genome is required for SFK1 to cause death, it is not required for SFK1 to hyperpolarize the mitochondria or for SFK1 to improve growth in high NaCl/FK506.

One drawback of using $DiOC_6$ to measure $\Delta\Psi_m$ is that it can be influenced by changes in the plasma membrane

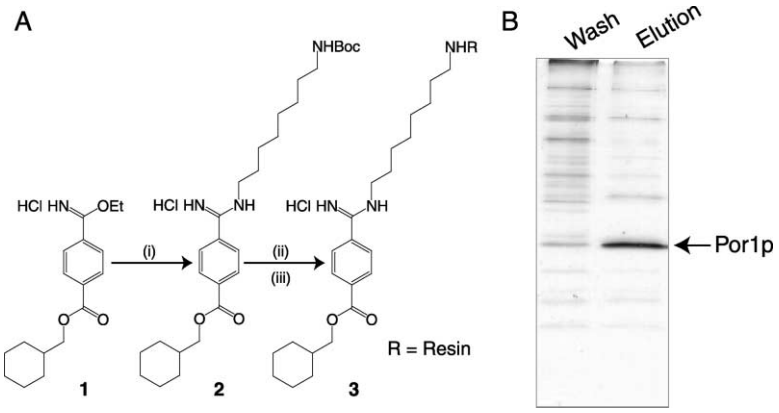


Figure 3. Synthesis of SFK1 Affinity Resin and Purification of SFK1-Associated Proteins

(A) Synthesis of SFK1 affinity resin. (i) $NH_2(CH_2)_8NHBoc$, EtOH, reflux; (ii) 4 M HCl in 1,4-dioxane, rt; (iii) NHS Hi-Trap Sepharose Resin, 2:3 EtOH: (aq.) 0.2 M $NaHCO_3$ (pH 8.3), 0.8% tween-20, rt.

(B) Identification of Por1p as an SFK1-associated protein. After incubation with a blank resin, cleared yeast lysate was incubated with the SFK1 affinity resin. The resin was then washed and eluted with wash solution containing 4.5 mg/ml $SFK1_4$. Both wash fractions ("Wash") and the elution fraction ("Elution") were run on a 15% SDS-PAGE gel and visualized by silver staining. For microsequencing, the elution fraction was dialyzed and concentrated 10-fold before SDS-PAGE. A 30 kDa band was excised from the gel, microsequenced, and identified as Por1p.

potential ($\Delta\Psi_p$). We believe that SFK1 hyperpolarizes the mitochondrial membrane (rather than the plasma membrane) for three reasons. Firstly, CCCP and, more importantly, NaN_3 can block the ability of SFK1 to hyperpolarize the cell. Secondly, SFK1 treatment does not significantly affect the initial rate of uptake of [^{14}C]methylammonium, an independent indicator of $\Delta\Psi_p$ (data not shown). Lastly, while SFK1 improves growth in high NaCl/FK506, hyperpolarization of the plasma membrane is thought to sensitize yeast to sodium stress by encouraging the influx of sodium and other cations. Hence, if SFK1 were able to hyperpolarize the plasma membrane, it would not be consistent with its ability to improve growth in high NaCl/FK506.

Systematic Screening of the Set of Viable Haploid Yeast Deletion Mutants for Sensitivity to SFK1

The set of deletion mutants generated by the *Saccharomyces* Genome Deletion Project offers a rapid means of characterizing the chemical genetic interactions of a small molecule on a genomic scale. The deletion mutants can be screened for strains that show selective sensitivity or resistance to a small molecule in order to characterize the genetic interactions between the target of the small molecule and the rest of the genome. This technique has been used successfully to characterize the small molecule rapamycin, an immunosuppressant that is known to target the target of rapamycin (TOR) proteins [23]. We screened the set of deletion strains for mutants that show selective sensitivity to SFK1. Strains were pinned into 96-well plates containing YPD, and SFK1 was added at 5 μM, a concentration that is growth inhibitory but not lethal. The corresponding control experiment was also performed in which vehicle (DMSO) was added instead of SFK1. The optical density (OD_{600}) of the cultures was recorded after 1 day of growth at 30°C, and the ratio of OD_{600}(SFK1) to OD_{600}(DMSO) for each strain was taken. Any strain for

