A small molecule screen reveals the essentiality of p38 kinase for choanoflagellate cell proliferation

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Author Contributions: F.U.N.R., A.C.D., and N.K. designed research; F.U.N.R. performed choanoflagellate research and A.P.S. synthesized sorafenib analogs; F.U.N.R., A.C.D., and N.K. analyzed data; and F.U.N.R., and N.K. wrote the paper.

Competing Interest Statement: None.

Classification: Biological Sciences – Biochemistry & Evolution

Keywords: p38 kinase, choanoflagellate, phenotypic screening, kinase enrichment, environmental stress response

Abstract

Choanoflagellates, the closest living relatives of animals, express diverse animal genes and may thereby provide insights into the ancestral roles of genes essential for modern animal cell biology and development. While efforts to study conserved gene families in choanoflagellates have been constrained by the relative inefficiency of currently available genetic tools, small molecule approaches are readily available and may provide a complementary and scalable approach for studying protein function. To study the physiological roles of choanoflagellate kinases, including animal kinase homologs, we established a high-throughput platform to screen the model choanoflagellate *Salpingoeca rosetta* with a curated library of human kinase inhibitors. We identified 36 diverse kinase inhibitors that disrupt *S. rosetta* cell proliferation. By exploring structure-activity relationships of one inhibitor, sorafenib, we identified a p38 kinase as a stress-activated regulator of cell proliferation in *S. rosetta*. This finding indicates a conserved p38 function between choanoflagellates, animals and fungi. Moreover, this study demonstrates that existing kinase inhibitors can serve as powerful tools to examine the ancestral roles of kinases that regulate modern animal development.

Significance Statement

Choanoflagellates are the closest living relatives of animals and are useful models for studying the ancestral functions of animal gene families, including kinases. In this study, we treated choanoflagellates with a library of small molecules to determine if currently available human kinase inhibitors could reveal choanoflagellate kinase function. This approach highlighted the essentiality of a specific kinase that is conserved between animals and choanoflagellates and provides tractable options for investigating protein functions in other emerging model systems with limited genetic tools.

Main Text

45 **Introduction**

Phenotypic screens with libraries of small molecules have revolutionized cell biology by providing chemical tools to study protein function (1–4). These screening efforts, which have largely focused on discovering novel therapeutics for human disease, have made available large libraries of small molecules (5) and diverse high-throughput
50 technologies for assaying phenotypes in cells and organisms (6–8). Despite the availability of these chemical tools and platforms, relatively few efforts outside of drug discovery have repurposed phenotypic screening to identify chemical probes.

We sought to test whether small molecules could serve as useful tools to study kinase function in choanoflagellates, the closest living relatives of animals.
55 Choanoflagellates possess homologs of diverse animal kinases (Figure S1) (9–11) and due to their phylogenetic placement are relevant for reconstructing the ancestral functions of animal cell signaling proteins (12, 13). Whether these predicted kinase homologs in choanoflagellates contribute to conserved physiological functions is not known.

60 Kinase signaling has profound effects on animal cell growth, proliferation, differentiation, and migration (14, 15). Kinases signal by catalyzing the phosphorylation of specific substrates, serving as protein scaffolds, and altering protein-protein interactions (16). Because aberrant kinase activity leads to human disease (14, 15), many tools to inhibit kinase activity and detect protein phosphorylation have been
65 developed. These tools may also allow the study of choanoflagellate kinases, which have conserved features within the kinase active site (9, 11). Although targeted genome

editing has been recently established in choanoflagellates (17), we inferred that the unique strengths of small molecules, including precise temporal control and discrete inhibition of enzymatic activity (18), might more readily reveal the physiological relevance of kinases, including those with essential functions, in choanoflagellates. Indeed, a previous study showed that two broad-spectrum kinase inhibitors disrupt cell proliferation in the choanoflagellate *Monosiga brevicollis* (19), but did not demonstrate whether kinases were directly targeted or identify the specific proteins involved.

Using a library of well-characterized kinase inhibitors that vary in their human kinase inhibition profile, we treated cultures of *Salpingoeca rosetta*, a model choanoflagellate, in a multiwell format. We found that treatment of *S. rosetta* cultures with a set of selective kinase inhibitors disrupted cell proliferation and led to global inhibition of *S. rosetta* phosphotyrosine signaling. Using the kinase inhibitor sorafenib as a probe and inactive analogs as negative controls, we found that a p38 kinase homolog is activated by environmental stressors and is an essential regulator of cell proliferation in *S. rosetta*.

Results

Screening of a human kinase inhibitor library reveals small molecules that inhibit* S. rosetta *kinase signaling and cell proliferation

To investigate whether kinase activity could regulate *S. rosetta* cell proliferation, we treated *S. rosetta* cultures with characterized human kinase inhibitors. Because the highest conservation between choanoflagellate and human kinases occurs in the kinase

90 domain (9–11), we focused on kinase inhibitors that bind in the kinase active site. As a proof-of-concept, we first assayed staurosporine, a well-characterized broad-spectrum kinase inhibitor and inducer of cell death in diverse organisms (20, 21), by flow cytometry analysis of treated *S. rosetta* in multiwell plates. Staurosporine significantly and reproducibly reduced *S. rosetta* cell density (Figure S2A-C) and tyrosine
95 phosphorylation in a dose-dependent manner (Figure S2C-D), which motivated us to expand our study to a screen of 1383 diverse compounds. Compounds in the library were curated to target diverse human kinases with varying coverage (Figure S1), and we included a few previously studied antimicrobials (e.g. genistein, rotenone) (19, 22) to evaluate the relative significance of kinase inhibitor-mediated disruption of *S. rosetta* cell
100 proliferation.

