Supplementary Data

Discovery of a small molecule targeting *IRA2* deletion in budding yeast and neurofibromin loss in malignant peripheral nerve sheath tumor cells.

Running title: A novel small molecule targeting NF1-deficient MPNST cells

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Supplemental Materials and Methods

High-Throughput Chemical Screening

Yeast cells were seeded to 384-well plates at an optical density of 0.05 in synthetic complete (SC) medium in the presence of test compounds at 18.6 μM in 3.7% DMSO. Inhibition was measured by optical density compared to DMSO control after 18 hours of incubation at 30°. Active compounds were re-screened in triplicate for confirmation. Hits were then assayed in triplicate wells at doses ranging from 0.01 μM to 200 μM for *erg6Δ* cells and 0.006 μM to 120 μM in *erg6Δ ira2Δ* cells, both in 3% DMSO and 3-fold increments. For the cell line screen, STS26T cells and T265 cells were grown in DMEM + L-Glutamine (Invitrogen) with 10% FBS (Sigma) and 1% Penicillin/Streptomycin (Fisher Scientific) at 37°C, 95% humidity, 7.5% CO_2. On day 1 of the assay, cells were harvested by trypsinization, pelleted by centrifugation, and resuspended in media, and then added to wells of a 384-well microtiter plate (CellStar tissue culture plate from Greiner). Each well received 50 μl of media containing 500 STS26T cells or 1000 T265 cells. Each microtiter plate contained only one cell type. Cells were grown at 37°C, 95% humidity, 7.5% CO_2 for 24 hours. On day two of the assay, test compound in DMSO solution was added to the appropriate wells. On day four of the assay 5 μl of resazurin (0.44 mM final) was added to each well, and cells were incubated for 4 hours. Fluorescence intensity was then measured (530 nm = λex, 580 nm = λem). Concentration in the assay was 0.025-12.65 μM for test compounds with 0.09-0.1% DMSO, depending on the screening tier. Test compounds were 10 μM for the first screening tiers. Test compounds were

0.025-12.65 μM for the dose response screens. Each plate contained controls of untreated cells, media only, and cells plus 5 μg/mL doxorubicin (Sigma-Aldrich), a known inhibitor. Fluorescence intensity measurements were normalized to untreated cells (maximum growth) and media only (minimum growth) on each microtiter plate. UC1 for laboratory studies was obtained from Chembridge Corporation.

Yeast RNA Extraction

5-10 x 10^6 cells were pelleted and suspended in 300 μL TriReagent and transferred to 1.5 mL screw-cap tubes containing 100-200 μL glass beads. Samples were homogenized in a bead beater 4x 45 seconds with 1 minute on ice in between. Extracts were transferred to 1.5 mL tubes, incubated 5' RT, then centrifuged 12,000 RCF 10' 4°. Clarified extracts were transferred to a fresh tube. 30 μL chloroform was added, tubes were shaken by hand for 15 seconds, then incubated 2' RT. Tubes were centrifuged 12,000 RCF 15' 4°. The aqueous layer (~100 μL) was transferred to a fresh tube. 150 μL RT isopropanol was added, samples were mixed by pipetting, and incubated for 10' RT. RNA was pelleted by centrifugation at 12,000 RCF 10' 4°. Pellets were washed with 300 μL 70% ethanol in DEPC-treated water, spun at 7,500 RCF 5' 4°, then ethanol was removed and pellets were air-dried for 10 minutes. RNA was resuspended by pipetting up and down with 45 μL DEPC-H2O then incubating at 50°-55° for 10'.