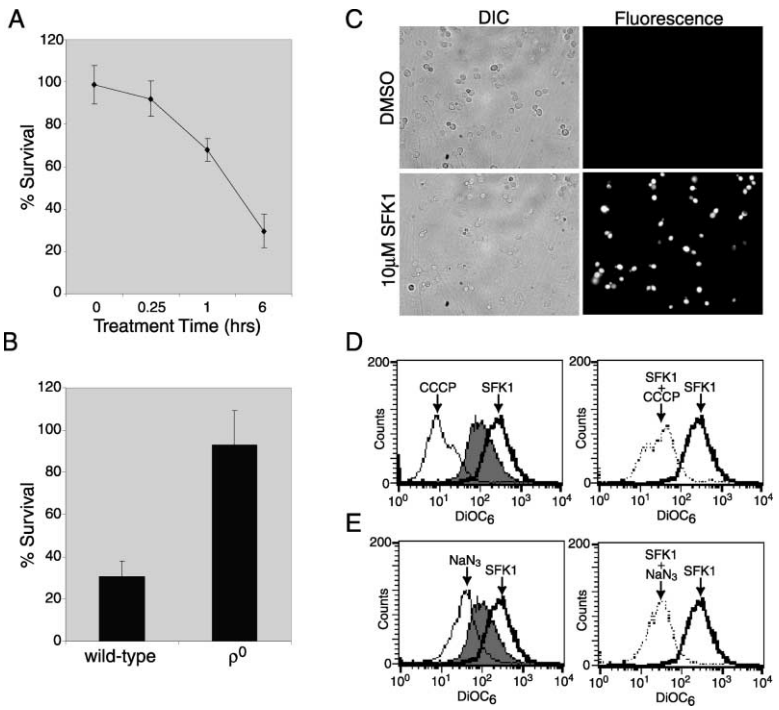


Figure 4. SFK1 Causes a Mitochondrially Induced Death

(A) Time course of SFK1-mediated death of wild-type yeast upon treatment with 10 μM SFK1.

(B) Percent survival of wild-type and ρ^0 yeast after treatment with 10 μM SFK1 for 6 hr.

(C) Visualization of ROS accumulation using H_2DCF-DA in wild-type yeast treated with either vehicle (DMSO) or 10 μM SFK1 for 1 hr.

(D) Use of $DiOC_6$ to monitor $\Delta\Psi_m$ of wild-type yeast upon treatment with either DMSO (filled), 50 μM CCCP (thin line), 5 μM SFK1 (thick line), or 5 μM SFK1 plus 50 μM CCCP (dotted line).

(E) Use of $DiOC_6$ to monitor $\Delta\Psi_m$ of wild-type yeast upon treatment with either DMSO (filled), 4 mM NaN_3 (thin line), 5 μM SFK1 (thick line), or 5 μM SFK1 plus 4 mM NaN_3 (dotted line).

Mitochondrial Function:
SOD2
YDL119C
TOM37
TCM62

Potassium Transport:
*TRK1**
*HAL5**

Aromatic Amino Acid Biosynthesis:
*TRP1**
*TRP2**
*TRP3**
TRP5
*ARO2**

Pentose Phosphate Cycle:
*TKL1**
RPE1

Chromatin/Transcriptional Regulation:
NUT1
NGG1
CSE2
*TOP1**
SPT4

Drug Resistance:
PDR1
PDR5
CYB5
SSZ1
ERG5

Other Genes:
DBF2
PTC1
RAM1
LSM1
PHO86
NBP2
YDL118W
*YDR008C**
YEL059W
YGR064W
YIL105C
*YLL033W**

Figure 5. Haploid Yeast Deletion Strains that Show Hypersensitivity to SFK1

Approximately 4700 viable haploid yeast deletion strains were pinned into 96-well plates and grown overnight in the presence of either DMSO or 5 μM SFK1. For a given strain, the ratio of growth in the presence and in the absence of SFK1 was taken. Those strains whose ratio was greater than two standard deviations below the mean were assembled in a smaller set of 96-well plates for retesting. The growth experiment was repeated three times for these condensed plates. If, for a given strain, the geometric average of the three ratios was less than 0.30, that strain was listed above as showing synthetic sensitivity to SFK1. An asterisk (*) indicates that the gene has been implicated in the response to sodium chloride stress.

which the ratio was greater than two standard deviations from the mean was then retested for selective sensitivity to SFK1.