We screened *S. rosetta* cultures with each of the 1383 small molecules in the library at 10 μM (Figure 1A, Table S1) and measured cell density at a 24-hour endpoint. This screen revealed 36 compounds (Figure 1A and Figure S3) that significantly decreased *S. rosetta* cell counts compared to vehicle (DMSO) control. We uncovered
105 inhibitors of *S. rosetta* cell proliferation that included ATP-competitive inhibitors of human protein kinases (e.g. tyrosine kinases, serine-threonine kinases) and lipid kinases (Figure S4A-C). Each compound varied in the diversity of human kinases that they target (Figure S4D), with some identified molecules being very selective (e.g. AT7867, everolimus, GSK461364) (Figure S4E). We also identified inhibitors of *S. rosetta* cell
110 proliferation that disrupt human kinase signaling indirectly, i.e. without binding to a kinase (Figure S3). For example, genistein, a natural product that indirectly alters kinase activity (23, 24), inhibited *S. rosetta* cell proliferation and was previously shown to inhibit the growth of the choanoflagellate *M. brevicollis* (19).

115 ***Inhibitors of* S. rosetta *cell proliferation also disrupt* S. rosetta *phosphotyrosine signaling***

Because our initial screen was designed to measure *S. rosetta* cell number at the 24-hour treatment endpoint and did not directly assess inhibition of kinase activity, we further monitored *S. rosetta* growth in response to compound treatment and used
120 biochemical methods to determine if *S. rosetta* cultures treated with the identified human kinase inhibitors showed decreased kinase signaling.

Antibodies that distinguish phosphotyrosine from non-phosphorylated tyrosine are widely used to monitor kinase activity in diverse organisms (19, 25–28) and have been used to detect tyrosine phosphorylation by heterogously expressed
125 choanoflagellate kinases (29–32). (Antibodies that recognize global phosphoserine or phosphothreonine are less often used because they lack the specificity and sensitivity needed to monitor changes in serine or threonine phosphorylation (26–28).) Because choanoflagellates have diverse tyrosine kinases with uncharacterized functions (9, 10, 33) and we identified inhibitors of tyrosine kinases that are used treat human disease
130 (34–36), we triaged our set of 36 identified molecules to focus on the 15 compounds that inhibit human tyrosine kinases (e.g. tyrosine kinase inhibitors, multi-class targeting kinase inhibitors) (Figure S3).

We focused our attention on three inhibitors -- glesatinib, masitinib and PP121 -- each of which have a narrower range of kinase targets than staurosporine (35–38). In
135 humans, glesatinib, masitinib and PP121 inhibit select receptor tyrosine kinases (RTKs); masitinib and PP121 also inhibit Src kinase and PP121 additionally inhibits PI3K (35, 36,

38). Although the extracellular domains of RTKs targeted by glesatinib, masitinib, and PP121 in humans (35, 36, 38) are not conserved in *S. rosetta*, specific residues shared in the kinase domains of human and choanoflagellate tyrosine kinases suggest that these inhibitors might still be effective in inhibiting choanoflagellate tyrosine kinases. For example, choanoflagellate tyrosine kinases have residues within the predicted active site that are necessary for kinase activity in other organisms (e.g. “K” in VAIK, “D” in HRD and DFG) (Figure S5A) (9, 39–41) and additional residues that confer sensitivity towards tyrosine kinase inhibitors (Figure S5B-D) (37, 42).

Of the three inhibitors tested, *S. rosetta* cultures were most sensitive to glesatinib. Although 1 μM masitinib and PP121 were sufficient to reduce *S. rosetta* cell proliferation over the first 40 hours of treatment (Figure 1B), masitinib- and PP121-treated cultures recovered within 85 hours. In contrast, glesatinib inhibited cell proliferation at 1 μM and 10 μM (Figure 1C) and throughout the 85-hour growth experiment at 1 μM (Figure 1B) by inducing cell lysis (Movie S1). Treatment of *S. rosetta* cultures with 1 μM glesatinib led to a global decrease in phosphotyrosine signal, while treatment with 1 μM masitinib or PP121 did not decrease phosphotyrosine levels as detected by western blot (Figure 1D).

These findings showed that glesatinib disrupts both cell proliferation and tyrosine kinase signaling. Together, these observations provide independent support for the hypothesis that kinase signaling regulates cell proliferation in choanoflagellates. Unfortunately, inactive analogs of glesatinib are not readily available for use in proteomic studies. Therefore, to identify specific *S. rosetta* kinases whose activity might regulate *S. rosetta* cell proliferation, we pursued further experiments to identify small molecules with

160 well-characterized structure-activity relationships in animals that could serve as useful probes in choanoflagellates.

