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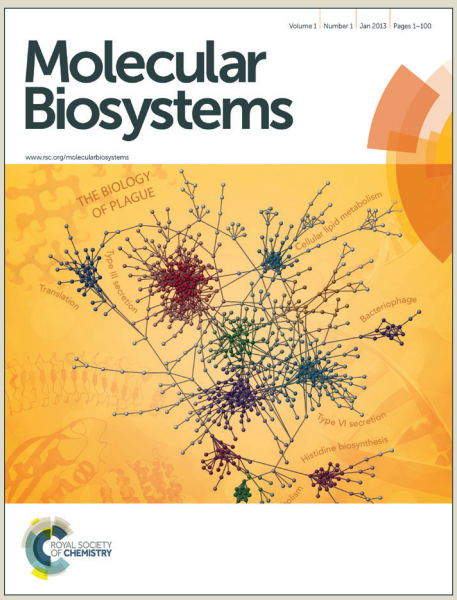

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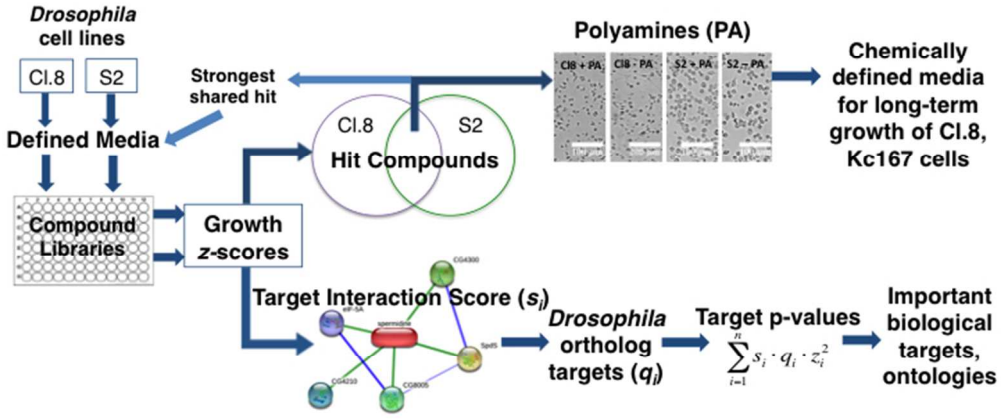

We describe a novel approach to screen for growth promoting compounds and score putative targets using a drug-protein interaction database.

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ARTICLE

An inverse small molecule screen to design a chemically defined medium supporting long-term growth of *Drosophila* cell lines

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Drosophila cell culture is used as a model system with multiple applications including the identification of new therapeutic targets in screens, the study of conserved signal transduction pathway mechanisms, and as an expression system for recombinant proteins. However, *in vitro* methods for *Drosophila* cell and organ culture are relatively undeveloped. To characterize the minimal requirements for long-term maintenance of *Drosophila* cell lines, we developed an inverse screening strategy to identify small molecules and synergies stimulating proliferation in a chemically defined medium. In this chemical-genetics approach, a compound-protein interaction database is used to systematically score genetic targets on a screen-wide scale to extract further information about cell growth. In the pilot screen, we focused on two well-characterized cell lines, Clone 8 (Cl.8) and Schneider 2 (S2). Validated factors were investigated for their ability to maintain cell growth over multiple passages in the chemically defined medium (CDM). The polyamine spermidine proved to be the critical component that enables the CDM to support the long-term maintenance of Cl.8 cells. Spermidine supplementation upregulates DNA synthesis for Cl.8 and S2 cells and increases MAPK signaling for Cl.8 cells. The CDM also supports the long-term growth of Kc167 cells. Our target scoring approach validated the importance of polyamines, with enrichment for multiple polyamine ontologies found for both cell lines. Future iterations of the screen will enable the identification of compound combinations optimized for specific applications—maintenance and generation of new cell lines or the production and purification of recombinant proteins—thus increasing the versatility of *Drosophila* cell culture as both a genetic and biochemical model system. Our cumulative target scoring approach improves on traditional chemical-genetics methods and is extensible to biological processes in other species.