The SFK1-sensitive deletion strains are listed in Figure 5. Several deletion strains with impaired mitochondrial function are selectively sensitive to SFK1 treatment. Consistent with the ability of SFK1 to stimulate ROS production, a strain deleted for Sod2, the mitochondrial form of superoxide dismutase (SOD), is highly sensitive to SFK1 treatment [24]. A strain deleted for Ydl119c, a member of the mitochondrial carrier family of membrane transporters, which are involved in the transport of a number of different metabolites across the inner mitochondrial membrane [25, 26], is sensitive to SFK1. A strain deleted for Tom37, a part of the TOM complex that is involved in the transport of proteins across the outer mitochondrial membrane [27], and a strain deleted for Tcm62, a chaperone-like protein that is required for the assembly of the mitochondrial succinate dehydrogenase complex [28], also show sensitivity to SFK1.

Several deletion strains with impaired ion homeostasis were also shown to be selectively sensitive to SFK1 treatment. A strain deleted for Trk1, a potassium transporter in the plasma membrane, and a strain deleted for Hal5, a positive regulator of Trk1 [16, 29], are strongly sensitive to SFK1. Trk1 and the homologous Trk2 form the high-affinity potassium uptake system in yeast [30]. The fact that a Δ*trk1* strain is sensitive to SFK1 suggests that SFK1 influences potassium transport. Interestingly, at sublethal concentrations, SFK1 is able to markedly improve growth in the presence of valinomycin, a small molecule that dissipates potassium gradients, independently confirming that SFK1 is involved in potassium transport. Valinomycin does not, however, affect the ability of SFK1 to improve growth on high NaCl plus FK506 (data not shown).

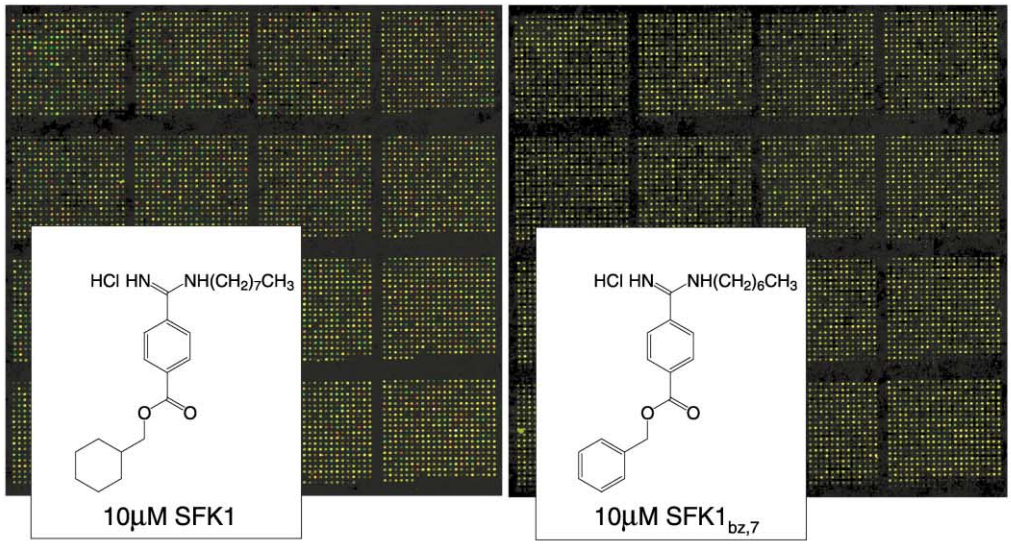
Strains deleted in genes either directly or indirectly involved in aromatic amino acid biosynthesis, including Tkl1, Aro2, Trp1, Trp2, Trp3, and Trp5 [31, 32], are also sensitive to SFK1. Although it is unclear why these strains are sensitive SFK1, a number of them are also selectively sensitive to sodium chloride stress. As expected, several strains deleted in genes involved in general drug resistance are also sensitive to SFK1 treatment. Pdr1, Pdr5, and Ssz1 are all involved in the pleiotropic drug response [33, 34]. Cyb5, which is involved in the catalytic cycle of cytochrome P450 [35], and Erg5, which is a cytochrome P450 sterol 22-desaturase [36], may also play a role in drug resistance.