Sorafenib inhibits* S. rosetta *phosphotyrosine signaling and cell proliferation

To select a small molecule that could help us identify specific *S. rosetta* kinases
165 that regulate *S. rosetta* cell proliferation, we tested a panel of 18 human TK inhibitors (Figure 2A, B) that have available inactive analogs and that share overlapping kinase targets with glesatinib. Of the 18 compounds tested, treatment with five different small molecules (sorafenib, regorafenib, AD80, milciclib, and vemurafenib) led to a global decrease in phosphotyrosine staining (Figure 2A) but not in serine and threonine
170 phosphorylation (Figure S6) as detected by Western blot. Treatment of *S. rosetta* with four of the five compounds, regorafenib, AD80, milciclib, and vemurafenib, resulted in a mild reduction in the rate of cell proliferation (Figure 2B). In contrast, sorafenib treatment entirely blocked *S. rosetta* cell proliferation throughout the 85-hour growth experiment (Figure 2B) and induced dynamic changes in *S. rosetta* morphology (including cell
175 elongation, Movies S2 and S3) compared to cells treated with vehicle (DMSO) control (Movie S4). Further testing of sorafenib revealed that it was linked to dose-responsive inhibition of phosphotyrosine that was similarly potent to glesatinib (Figure 2C) and sorafenib also inhibited extracellular signal-regulated kinase (e.g. pERK) signaling (Figure 2D), a signaling pathway that regulates cell proliferation in fungi and animals
180 (43). Moreover, many analogs of sorafenib have been studied to reveal specific chemical features that are necessary for sorafenib's inhibition of kinase activity (44) and cell

viability (45). Therefore, we prioritized sorafenib as a tool to identify specific *S. rosetta* kinases that regulate cell proliferation.

185 ***Sorafenib binds to* S. rosetta *p38 kinase***

To identify *S. rosetta* kinases that might be inhibited by sorafenib, and therefore important for the regulation of cell proliferation, we focused on a kinase-enrichment strategy that previously has been used in animals and plants to enrich kinases out of prepared lysates (46, 47). We used ActivX ATP and ADP probes (46, 47) to covalently
190 enrich for kinases and high-affinity kinase-binders present within *S. rosetta* lysates after pretreatment with vehicle (DMSO) or sorafenib. Because sorafenib and ActivX probes competitively bind to kinase active sites, kinases that were more often enriched with DMSO pretreatment and less enriched with sorafenib pretreatment were likely sorafenib binders. We quantified absolute protein abundance (48) and focused our attention on
195 kinases that were present in both DMSO and sorafenib pretreated samples but were enriched in multiple DMSO replicates, represented by higher ProteinLynx Global Server (PLGS) protein scores (48). Through this approach, we identified a predicted *S. rosetta* p38 kinase that bound 10-fold less to ActivX probe in the presence of sorafenib (Figure 3A, Table S2). Therefore, we infer that sorafenib binds directly to *S. rosetta* p38 kinase.
200 In animals and fungi, p38 kinases regulate cell proliferation and are activated in response to environmental stressors (49–52).

Although previous studies have predicted multiple p38 kinases in *S. rosetta* (50, 53), only the p38 recovered from the ActivX binding assay (EGD79605) has the

diagnostic “TGY” motif that distinguishes p38 kinases from other related kinases in humans and yeast (Figure S7A) (54). Hereafter we refer to this protein as Sr-p38.

ActivX probes and other proteomic approaches have previously identified human p38 kinases as primary targets of sorafenib in humans (55, 56). In animals, sorafenib preferentially binds to kinases with threonine gatekeeper residues (56), including tyrosine kinases and two of four vertebrate p38 kinase paralogs with threonine gatekeepers (56) (Figure S7A). In addition to having the diagnostic “TGY” motif of p38 kinases, Sr-p38 contains a threonine gatekeeper (Figure S7A) and lysine residues necessary for sorafenib and ActivX probe binding respectively.

Sr-p38 is responsive to heat and oxidative stress

Choanoflagellates live in sun-lit water zones that undergo daily and yearly fluctuations in temperature and nutrients (57–59). Because environmental stressors activate p38 kinases in animals and fungi (49–52, 60–63), we wondered if stressors relevant to choanoflagellates would activate Sr-p38 kinase signaling. All 23 choanoflagellate species sequenced thus far (10, 13, 53) express at least one predicted p38 kinase with a conserved “TGY” motif (Figure S7B). Although a previous study identified a nutrient-sensitive protein in *M. brevicollis* with a molecular weight similar to p38 kinases (19), the identity and function of this protein were not directly studied. To investigate if Sr-p38 is activated in response to environmental stressors, we tested heat shock and oxidative stress, two environmental stressors that would be common to all choanoflagellate habitats.

When *S. rosetta* cells cultured at ambient temperature were subjected to heat shock and oxidative stress, we observed an increase in p38 phosphorylation. Upstream kinases activate p38 by phosphorylation at the “TGY” motif (54), and this phosphorylation is recognized by several phospho-specific antibodies (Figure S7C).
230 Within 30 minutes of heat shock at 37°C, the phospho-p38 signal from at least two protein species increased relative to pre-treatment (Figure 3B). We also observed an increase in signal for at least one protein using the phospho-specific p38 antibody on cell lysates that were treated with 0.5M hydrogen peroxide (Figure 3C). Thus, the connection between environmental stress and the activation of p38 appears to be conserved
235 between yeast, animals, and choanoflagellates.

Sr-p38 *kinase phosphorylation is inhibited by sorafenib and is essential for* S. rosetta *cell proliferation*

Previous studies have connected tyrosine kinase signaling and p38 kinase
240 activation in animals in response to multiple stimuli (e.g. growth factors, ultraviolet light) (49, 64, 65). Sorafenib blocks this signaling axis by inhibiting p38 and diverse tyrosine kinases that contain threonine gatekeepers (44, 56). To examine if the signaling axis between tyrosine kinases and p38 is conserved in *S. rosetta,* we tested whether sorafenib could reduce the observed phosphorylation of p38 in *S. rosetta* cultures
245 subjected to heat shock. As a control for non-kinase inhibition by sorafenib (45), we used APS6-46, an analog that shares sorafenib's non-kinase inhibitory activity (45) but does not inhibit *S. rosetta* tyrosine phosphorylation or cell division (Figure S8). *S. rosetta* cultures pretreated with sorafenib had reduced p38 phosphorylation, whereas cultures

pretreated with APS6-46 retained p38 phosphorylation comparable to the DMSO
250 (vehicle) control (Figure 3D).