Introduction

Chemical genetics has recently emerged as a complimentary method to traditional genetics where the central theme is the use of small molecules for studying biological systems[1–4]. One especially promising form of chemical genetics is inverse drug screening, where known bioactive compounds are screened for phenotypes[1,3–6]. This inverse approach is analogous to RNA interference (RNAi) screens in that the compounds all have known or putative targets, and thus compound "hits" provide insights into the biological pathways involved in the process of interest[1,3–6]. Some advantages of the small molecule approach are speed, reversibility, wide applicability across species, and efficiency (one compound can probe multiple putative targets)[4]. These screens have been harnessed to investigate multiple aspects of biology including mitosis, pigmentation, development, insulin signaling, and wound healing[7–11]. In particular, *Drosophila melanogaster* has been used for whole organism scale small molecule screens to study various biological processes[12,13].

Drosophila is a versatile model system used to understand the development and physiology of multiple tissue types[14–17]. Traditionally, the unsurpassed genetic and molecular tools available for *in vivo* studies has relegated the development of *in vitro* tools to a secondary role[18]. However, for increased throughput and ease, *Drosophila* cell and organ culture is becoming more widespread, especially in genetic and chemical screens (Fig. 1A-B)[17–21]. Still, *Drosophila* cell culture tools are relatively undeveloped, limiting the utility of *Drosophila* cell

culture as a model system. In particular, there is no chemically defined medium (CDM) available for the culture of *Drosophila* cell lines; they all either include undefined extracts (e.g., yeast extract) or require supplementation with undefined and highly variable serum such as fetal bovine serum (FBS) or fly extract (FEX)[22]. These undefined supplements limit the control and reproducibility of cell culture experiments, and due to their complex nature hinder proteomic analyses. While several companies offer serum-free media for the growth of insect cells, these formulations are proprietary.