Transcriptional Profiling of SFK1 Suggests that SFK1 Affects Mitochondrial Function

In order to determine whether SFK1 treatment affects gene expression, we used DNA microarrays to profile the transcriptional changes that occur when wild-type cells are treated with SFK1. Treatment with SFK1 results in a very dynamic transcriptional response, with 719 upregulated genes and 700 downregulated genes (Figure 6A). In agreement with its reduced activity, treatment with the analog $SFK1_{bz,7}$ results in a weak transcriptional response, with only 15 upregulated genes and 5 downregulated genes (Figure 6A). SFK1 stimulates the transcription of a number of calcineurin-target genes, including *ENA1*, *GSC2*, *PMC1*, and *PMR1* [7] (Figure 6B). SFK1 is not able to upregulate these genes in a calcineurin-deleted (Δ*cna1* Δ*cna2*) strain, suggesting that SFK1 stimulates the transcription of these genes via calcineurin. Furthermore, SFK1 does not increase the level of *ENA1* transcription in calcineurin-deleted yeast treated with 0.7 M NaCl (Figure 6C). These results suggest that the ability of SFK1 to improve the growth of calcineurin-deleted yeast in high NaCl cannot be explained by increased *ENA1* expression.

To analyze the expression changes that occur as a result of SFK1 treatment under conditions where it improves growth, we used a vector-based analysis [37, 38] to compare the profiles of calcineurin-deleted yeast treated with 0.7 M NaCl and of calcineurin-deleted yeast treated with 0.7 M NaCl and SFK1 (Figure 6D). We compared the expression changes in the two profiles both globally and within select functional gene categories. Categories were defined according to the Munich Information Center for Protein Sequences (MIPS) and were chosen because they were highly modulated transcriptionally by treatment with 0.7 M NaCl. In this analysis the overall angle between the two profiles (26.0°) suggests that the two profiles are quite similar. That is, if a given gene is induced in one profile, it is likely induced in the other, and similarly, if a given gene is repressed in one profile, it is likely repressed in the other. The overall magnitude between the two profiles (1.0) also suggests that the magnitude of the expression changes is quite similar between the two profiles. How-

A



B

Strain:	wild-type	Δ*cna1* Δ*cna2*
Treatment:	5μM SFK1	5μM SFK1
ENA1	2.9	1.3
GSC2	2.6	-1.5
PMC1	2.8	1.2
PMR1	2.1	1.2

C

Strain:	Δ*cna1* Δ*cna2*	Δ*cna1* Δ*cna2*
Treatment:	0.7M NaCl + DMSO	0.7M NaCl + 5μM SFK1
ENA1	14.8	8.4
GSC2	-1.8	-1.4
PMC1	3.2	1.9
PMR1	1.1	1.2

D

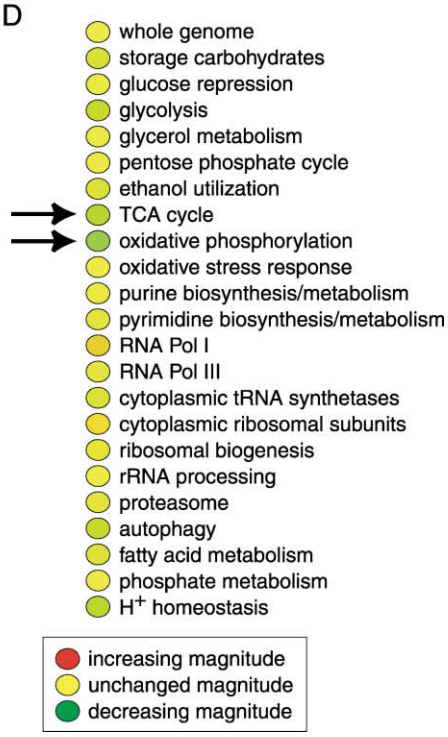


Figure 6. Transcripitional Profiling of SFK1

(A) Hybridized, spotted DNA microarrays indicating the transcriptional response of wild-type yeast to treatment with either 10 μM SFK1 or 10 μM $SFK1_{bz,7}$. SFK1 treatment results in a much more dramatic transcriptional response (719 genes upregulated, 700 downregulated) than $SFK1_{bz,7}$ treatment (15 genes upregulated, 5 downregulated).

(B) Induction of calcineurin-target genes by 5 μM SFK1 in wild-type and Δ*cna1* Δ*cna2* yeast. A similar pattern was observed when the profiles were performed using 10 μM SFK1.

(C) Induction of calcineurin-target genes in Δ*cna1* Δ*cna2* yeast by 0.7 M NaCl plus DMSO and by 0.7 M NaCl plus 5 μM SFK1. A similar pattern was observed when the profiles were repeated.