Two p38-selective human kinase inhibitors, skepinone-L and BIRB 796, reduced *S. rosetta* cell proliferation at 1 μM (Figure 3E) and almost entirely abrogated cell proliferation at 10 μM (Figure 3F) during an 80-hour growth course, providing additional evidence that p38 is required for cell proliferation. At 10 μM, skepinone-L induced
255 changes in cell morphology similar to sorafenib (Movie S5). In contrast, skepinone-L and BIRB 796 did not inhibit p38 phosphorylation (Figure 3G), consistent with the small molecules acting directly on p38 rather than an upstream kinase. We concluded that disruption of p38 kinase activity is necessary to inhibit cell proliferation in *S. rosetta*, and *S. rosetta* p38 kinase is phosphorylated by upstream tyrosine kinases in response to
260 heat shock (Figure 3H).

Discussion

To investigate kinase activity in choanoflagellates and the relevance of kinase
265 signaling in choanoflagellate physiology, we have established a high-throughput phenotypic screen with a small molecule library that provides several features not available with current genetic methods in choanoflagellates (17). By treating *S. rosetta* cultures with small molecules from a library of validated human kinase inhibitors, we uncovered a kinase inhibitor pharmacopeia that revealed the physiological relevance of
270 p38 kinase for the regulation of *S. rosetta* cell proliferation. Moreover, we identified biologically-relevant environmental stressors that activate p38 kinase.

The environmental stress response is comprised of stress-activated kinases, including p38, that evolved before the divergence of animals and fungi (50). Recent work by Shabardina et al. (2022) found that the *S. rosetta* p38 kinase we identified by mass spectrometry, Sr-p38, is closely related to metazoan p38 kinases that respond to diverse environmental stressors (52). Our approach of using small molecules to uncover Sr-p38's role in *S. rosetta* was useful, as p38 likely would have evaded genetic knockout due to its known essentiality in other organisms (49). Our current study provides the first demonstration, to our knowledge, of a direct connection between p38 kinase activity and choanoflagellate cell physiology.

Choanoflagellates have dynamic life histories and diverse kinases families that are not found in animals (9, 11, 53, 66). Whether kinases regulate additional aspects of choanoflagellate physiology, including life history transitions, remains to be investigated. We infer that fast-acting inhibition of kinase activity will be a powerful approach to study the roles of kinases in the regulation of cell state transitions in choanoflagellates. Because kinase inhibitors allow the enzymatic activity of kinases to be disrupted while preserving kinase localization and scaffolding functions, using small molecules as tools can distinguish whether kinase catalytic activity or other kinase functions are necessary for choanoflagellate development (16, 18). Insight into the roles of individual kinases during the emergence of new cell-cell signaling networks (e.g., receptor tyrosine kinase signaling in holozoans) will be fundamental to understanding the contributions of kinase signaling to the origin of animal multicellularity.

Materials and Methods

Detailed methods are provided in Supplementary Materials and Methods.

300

Choanoflagellate husbandry and compound treatment

S. rosetta cells (strain SrEpac (67)) and their bacterial prey were propagated in high nutrient media (5% Sea Water Complete (68) or 4% Sea Water Complete with 4% Cereal Grass (17)) and diluted at exponential phase (~$5x10^5$ – $9x10^5$ cells/mL) to lower

305 density (~$1x10^4$ - $2x10^4$ cells/mL) for compound treatment.

Characterization of* S. rosetta *physiology after compound treatment

S. rosetta physiology after compound treatment was monitored by cell counting or western blotting. *S. rosetta* cell counts were automated with flow cytometry or brightfield

310 imaging. For western blotting, *S. rosetta* proteins from cell lysates were separated by SDS-PAGE gel chromatography, transferred to nitrocellulose, probed by primary antibodies to the target of interest, followed by incubation with infrared fluorescent dyes, and imaged with near-infrared fluorescence imaging.

315 ***Identification of sorafenib targets using mass spectrometry***

To identify peptides from *S. rosetta* proteins that were covalently attached to ActivX probes in the presence or absence of sorafenib, SrEpac cultures or lysates were pre-treated with sorafenib or vehicle (DMSO) followed by incubation with ActivX probe. Proteins were enriched by streptavidin pulldown and peptides were generated by

320 trypsinization and separated by tandem mass spectrometry (LC-MS/MS).

Acknowledgments

We thank D. Booth, M. Carver, M. Coyle, A. Garcia De Las Bayonas and J. Reyes-Rivera for critical reading of the manuscript; K. Shokat (University of California, San Francisco) for the contribution of kinase inhibitors in the screened library and Shokat lab members for discussions; M. West and P. He of the High-Throughput Screening Facility (QB3 HTSF) at UC Berkeley who provided access to the Agilent V11 Bravo liquid handling system for screening plate generation through funding from the National Institutes of Health (S10OD021828); H. Nolla, A. Valeros and the CRL-FACS facility and staff at UC Berkeley for their support and providing access to the BD LSRII flow cytometer and BD HTS sample loading robot; A. Andaya of the mass spectrometry facility at UC Davis. The A.C.D. laboratory receives support from the NIH (RO1s CA227636, CA258736, CA256480, and R56 AG066712), the Mark Foundation for Cancer Research (20-030-ASP, 21-039-ASP) and Alex's Lemonade Stand Foundation for Childhood Cancer. N.K. and F.U.N.R.'s work was supported by an Investigator award (N.K.) and the Hanna H. Gray Fellows Program (F.U.N.R.), both from the Howard Hughes Medical Institute.