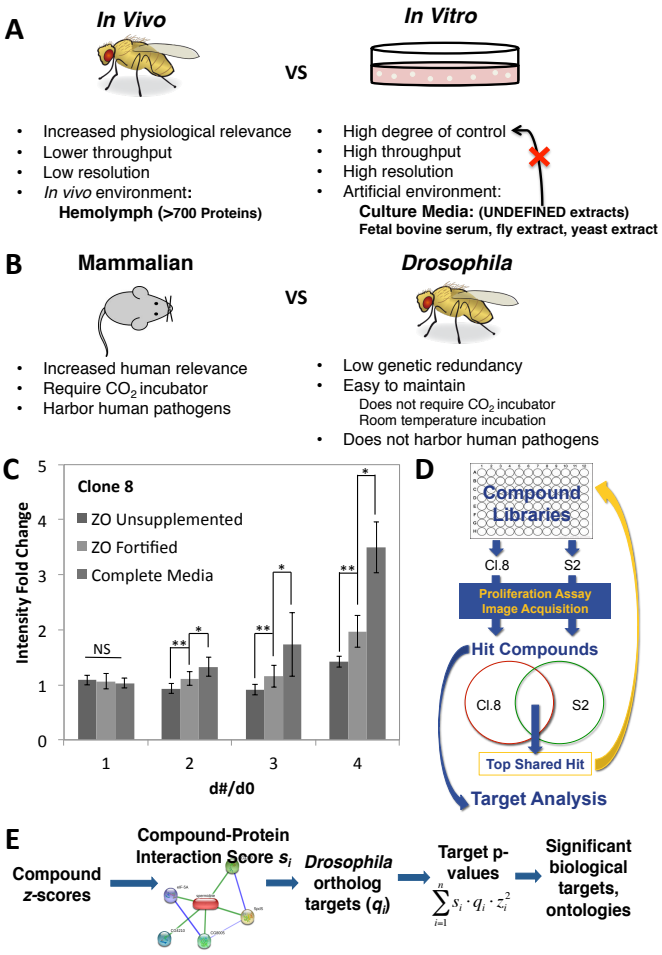

Figure 1: (A): Advantages and limitations of *in vivo* and *in vitro* experimentation. The precise control offered by *in vitro* culture is abrogated by the required medium supplementation with undefined extracts. (B): Comparison of mammalian versus *Drosophila* cell culture. (C): Comparison of Cl.8 growth kinetics in complete serum containing media, ZO media unsupplemented, and "ZO Fortified." ZO Fortified supports initial attachment and proliferation of Cl.8 cells whereas ZO unsupplemented does not (Supplementary Fig. 1). Single and double asterisks denote $p < 0.05$ and $p < 0.01$, respectively, for two-tailed t-test for unmatched pairs. (D): Screening pipeline. Small molecule libraries are screened on two cell lines for their ability to promote proliferation in a minimal, serum-free medium. Putative hit compounds are then compared between cell lines to minimize false positives, and analysis on putative targets is conducted. The screen can be iterated upon to identify compound synergies by incorporating hit compounds into the background media and rescreening. (E): Target scoring pipeline. Compound-protein interaction scores (s_i) are identified from a database and linked to compound *z*-scores. Non-*Drosophila* protein targets are "translated" to their *Drosophila* orthologs retaining their percent identity match score, q_i. *Drosophila* protein target p-values are then calculated by summing for each interaction with a compound its compound-protein interaction score, percent identity match scores, and squared compound z-scores (which follows a chi-squared distribution). This analysis yields a list of significantly targeted proteins for a process of interest. Protein target lists can be also converted to their encoding genes and gene ontology enrichment can be performed. More details can be found in the materials and methods.

The most recent attempts to rationally design chemically defined media for *Drosophila* cell culture were made over 30 years ago, before the development and spread of high-throughput screening techniques[22,23]. Wyss' systematic attempt was based on the requirements of two embryonic cell lines, Kc and *Ca*[22,24–26]. The resulting formulation, ZO media, could support Kc cell growth upon inclusion of fly extract, and was also used to create an epithelial-like cell line from *Chironomus tentans* upon supplementation with FBS, yeast extract, and insulin[22,27].

Applied to culture media design, small molecule screens have previously been used to identify media supplements enabling self-renewal of embryonic stem cells and to find inducers of B-cell expansion[28,29]. Similarly, a compound cocktail was optimized to support long-term growth of human embryonic stem cells using five predefined candidate molecules associated with known pathways[30]. However, to our knowledge no systematic attempt has been made to both identify growth promoters and optimize *Drosophila* culture media in a high-throughput fashion. We hypothesized that by performing a pilot inverse small molecule screen on *Drosophila* cells we could identify 1) novel compounds and compound synergies stimulating cell proliferation, 2) genetic targets and biological pathways important for growth, and 3) a combination of compounds sufficient for long-term growth and maintenance of *Drosophila* cell lines in a chemically defined medium.

Here, we demonstrate a high-throughput inverse drug-screening platform to identify novel compounds and genetic targets important for proliferation of *Drosophila* cells. By systematically identifying and scoring protein targets of the screened compounds, we can identify genes and pathways in addition to compounds important for growth. We have developed an approach that harnesses a chemical-protein interaction database to "translate" cumulative small molecule scores to gene target scores to elucidate targets with small effects (e.g. small effects from multiple compounds with same target) (Fig. 1E). This approach is an improvement over traditional methods where only "hit" compounds' targets are investigated, and can be applied to screens in other model organisms for which databases are available. The pipeline can also be expanded to identify compound synergies that can be exploited to design CDM capable of supporting long-term growth of multiple *Drosophila* cell lines (Fig. 1D). The protocol can be used as a template for the rational design of media, to identify growth-promoting factors, and implicate signaling pathways important for growth.

In this proof-of-principle screen, we focus on two standard *Drosophila* cell lines, the adherent Clone 8 (Cl.8), which has

previously been used to identify novel insect-specific growth factors, and S2-DRSC (S2), which is frequently used for recombinant protein production and grows in suspension[31–34]. The screen led to the identification of multiple candidate molecules relevant for stimulating growth and viability of both cell lines. In particular, the pilot screen revealed polyamines as the critical missing component of a CDM for *Drosophila* cells, and sufficient for enabling long-term growth of Cl.8 and Kc167 cells without requiring any weaning of the cells from sera. To our knowledge this is the first successful attempt to harness a small molecule screen to systematically define the minimal requirements for long-term *Drosophila* cell growth in a chemically defined environment.