Supplemental Figure Legends

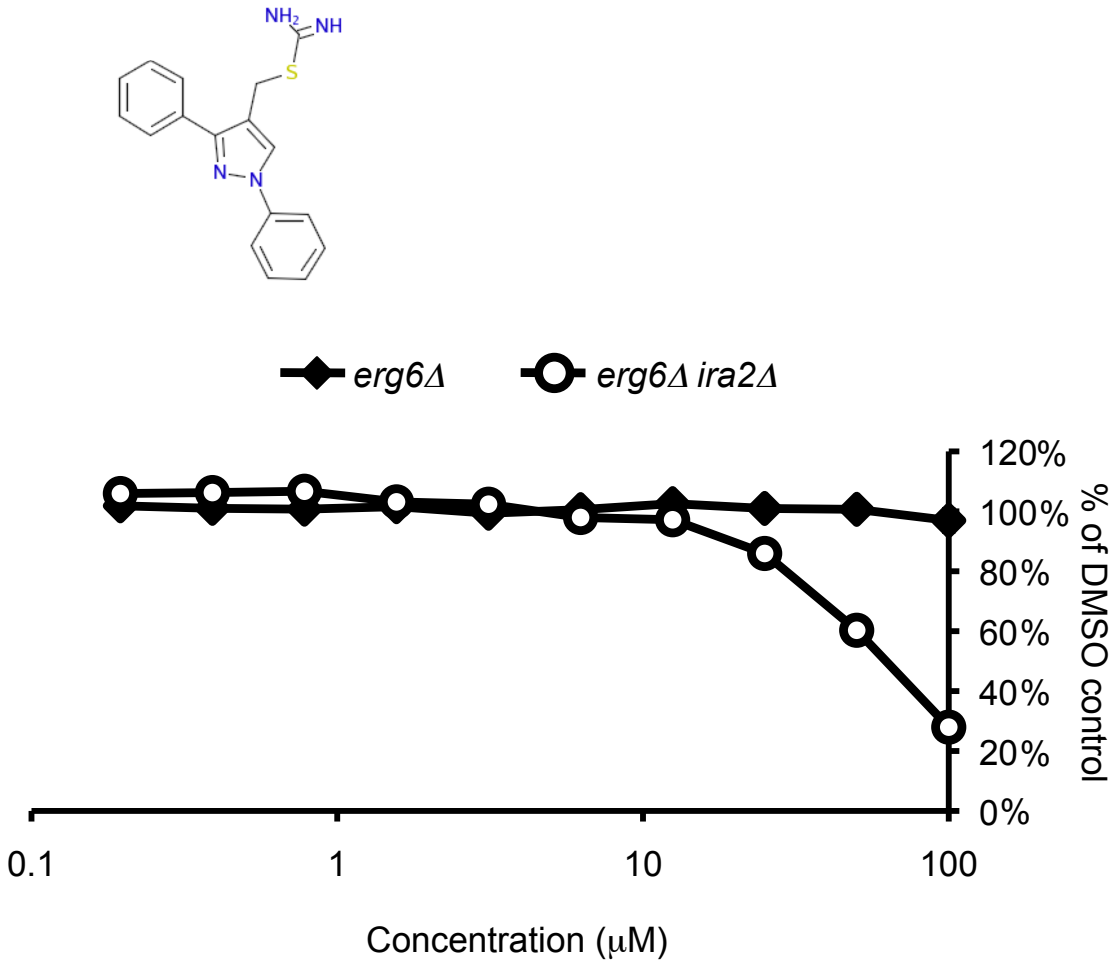
Supplemental Figure 1 – Analysis of a UC1-like compound. A 96-well plate assay was performed on a compound with similar structure to UC1, except lacking a bromine at the meta position. The assay was performed on three independent cultures of *erg6Δ* and *erg6Δ ira2Δ*, with growth measured after 18 hours of incubation as in UC1 assays. Values are the mean % of DMSO for three cultures. Error bars are obscured by the data points.