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Figures and Tables

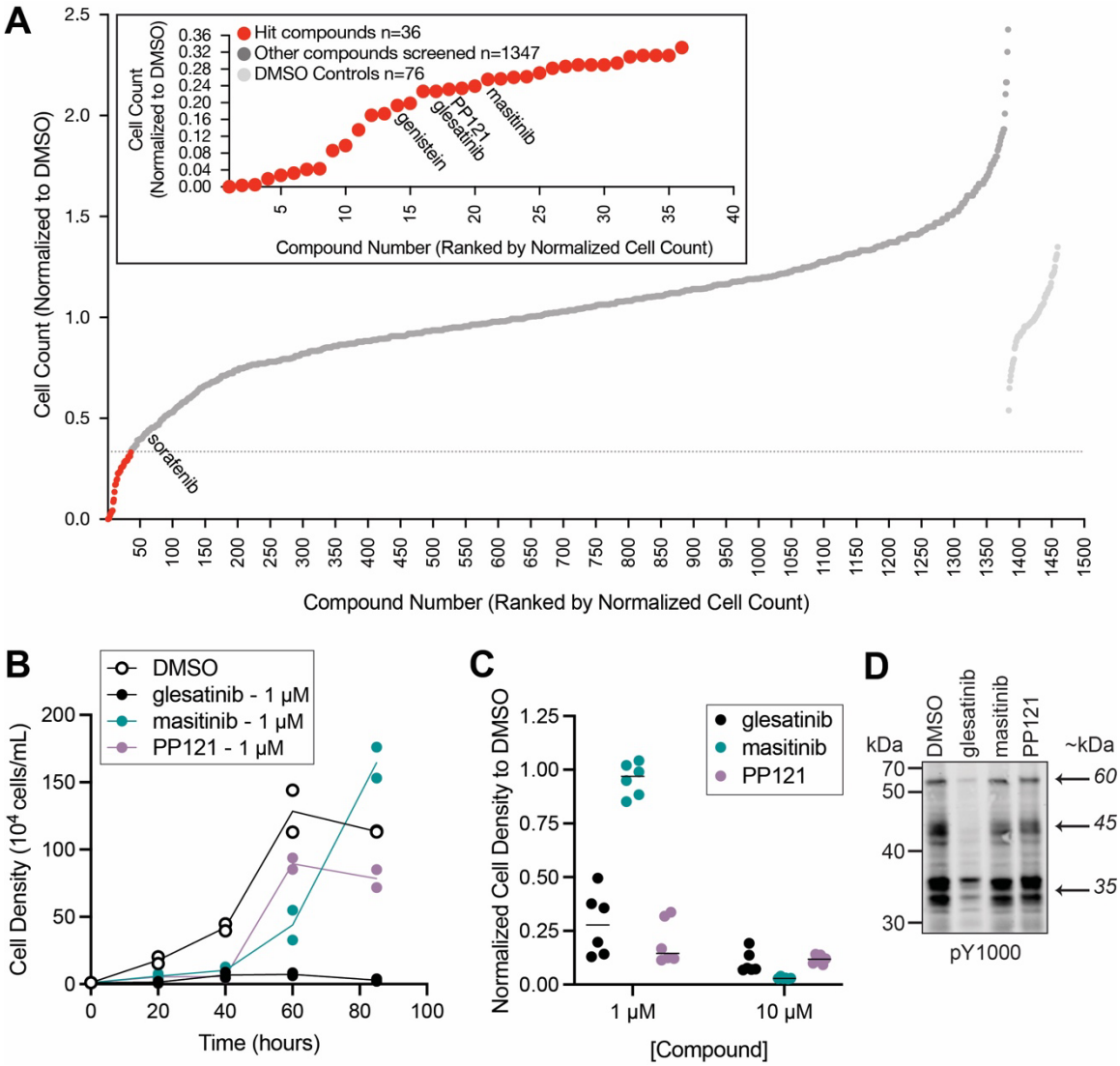

Figure 1. High-throughput screening of a small molecule library revealed that glesatinib disrupts *S. rosetta* cell proliferation and tyrosine phosphosignaling

(A) Treatment of *S. rosetta* cultures with 1383 different small molecules (see Table S1 for list of compounds and Figure S1 for annotated kinase targets of human kinase inhibitors in the library) resulted in a distribution of cell counts at the 24-hour endpoint. Cultures were treated in 384-well plates with each compound as a single replicate and cell counts were determined by flow cytometry (Also see Figure S2). *S. rosetta* cell counts were normalized to DMSO controls (light grey). Compounds determined to significantly inhibit *S. rosetta* cell proliferation (based on two-tailed p-value < 0.05 calculated from z-score) are indicated in red, and compounds that were not significant inhibitors are indicated in dark grey, even though some (e.g. sorafenib) may inhibit cell proliferation in other assays. The graph inset shows the range of normalized cell counts for compounds that

significantly inhibited *S. rosetta* cell proliferation. Structures, mechanisms of action, and human kinase selectivities of the identified inhibitors of *S. rosetta* proliferation are shown in Figures S3 and S4. Compounds that were the focus of further study in this project -- sorafenib, genistein, glesatinib, PP121, masitinib -- are labeled.