(D) Using a vector-based analysis, the transcriptional profiles of Δ*cna1* Δ*cna2* yeast treated with 0.7 M NaCl plus DMSO and of Δ*cna1* Δ*cna2* yeast treated with 0.7 M NaCl plus 5 μM SFK1 were compared both globally and within select gene categories. Red indicates that SFK1 increased the magnitude of gene expression changes induced by NaCl, yellow indicates that SFK1 did not affect the magnitude, and green indicates that SFK1 decreased the magnitude. Arrows point to two categories, TCA cycle and oxidative phosphorylation, in which SFK1 selectively suppressed the gene expression induced by NaCl. A similar pattern was observed when the profiles were repeated.

ever, SFK1 does modulate the magnitude of specific categories. For example, oxidative phosphorylation genes are highly upregulated in calcineurin-deleted yeast treated with 0.7 M NaCl, but the genes are less upregulated in calcineurin-deleted yeast treated with 0.7 M NaCl plus SFK1. SFK1 targeting of the mitochondria is reflected in its inhibition of the transcription of genes associated with mitochondrial functions, specifically the TCA cycle and oxidative phosphorylation.

Discussion

One of the greatest barriers to the development of small molecules tools for the dissection of biological processes is the lack of general and efficient methods to identify the targets of those small molecules. Many tools exist in yeast for the rapid characterization of the activities of small molecules, including the identification of chemical genetic interactions via the yeast deletion set and genome-wide expression profiling. Here, we have used these tools to characterize a small molecule, SFK1, which interacts biochemically with Por1p, a marker protein for the OMM. The biochemical interaction of SFK1 with Por1p may indicate one of several possibilities. First, it is possible that Por1p is a direct and relevant cellular target of SFK1. Second, SFK1 may possibly bind a complex that contains both its biologically relevant target as well as Por1p. In this scenario, it is also possible that Por1p may be present in that complex of proteins with a high stoichometric ratio and, thus, would be the most predominant protein isolated in the biochemical experiment. A third possibility is that SFK1 may be sequestered in mitochondrial membranes, and hence, the biochemical experiment with SFK1 might bring down mitochondrial membrane fragments that contain Por1p, as well as other proteins. The high levels of Por1p in the OMM might then make it the most abundant protein isolated. SFK1's biochemical association with Por1p suggests that SFK1 may at least in part be localized to the mitochondria, and it suggests that SFK1's modulation of the ionic balance of the cell may possibly occur via the mitochondria. Under low-salt conditions, SFK1 induces a mitochondrially mediated death that is accompanied by the release of ROS. In the presence of either KCl or NaCl, SFK1 does not inhibit growth. Finally, under conditions of high NaCl plus FK506, SFK1 actually improves growth.

One possible direct protein target for SFK1 (other than Por1p) would be an ion channel/transporter (or a regulator of such a channel/transporter) in the mitochondria that is involved in the transport of sodium and/or potassium. In support of this hypothesis, moderate amounts of either NaCl or KCl rescue cells from SFK1-mediated growth inhibition. Trk1-deleted and Hal5-deleted cells, which have an impaired potassium import mechanism, are selectively sensitive to SFK1. Furthermore, at sublethal concentrations, SFK1 markedly improves growth in the presence of valinomycin. Hence, SFK1 may be involved in creating the potassium gradients that are dissipated by valinomycin. Consistent with its ability to build up potassium gradients, SFK1 induces a hyperpolarization of the inner mitochondrial membrane. However, because valinomycin does not affect the ability of SFK1 to improve growth of yeast in high NaCl plus FK506, it is unlikely that SFK1 only affects the transport of potassium. Given that many of the same transporters that are involved in Na^+ transport are also involved in K^+ transport, SFK1 may influence the balance of both cations by targeting the same transporter. By inhibiting a Na^+/K^+ transporter, for example, SFK1 might simultaneously inhibit growth in low potassium (a nontoxic salt required by the cell), but improve growth in the presence of sodium (a toxic salt).