Materials and methods

Cell culture

Cl.8, S2, and Kc167 (Kc) cells were expanded in optimized serum-containing media, Cl.8, S2, and Kc167 media, respectively. Cl.8 media contains M3 media (Sigma-Aldrich®) supplemented with FBS (2%), insulin (5 μg/m), and fly extract (2.5%). S2 media is based on Schneider's media (Gibco® Life Technologies) supplemented with FBS (10%). Kc media containing M3 media supplemented with yeast extract (10 mg/mL), bactopeptone (25 mg/mL), and 5% FBS. All cell lines were obtained and cultured according to instructions from the *Drosophila* Genomics Resource Center (DGRC). Fly extract was prepared from adult *yw* flies as described by the DGRC. For culture in chemically defined media, cells were rinsed three times in PBS to remove residual serum and seeded at around 70% confluency in ZO Fortified or ZB Media (ZO Fortified with 1 μM spermidine added at time of passage). Upon reaching confluency, cells were passaged 1:2 retaining half the spent media, similar to routine maintenance in complete media, with cells passaged in ZB Media receiving fresh doses of 1 μM spermidine.

Basal media

While Wyss' ZO medium was never widely adopted, we selected it as the starting point for designing a completely chemically defined basal medium as it represents the most recent and thorough systematic effort to create a chemically defined medium for *Drosophila*[22]. ZO media was initially acquired commercially (Sweden National Veterinary Institute) but is currently prepared in our lab in small batches as described by Wyss with similar results[25,27]. Preliminary efforts focused on testing compound candidates from the literature for their effect on proliferation (data not shown). Various proliferation assays were tested, with CyQUANT® Direct Proliferation Assay (Life Technologies) yielding the best calibration between fluorescent intensity and cell number. CyQUANT is a DNA content-based assay that uses a background suppressing dye that is selectively permeable to dead cells (lacking membrane integrity), enabling specific labeling of live cells[17]. Conveniently, this assay yields good calibrations between cell number and fluorescent intensity ($r^2 > 0.95$) even when used at 0.25x the suggested working concentration (data not shown).

Using this approach we developed an intermediate "ZO Fortified" medium, made up of ZO media supplemented with insulin (5 μg/mL), trehalose (26.4 mM), L-alanyl-L-glutamine (ala-gln, 12 μM), and L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (A2P, 0.08 μM), with pH adjusted to 6.75. Insulin is a growth stimulator through insulin receptor signaling that is frequently used as a media supplement; trehalose is a disaccharide present in insect hemolymph that has roles in protection from environmental stresses such as temperature and oxidation; ala-gln is a stabilized dipeptide version of the essential amino acid L-glutamine; A2P is a stable form of L-ascorbic acid which is essential for many insects[25,27,35–38]. Whereas ZO unsupplemented does not support proliferation of Cl.8 cells, ZO Fortified supports short-term cell growth and initial attachment (Fig. 1C, Supplementary Fig. 1). ZO Fortified is capable of supporting slow growth of Cl.8 cells through approximately 3 passages.

Pilot screen

To more efficiently identify compounds important for cell growth in serum-free media, we selected five small molecule libraries for pilot screening: the Wnt Pathway Library (75 compounds), Autophagy Library (97 compounds), Kinase Inhibitor Library (80 compounds), Phosphatase Inhibitor Library (33 compounds), and Ion Channel Ligand Library (72 compounds) (Enzo® Life Sciences). The Wnt pathway is important for regulating cell proliferation and differentiation[39]. The Autophagy Library encompasses the target of rapamycin (TOR) pathway, involved in the regulation of growth and apoptosis with respect to nutrition, and is important in cancer[35,40–42]. The Phosphatase and Kinase Libraries target tumor suppressors and oncogenes involved in TOR signaling, as well as other kinases and phosphatases involved in cell growth, proliferation, and survival[35,40]. Ion channels have well-known roles in cell proliferation and cancer[43,44]. These libraries come in a convenient 96-well format in dimethyl sulfoxide (DMSO) at a concentration of 10 μM.