(B) Treatment of *S. rosetta* cultures with 1 μM glesatinib led to a complete block of cell proliferation, while treatment with 1 μM masitinib or PP121 led to a partial reduction in cell proliferation relative to DMSO-treated cultures. Two biological replicates were conducted per treatment, and each point represents the mean of three measurements from each biological replicate. For timepoints at 40, 60, and 85 hours, cell densities of inhibitor treated cultures were significantly different from vehicle (DMSO) (p-value <0.01). Significance was determined by a two-way ANOVA multiple comparisons test.

(C) *S. rosetta* cultures treated with 1 μM or 10 μM glesatinib or PP121 for 24 hours had reduced normalized cell density whereas masitinib only had reduced normalized cell density at 10 μM. Normalized cell densities were determined to be reduced if differences between treatments and vehicle (DMSO) were significant (p-value <0.01) Significance was determined by determined by a two-way ANOVA multiple comparisons test. Movie S1 shows *S. rosetta* cells treated with 10 μM glesatinib.

(D) Glesatinib treatment inhibits *S. rosetta* tyrosine phosphorylation. Western blot analysis of *S. rosetta* cultures treated with 1 μM of glesatinib, masitinib or PP121 for 1 hour showed a decrease in tyrosine phosphorylation (as detected with pY1000 anti-phosphotyrosine antibody) compared to vehicle (DMSO) control. Glesatinib treatment resulted in a decrease of tyrosine phosphorylation of proteins at ~60kDa, ~45kDa, and ~35kDa, indicated by arrows. Masitinib and PP121 did not reduce the phosphotyrosine signal. Raw blot image and details on western blot cropping are available at: https://doi.org/10.6084/m9.figshare.20669730.v1

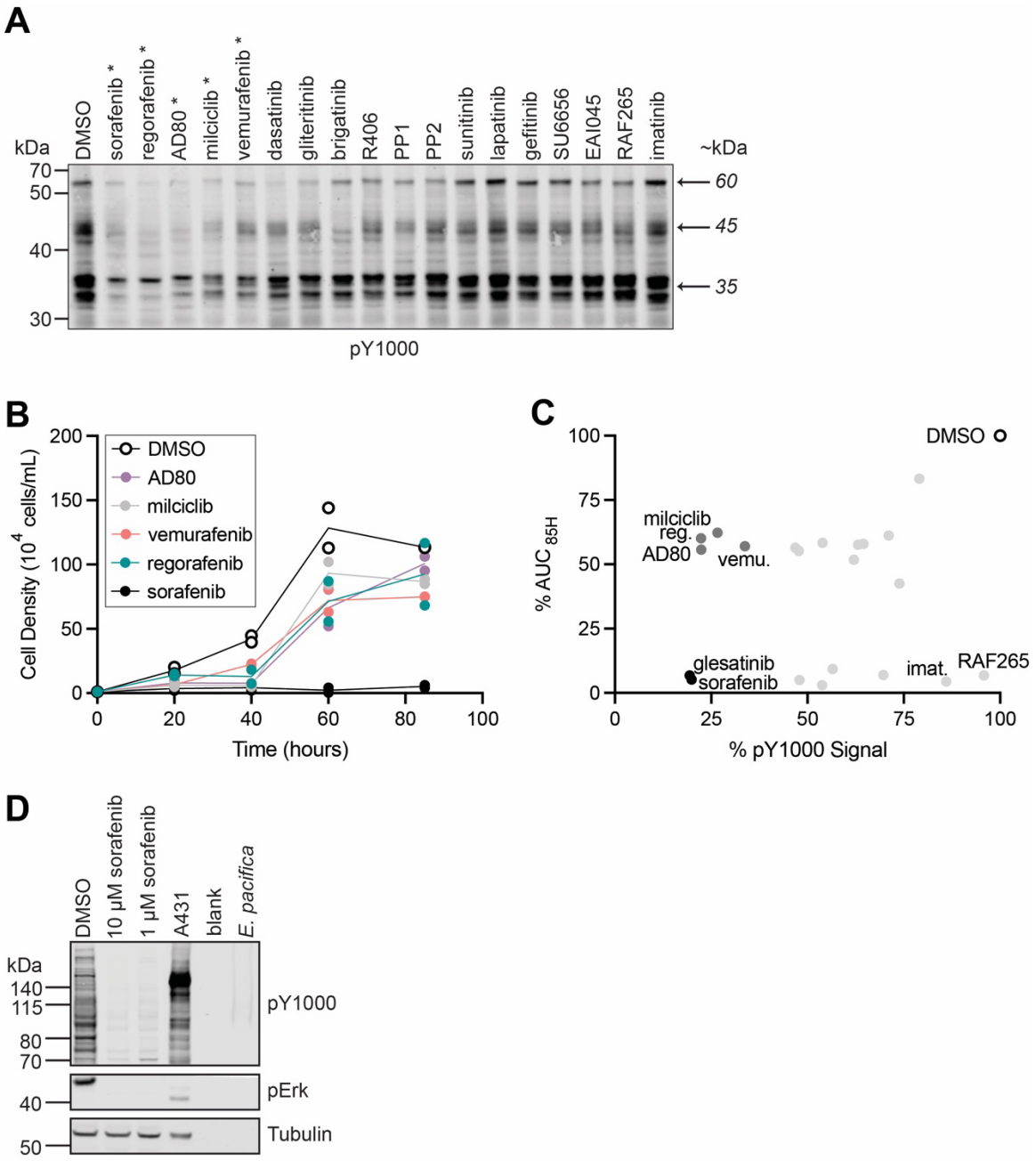

Figure 2. Sorafenib, a versatile probe for inhibiting *S. rosetta* cell proliferation and tyrosine kinase activity