Our preliminary results suggest that SFK1 reduces the overall level of sodium in the cell, either by preventing sodium uptake or promoting sodium extrusion (data not shown). These results provide an explanation of how SFK1 improves growth on high NaCl plus FK506. Much of our evidence thus far suggests that SFK1 targets a transporter/channel in the mitochondria. In order to incorporate these new results into our current model, one could envision two possibilities. Perhaps SFK1 targets a transporter/channel that is present in both the mitochondria and the plasma membrane and, hence, influences the transport of ions across both membranes. Another less probable possibility is that SFK1 targets the mitochondria and that a signal is transmitted from the mitochondria to the plasma membrane, resulting in changes in ion fluxes at that membrane. To elucidate further the molecular target(s) of SFK1, it will be necessary to perform further phenotypic characterization, such as further characterization of SFK1's effects on sodium and potassium flux, as well as genetic screens looking for genes that when overexpressed suppress the ability of SFK1 to cause cell death.

By creating an imbalance in ion homeostasis, SFK1 causes death via the mitochondria. This death shares several characteristics with programmed cell death (PCD), in that it requires mitochondrial function and is associated with ROS production. Yeast are known to undergo PCD in response to a number of stresses such as H_2O_2 [39], aging [40], acetic acid [41], and high levels of mating factor [19], as well as the expression of the proapoptotic mammalian protein Bax [42–44]. Although it is unclear why a unicellular organism like yeast might actively undergo PCD, it has been suggested that PCD is an altruistic action that benefits the population as a whole by removing unfit individuals. Yeast lack much of the machinery that is associated with mammalian apoptosis, such as members of the Bcl-2 family, but were recently shown to possess a caspase [45]. Intriguingly, calcineurin has been implicated in PCD in yeast. The calmoldulin-calcineurin pathway has been shown to oppose mating factor-induced PCD [19]. Recently, it was also suggested that NaCl causes PCD in yeast as a result of ion disequilibrium and that calcineurin opposes this death [46]. It remains to be seen, however, whether this NaCl-induced death possesses all of the requisite markers of PCD.

Many of the yeast deletion strains that show hypersensitivity to SFK1 have been implicated in cell death in other contexts. Yeast deleted for the mitochondrial form of SOD, Sod2, are hypersensitive to SFK1, suggesting that Sod2 protects the cell from the SFK1-induced ROS production. In mammalian cells, overexpression of the mitochondrial form of SOD has been shown to prevent apoptosis, at least in part by preventing ROS accumulation [47, 48]. Yeast deleted for the TOM complex member Tom37 are hypersensitive to SFK1. Tom37 is homologous to the mammalian metaxin gene, which is located in the OMM

and which has been implicated in tumor necrosis factor-mediated death [49]. Lastly, yeast deleted for the succinate dehydrogenase chaperone, Tcm62, are sensitive to SFK1 treatment. Interestingly, Tcm62 was isolated in a screen for yeast mutants with a defect in growth on a nonfermentable carbon source that is corrected by the expression of the antiapoptotic Bcl-x_L. Bcl-x_L is thought to facilitate the transition from fermentative to nonfermentative growth in yeast. Tcm62 was also shown to protect mammalian cells from death upon growth factor withdrawal [50]. In the future, it will be interesting to explore whether SFK1 has any effect in higher organisms, in particular whether it has any effect on mitochondrial function, ROS production, or cell death.

Several intriguing similarities exist between SFK1-mediated death and Bax-mediated PCD in yeast. Bax-mediated PCD is thought to require respiring mitochondria. Just as ρ^0 cells are resistant to SFK1-mediated death, they are resistant to Bax-mediated death [43, 44]. Although VDAC, in association with Bax, has been shown to play a role in apoptosis in mammalian cells [51], the VDAC proteins (Por1p/Por2p) are not required for Bax-mediated death in yeast [43, 44], just as they are not required for SFK1-mediated death. Similar to SFK1 treatment, Bax expression results in a prolonged hyperpolarization of the mitochondrial membrane as measured by $DiOC_6$, even in ρ^0 cells [44]. Also similar to SFK1 treatment, Bax expression results in increased production of ROS [44]. Because NaCl and KCl, but not sorbitol, rescue cells from SFK1-mediated death, mitochondrially mediated death in yeast may be a response to ionic rather than osmotic stresses. Interestingly, expression of the antiapoptotic mammalian protein Bcl-2 in yeast has recently been shown to improve growth of calcineurin-deleted yeast in high NaCl, but not to improve growth in high sorbitol [46]. This result further links the mitochondria and salt stress and suggests that calcineurin plays a role in mediating this connection.