Because most compound targets are identified using mammalian models, their efficacy and specificity to *Drosophila* are largely unknown. Further, many of the compounds tested are likely growth inhibitors rather than stimulators. To supplement the commercial libraries, we thus created a custom library consisting of supplements we hypothesized to be important for cell growth (39 unique compounds/supplements, 53 total, electronic supplementary information). Preliminary investigations yielded working concentrations for some of these compounds; therefore, custom library stocks were prepared either at 10 μM (for un-tested compounds) or 10,000x their working concentration (based on preliminary tests). The vast majority of these compounds stocks were prepared in DMSO,

but some were prepared in water or ethanol based on solubility (electronic supplementary information). Based on the screening concentrations selected, the final DMSO dilution is 1:1000 or greater, and thus not expected to impact results. These 6 libraries served as the basis for the pilot screen. The combined contents of the tested libraries along with raw scores from the screening experiments are listed in the electronic supplementary information (electronic supplementary information).

Five stock plates of randomized compounds in singlet (410 total) were prepared at 75 μM in water (1:133 dilution) using an automated liquid handler (Eppendorf epMotion® 5075). Ten strategically located negative controls were incorporated into each stock plate by dosing wells with DMSO (1:133, carrier only control)[45]. Four randomly placed positive control wells were also included on each plate by dosing wells with DMSO (1:133) for consistency, but for flexibility positive control treatments were prepared in the working stock. Working stocks at 2 and 20 μM (2x) were then prepared in ZO Fortified by diluting the 5 stock plates 1:3.75 (high concentration working stock) and then serially diluting 1:10 (low concentration working stock). Positive control wells were prepared by replacing 10% of the media with 100% fly extract (FEX)[17,22,25].

Cells were rinsed three times in phosphate buffered saline (PBS) and seeded at 50,000 cells/well (50 μL) in ZO Fortified. Working stocks, 50 μL, were then immediately added on top of the cells (day 0). Plates were incubated at 25° C for four days, and then imaged at 10x magnification on a Nikon microscope using automated screen acquisition in MetaMorph® software. Plates were then treated with CyQUANT® (0.25x final concentration) for one hour at 25°C, and fluorescent intensity was measured with a plate reader (Biotek Synergy H2).

Screen analysis and hit selection

Strictly standardized mean difference (SSMD) scores for classifying assay quality were calculated with respect to negative controls on a plate-wise basis, with the average assay quality being excellent for both cell lines and screened concentrations[45]. We investigated the effect of performing positional corrections (median polishing, etc.) to account for plate-location effects and found that screen quality, as assessed by the SSMD, were overall best when no positional corrections were made[45,46]. As a hit selection metric we used z-scores, which measure the number of standard deviations from the negative control that a small molecule treatment causes. Z-scores were calculated on a plate-wise basis, and hit cutoffs of $z = \pm 3$ were selected[45]. Visual inspection of images was used to remove false-positive hits, which generally occurred due to autofluorescence of a few of the compounds.

Growth kinetics experiments

Cells were rinsed three times in PBS and seeded in 96-well plates at a concentration of 50,000 cell/well (by plating 5 μL of a $1x10^7$ cell/mL suspension in PBS on top of 95 μL of media). Three replicates per media were assayed with CyQUANT (0.25x final concentration) daily, starting immediately after seeding (day 0). After addition of CyQUANT, cells were incubated for one hour at 25°C, and fluorescent intensity was measured with a plate reader (Biotek Synergy H2) at a gain of 70 to facilitate day-by-day comparisons in intensity. A fold change in intensity of >1 indicates proliferation of cells.

Polyamine-depleted spermidine dose-response measurements

Cl.8 cells were rinsed three times in PBS and seeded in ZO Fortified at $1x10^6$ cell/mL. After nine days of culture in ZO Fortified, cells were harvested and seeded in tissue culture flasks in ZO Fortified supplemented with 0, 0.1, 1, or 10 μM spermidine (in triplicate). After 6 days of culture, cells were manually counted.