(A) Five small molecule inhibitors of human tyrosine kinases – sorafenib, regorafenib, AD80, milciclib, and vemurafenib (indicated with asterisks) – inhibited tyrosine kinase activity in *S. rosetta. S. rosetta* cultures were treated with a panel of small molecules that inhibit human tyrosine kinases at 1 µM for 15 minutes and assessed for changes in tyrosine phosphorylation. The treated lysates were analyzed by western blot with a phosphotyrosine antibody (pY1000)

to identify if any changes in tyrosine phosphorylation occurred. The resulting level of tyrosine phosphorylation of proteins at ~60kDa, ~45kDa and ~35kDa is indicated by arrows. No changes in relative serine or threonine phosphorylation were observed by western blot (Fig. S6). Raw blot image and details on western blot cropping are available at: https://doi.org/10.6084/m9.figshare.20669730.v1

(B) *S. rosetta* cultures treated with 1 μM sorafenib, regorafenib, AD80, milciclib and vemurafenib had reduced cell density at 40 hours in comparison to vehicle (DMSO) control but only sorafenib treatment was significant (p-value <0.05) after 40 hours. Two biological replicates were conducted per treatment and each point represents the mean of three measurements from each biological replicate. For timepoints at 40, 60, and 85 hours, cell densities of inhibitor treated cultures were considered significant if different from vehicle (DMSO) (p-value <0.05). Significance was determined by determined by a two-way ANOVA multiple comparisons test. Movies S2 and S3 show *S. rosetta* cells treated with 10 μM sorafenib in comparison to Movie S4 of cells treated with vehicle (DMSO).

(C) Sorafenib inhibited *S. rosetta* cell proliferation and tyrosine phosphorylation similarly to glesatinib. For each of the 18 human tyrosine kinase inhibitors tested in panel A and Figure 1D, the percent inhibition of phosphotyrosine signal (as detected with the pY1000 antibody, %pY1000) normalized to total protein stain was plotted vs the percentage of the area under the curve over an 85-hour (% AUC_{85}) treatment. Sorafenib and glesatinib most strongly inhibited global phosphotyrosine and cell proliferation. AD80, regorafenib (reg.), milciclib and vemurafenib (vemu.) were strong inhibitors of global phosphotyrosine but moderate inhibitors of proliferation. Imatinib (imat.) and RAF265 did not inhibit phosphotyrosine staining or cell proliferation.

(D) *S. rosetta* cultures treated with 10 μM and 1 μM sorafenib for 15 minutes had a dose-dependent decrease in phosphotyrosine (pY1000) and complete decrease of phospho-Erk (pErk). Erk kinase belongs to the mitogen-activated protein kinase (MAPK) signaling network that is the core signaling regulator of cell growth in eukaryotes (69). EGF-stimulated A431 lysate was used as a positive control for phosphotyrosine (pY1000) and phospho-Erk (pErk). Tubulin (anti α-tubulin, YOL1/34) was used as a loading control, and lysate from *E. pacifica*, a bacterial prey of *S. rosetta* was used to control for minor *E. pacifica* protein staining with each antibody. Raw blot image and details on western blot cropping are available at: https://doi.org/10.6084/m9.figshare.20669730.v1