Significance

We identify a small molecule suppressor of FK506 (SFK1) that improves growth in high concentrations of NaCl in the presence of FK506, but that induces death via the mitochondria in low salt. We show that SFK1 interacts either directly or indirectly with Por1p in the OMM, suggesting that SFK1 targets the mitochondria. By screening the haploid yeast deletion strains for hypersensitivity to SFK1, we map the chemical genetic interactions of SFK1 on a genome-wide scale. These interactions provide additional evidence that SFK1 affects both mitochondrial function and ionic balance. Our data suggest that the mitochondria act as a sensor for ionic balance and implicate calcineurin as a mediator in this sensing network. Our data also suggest that mitochondrially mediated death in yeast, unlike that in mammalian cells, may be induced by ionic rather than osmotic stresses.

Experimental Procedures

Yeast Strains, Media, and Titration Experiments

The primary yeast strains used in this study were YPH499 (wild-type; *MAT***a** *ura3-52 lys2-801 ade2-101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1*) and MCY300-1 (calcineurin-deleted; *MAT***a** *ura3-52 lys2-801 ade2-101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 cna1*Δ*1::hisG cna2*Δ*1::HIS3*) obtained from Martha Cyert, Stanford University. Rich medium (YPD) contained 1% yeast extract (Difco), 2% Bacto Peptone (Difco), and 2% glucose. NaCl, KCl, and sorbitol were added as indicated. Low-pH YPD contained 50 mM succinic acid and was adjusted to pH 3.5 with HCl. All titration experiments were performed in 96-well plates for 48 hrs at 30°C.

Screening of Chemical Libraries

Chemical libraries included ChemBridge DIVERSet E (ChemBridge Corp.), National Cancer Institute Open Collection I, and compounds synthesized in-house by diversity-oriented organic synthesis, including a library of dihydropyrancarboxamides [52]. Typically, compounds were screened at 20–40 μM. 384-well plates were filled with YPD containing 1.25 × 0.7 M NaCl (40 μl per well), and compounds were robotically pinned into the media. Cultures of either YPH499 or MCY300-1 were grown at 30°C to OD_{600} of 1, diluted 20-fold in YPD, and added to the plates (10 μl per well). If wild-type yeast were used, 125 nM FK506 was included to inhibit calcineurin.

Synthesis of SFK1 Derivatives and SFK1 Resin

The synthesis of all SFK1 derivatives, except for SFK1-NHBoc (**2**), has been described previously [53, 54]. To synthesize SFK1-NHBoc (**2**), cyclohexylmethyl *p*-carbethoxyimidobenzoate (**1**) was reacted with mono-Boc-1,8-diaminooctane using a procedure similar to that of Chow et al. [54]. SFK1-NHBoc (**2**)- ^{1}H-NMR (500 MHz, $(CD_3)_2SO$): 8.15 (d, 2H, J = 9 Hz); 7.86 (d, 2H, J = 9 Hz); 6.78 (t, 1H, J = 6 Hz); 4.16 (d, 2H, J = 7 Hz); 3.38 (t, 2H, J = 7 Hz); 2.90 (dt, 2H, J = 7 Hz,7 Hz); 1.79-1.71 (m, 5H); 1.67-1.62 (m, 3H); 1.41-1.33 (s, 9H); 1.41-1.13 (m, 13H); 1.07 (q, 2H); LC-ESI-MS calculated for $(C_{28}H_{45}N_3O_4)H^+$ 488.4, found 488.5. To synthesize mono-Boc-1,8-diaminooctane, di-*tert*-butyl dicarbonate (7.4 g, 34.0 mmol) was dissolved in 50 ml MeOH/H_2O and added dropwise over 30 min to a solution of 1,8-diaminooctane (4.9 g, 34.0 mmol) in 50 ml MeOH/H_2O (1:1). The reaction was stirred overnight at room temperature. 100 ml H_2O was added, and the white precipitate that formed was removed by filtration. The filtrate was made basic (pH 12.5) with NaOH and extracted twice with ether. The ether was washed with saturated NaCl, dried with $MgSO_4$, and evaporated to give the product. SFK1-NHBoc (**2**) was deprotected by treatment with 4 M HCl in 1,4-dioxane for 1 hr to yield SFK1-NH_2 HCl. SFK1-NH_2 HCl- LC-ESI-MS calculated for $(C_{23}H_{37}N_3O_2)H^+$ 388.3, found 388.4. SFK1 resin (**3**) was made by reacting 1 ml Hi-Trap NHS-activated sepharose resin (Amersham Pharmacia Biotech) with SFK1-NH_2 HCl (10.5 mg, 20 μmol) in 3 ml 2:3 EtOH: (aq.) 0.2 M $NaHCO_3$ (pH 8.3), 0.8% Tween 20, overnight at room temperature. Blank resin was made by the same procedure using ethanolamine. The resins were capped according to the manufacturer's protocols.