Supplemental Figure 2 – Plasmid shuffle system for high-efficiency transformation of *erg6Δ ira2Δ* yeast cells. The "library background" strain bears genomic deletion of *ERG6* and *IRA2* using the *HIS3* and *URA3* markers, respectively, with the *URA3* marker reverted by selection on 5-FOA medium. The strain has *leu2-3* and *ura3-52* markers available for plasmid selection. Construct pMW001, a *URA3* plasmid containing the *ERG6* gene, was introduced by electroporation and enables high-efficiency transformation with the *LEU2*-marked YEp13 yeast genomic library. Selection on C-Leu 5-FOA isolates only those cells containing a library (*LEU2*) plasmid and lacking the *ERG6 (URA3)* plasmid. When selection on UC1 agar, cells that are resistant will form colonies. The high-copy suppressor background MDW230 was generated by back-crossing the *leu2-3* and *his3-11* auxotrophic markers to the Σ1278b genetic background containing 5-FOA selected *ira2Δ*. *ERG6* was then deleted by one-step replacement with *HIS3*. The *pRS416-ERG6* rescue plasmid was generated

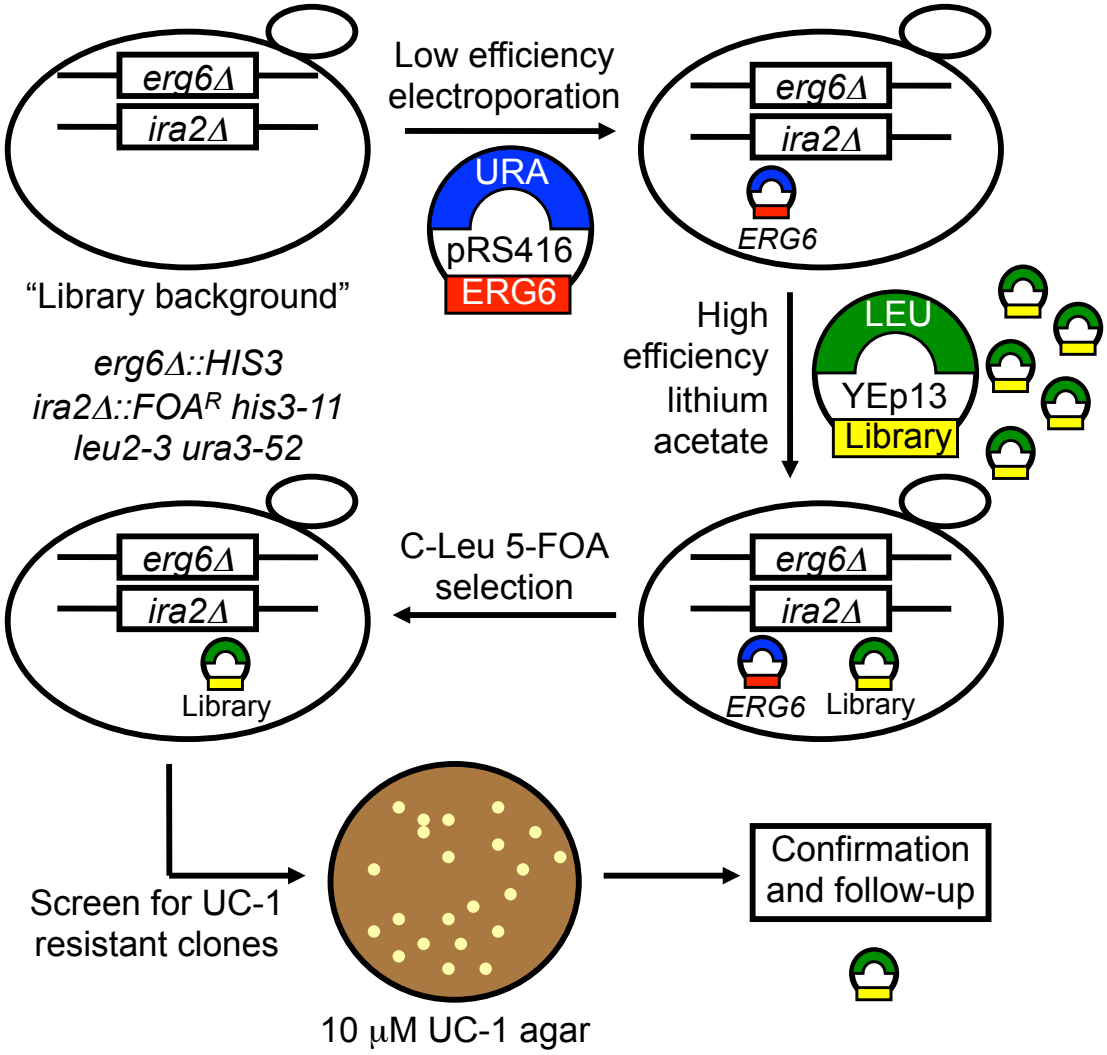
by PCR of the *ERG6* gene from genomic DNA followed by insertion into the SmaI restriction site of pRS416. The plasmid was introduced by electroporation as described in (42), except DTT was omitted in the preincubation, and cells were plated to selective media without sorbitol. Library plasmids were then introduced to MDW230 by chemical transformation (43). Transformants were selected on C-Leu, and the *ERG6* rescue plasmid was counter-selected on C-Leu 5-FOA. Colonies were pooled and plated onto 10 μM UC1 to select for resistant colonies.