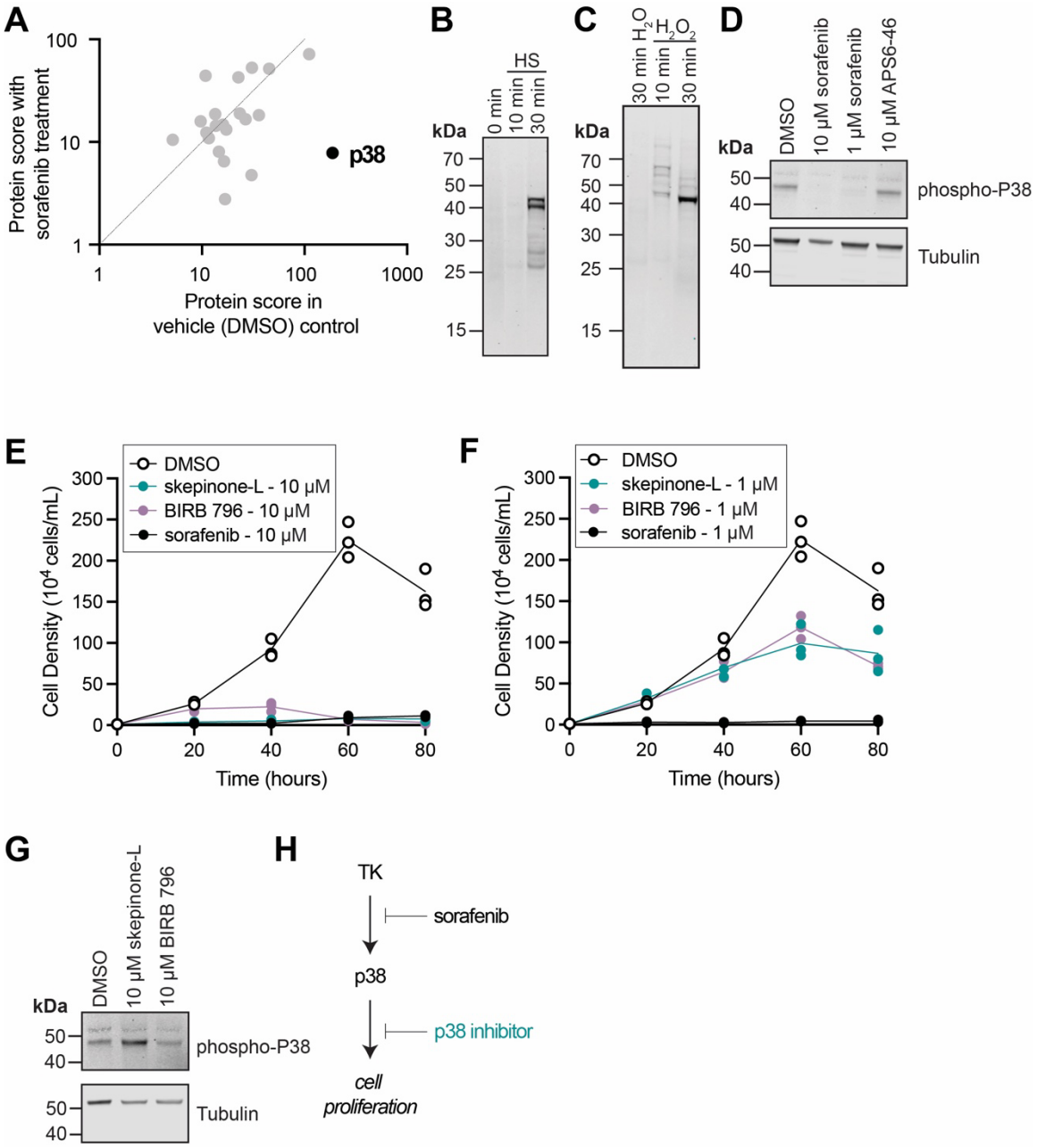

Figure 3. Sr-p38 binds to sorafenib, is activated by environmental stressors, and regulates *S. rosetta* cell proliferation.

(A) Sorafenib binds to Sr-p38. The ActivX ATP probe was used to pull down kinases from *S. rosetta* lysates that were pretreated with either DMSO or the ATP-competitive inhibitor sorafenib. We found that pretreatment with sorafenib reduced the level of Sr-p38 recovered using the ActivX ATP probe, indicating that sorafenib and Sr-p38 interact and outcompete ActivX ATP probe binding. Kinases plotted are only those that were identified in both vehicle and sorafenib pre-

treatments. For full kinase enrichment list, see Table S2, and for alignment of Sr-p38 with those from animals and fungi, see Fig. S7.

(B-C) Sr-p38 kinase is activated by heat shock and oxidative stress. *S. rosetta* cells, normally cultured at 22°C were incubated at 37°C or treated with hydrogen peroxide for 10 min. or 30 min. Lysates from the treated cultures were analyzed by western blot with a p38 antibody specific for phosphorylated p38 kinase (phospho-p38) to identify if any changes in p38 phosphorylation occurred. (B) 30 minutes of heat shock was sufficient to induce p38 phosphorylation as was (C) 10 min of treatment with 0.5M H_2O_2. A 12% Bis-Tris SDS-PAGE gel was used to resolve the western bands observed. An anisomycin-treated human cell lysate was used as a positive control to validate the phospho-p38 antibody in Figure S7C. Raw blot images and details on western blot cropping are available at: https://doi.org/10.6084/m9.figshare.20669730.v1

(D) Sr-p38 phosphorylation is inhibited by sorafenib, but not by the sorafenib analog APS6-46. *S. rosetta* cultures pretreated with 10 μM or 1 μM sorafenib for 30 minutes followed by 30 minutes of heat shock at 37°C had decreased p38 phosphorylation. APS6-46 treated cultures were not different from vehicle (DMSO) control. Data from all sorafenib analogs tested are shown in Figure S8A-B. Treatment growth curves, dose response, and tyrosine phosphorylation analysis with APS6-46 treated cultures are in Figure S8C-E. Raw blot image and details on western blot cropping are available at: https://doi.org/10.6084/m9.figshare.20669730.v1

(E-F) Selective inhibition of Sr-p38 disrupts *S. rosetta* cell proliferation. *S. rosetta* cultures were treated with sorafenib or one of two p38-specific inhibitors, skepinone-L or BIRB 796, in 24-well plates over an 80-hour growth course. (E) At 40 hours, cells treated with 10 μM skepinone-L, BIRB 796 or sorafenib showed little evidence of cell proliferation in comparison to vehicle (DMSO) control (p-value <0.01). (F) Cells treated with 1 μM skepinone-L or BIRB 796 had reduced cell density in comparison to vehicle (DMSO) control (p-value <0.01) at 60 hours. Three biological replicates were conducted per experiment and significance was determined by determined by a two-way ANOVA multiple comparisons test. Movie S5 shows a timelapse of *S. rosetta* cells treated with skepinone-L.

(G) Sr-p38 phosphorylation is not inhibited by the p38-specific inhibitors skepinone-L or BIRB 796. *S. rosetta* cultures pretreated with 10 μM of skepinone-L and BIRB 796 for 30 minutes followed by 30 minutes of heat shock at 37°C were not different from vehicle (DMSO) control. Raw blot image and details on western blot cropping are available at: https://doi.org/10.6084/m9.figshare.20669730.v1

(H) Proposed mechanism for regulation of Sr-p38 by tyrosine kinases and the essentiality of Sr-p38 for *S. rosetta* cell proliferation. Sr-p38 kinase is phosphorylated by upstream tyrosine kinases and is necessary for cell proliferation. Sorafenib inhibits Sr-p38 phosphorylation by blocking the activity of upstream tyrosine kinases. p38 inhibitors that do not inhibit these upstream

645 tyrosine kinases also do not reduce Sr-p38 phosphorylation but do block Sr-p38 kinase activity and thereby block *S. rosetta* cell proliferation.