Identification of SFK1-Associated Proteins

YPH499 cells (280 ml) were grown to OD_{600} ~1.2, lysed with glass beads in MIPP with 200 μM PMSF, 1 mM DTT, and 1 μg/ml leupeptin and pepstatin. The lysate was centrifuged at 12,000 rpm for 10 min. The lysate was then incubated with blank resin for 1 hr at 4°C. The cleared lysate was incubated with the SFK1 resin for 2 hr at 4°C. The resin was then washed with 5 × 2 ml of PBS with 200 mM NaCl and 40% DMSO. Bound proteins were then eluted with the same wash solution containing 4.5 mg/ml $SFK1_4$. The elution fraction was dialyzed against PBS, concentrated 10-fold by microcon, separated by 15% SDS-PAGE, and visualized by Coomassie-colloidal staining. A 30 kDa band was excised from the gel, washed in 50% acetonitrile/water, and microsequenced. Sequence analysis was performed at the Harvard Microchemistry Facility by microcapillary reverse-phase HPLC nanoelectrospray tandem mass spectrometry on a Finnigan LCQ DECA quadrupole ion trap mass spectrometer.

Yeast Cell Survival

Either YPH499 cells or ρ^0 cells (generated from YPH499 cells) were grown to OD_{600} 0.2 in YPD, centrifuged, and resuspended in YPD containing either DMSO or 10 μM SFK1. At a given time point, ~3 × 10^6 cells (based on OD_{600}) were centrifuged, washed twice with YPD, and diluted 1:400 in YPD. 75 μl of the dilution was plated on YPD-

agar, and the plates were grown for 2–3 days at 30°C. Percent survival was calculated by dividing the number of colonies from the SFK1-treated cells by the number of colonies from the DMSO-treated cells.

Assessment of ROS Production and Mitochondrial Potential

To assess ROS production, cells were grown to OD_{600} 1 in YPD, centrifuged, and resuspended in YPD containing either DMSO or 10 μM SFK1. Cells were treated for 50 min at 30°C, and then 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) was added for an additional 10 min at 30°C. Cells were imaged in the DIC and FITC channels. To assess mitochondrial potential, cells were grown to OD_{600} 1 in YPD, centrifuged, and resuspended in YPD containing either DMSO, 5 μM SFK1, 50 μM CCCP, 4 mM NaN_3, or a combination of treatments. Cells were treated for 20 min at 30°C, and then 20 nM $DiOC_6$ was added for an additional 10 min. Cells were subjected to FACS analysis.

Sensitivity of Yeast Deletion Strains to SFK1

Approximately 4700 viable haploid yeast deletion strains from the *Saccharomyces* Genome Deletion Project were pinned from stock plates using a 96-pin pinning tool into 96-well plates containing YPD (100 μl per well) and either DMSO or 5 μM SFK1. The plates were incubated overnight at 30°C. The OD_{600} of each well was read using a Spectramax Plus 384 plate reader (Molecular Devices). For a given strain, the ratio of growth in the presence and in the absence of SFK1 was taken. Those strains whose ratio of OD_{600}(SFK1) to OD_{600}(DMSO) was more than two standard deviations below the mean were collected in a smaller set of 96-well plates for retesting. The growth experiment was repeated three times for these condensed plates. If, for a given strain, the geometric average of the three growth ratios of OD_{600}(SFK1) to OD_{600}(DMSO) was less than 0.30, that strain was listed as showing synthetic sensitivity to SFK1.

Transcriptional Profiling

YPH499 cells were grown in YPD to OD_{600} 1 and treated with the indicated amount of either DMSO, SFK1, or $SFK1_{bz,7}$ for 30 min at 30°C. For NaCl treatment profiles, MCY300-1 cells were grown in YPD to OD_{600} 1, treated with H_2O or 0.7 M NaCl, using a 5 M NaCl stock, plus either DMSO or 5 μM SFK1, for 30 min at 30°C. mRNA isolation, reverse transcription, labeling, hybridization, and scanning have been described previously [55]. Vector analysis of expression profile data has also been described previously [37, 38].

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