Supplemental Figure 3 – Phenotypic screening of UC1-resistant colonies. Library transformants that grew on 10 μM UC1 were suspended in SC medium and dropped to a series of plates. Plates were incubated for three days at 30° before classifying colonies based on their growth patterns, as indicated in the schematic diagram. Strains with low PKA activity stain positive with iodine vapor. These transformants typically had regained PKA regulation by a plasmid containing the phosphodiesterase *PDE2* which lowers cAMP levels. Deletion of *ERG6* confers resistance to the antifungal agent Nystatin. Transformants that became UC1-resistant by regaining *ERG6* were identified by Nystatin sensitivity. Suppressing constructs that were not specific for UC1 were identified by cross-resistance to a structurally unrelated compound (CmpdY).

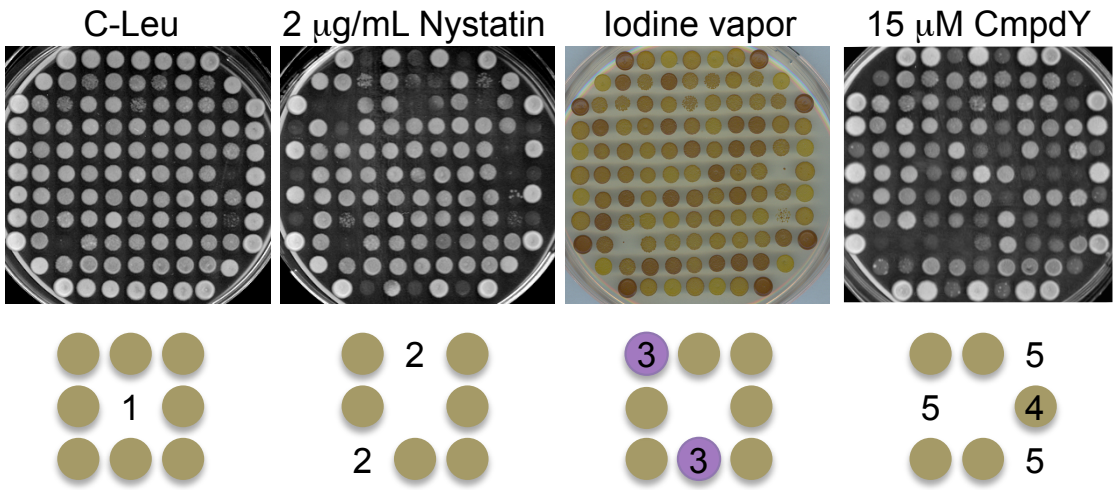
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



1 – Failure on C-Leu: spontaneously resistant colony, no plasmid.
2 – Failure on Nystatin: colony regained *ERG6.*
3 – Positive iodine vapor: colony restored PKA regulation.
4 – Growth on CmpdY: colony acquired a non-specific suppressor.
5 – Leu+, Nystatin^{R}, Iodine-, CmpdY^{S}: Candidate UC1 suppressor.