Cell immunostaining and western blots

For EdU incorporation, Cl.8 and S2 cells were rinsed three times in PBS and seeded in optical grade 96-well plates at a concentration of 50,000 cell/well (by plating 5 μL of a $1x10^7$ cell/mL suspension in PBS on top of 95 μL of media). After four days of culture, cells were assayed with Invitrogen's Click-iT® EdU Alexa Fluor® 647 Imaging Kit according to their instructions, with a three hour EdU incorporation time. Cells were then stained with DAPI (1:1000) and two positions per well were imaged at 40x magnification on an EVOS® fluorescent microscope (AMG). CellProfiler, a customizable image analysis package was used to quantify total number of cells (from DAPI images) and number of EdU positive cells (from channel 647 images) within each image[47,48]. A minimum of 375 cells was analyzed for each condition tested.

For western blots, Cl.8 and S2 cells were rinsed three times in PBS and seeded in tissue culture flasks at a concentration of 1,000,000 cell/mL in the various media. Cells were cultured until positive control samples (complete media) reached ~90% confluency, at which point all cell samples were lysed for 30 minutes on ice in a buffer containing 50 mM Tris, 150 mM NaCl, 1% Nonidet P 40 substitute (Sigma 74385), and 1% Sigma protease inhibitor cocktail (P8340). Nucleic acid and cell debris were cleared by centrifugation at 12,000 rpm for 20 minutes at 4°C. Because polyamines are known to play roles in initiation of transcription and translation, "housekeeping" genes routinely used as loading controls for western blots are likely inappropriate for this application[49–52]. Thus samples were normalized by total loaded protein. Sample protein concentrations were determined three independent times per sample using Coomassie Plus (Bradford) Assay (Thermo Scientific 23238). ANOVA comparison of loading concentrations across all samples found no significant variation between samples ($p > 0.34$). Proteins were resolved by SDS-PAGE in reducing conditions, electroblotted to PVDF membranes (Hybone 10600087), and probed with either 1:1000 anti-rabbit p44/p42 MAPK (Erk1/2) (Cell Signaling) or 1:1500 anti-mouse MAPK (dpERK1/2) (Sigma). Blots were developed using WesternBreeze chromogenic western blot

immunodetection kit (Invitrogen) and quantification was done in Fiji[53].

Compound target analysis

The Search Tool for Interactions of Chemicals (STITCH) database was used to identify protein targets of the tested compounds[54–56]. STITCH integrates data from multiple databases and catalogs the interactions between more than 300,000 compounds and 2.6 million proteins in 1,133 different species[56]. The STITCH download file of chemical-protein links was queried for all of the screened compounds, and interactions and combined interaction scores for human (*Homo sapiens)*, mouse (*Mus musculus*), and *Drosophila melanogaster* were compiled, resulting in a list of 23,158 uniquely targeted proteins. To assign one score per interaction and penalize inconsistent results, *z*-scores at high and low concentration were averaged. Drugs yielding no STITCH results were removed from the analysis (78 compounds total). *Drosophila* orthologs and % identity match scores for the human and mouse proteins were then found using Ensembl[57]. Proteins yielding no orthologs were removed from analysis (9,491 unique proteins).

This analysis resulted in a list of 6,090 proteins (~22% of the *Drosophila* protein-coding genome) that were potentially targeted by the small molecule screen[57,58]. In order to "translate" the compound scores to a scored protein list, we began by linking the absolute value of each average *z*-score to each compound-protein interaction. The average of the two concentration z-scores was used to penalize compounds that yielded inconsistent results. Because the database (STITCH) is rooted in text-mining, interaction scores do not provide any information about whether a given compound acts as an inhibitor or activator of its target protein. Thus absolute values were used. To generate protein lists of targets important for stimulating versus inhibiting proliferation, compounds were separated into potential proliferation agonists or antagonists (average *z*-score positive or negative, respectively), and targets were scored for these two sets of compounds separately.

Specifically, for each n compound related to d *Drosophila* proteins, each i^{th} relation has an interaction score (s_i) and % identity match score (q_i) between 0 and 1, and *z*-score z_i. Approximately then, the protein score, Σ ($s_i \bullet q_i \bullet z_i^2$), follows a chi-squared distribution with degrees of freedom n, and p-values can be calculated accordingly. These protein p-values are thus based on: 1) the strength with which their interactor compounds affected proliferation (absolute z-score), 2) the confidence level of the compound-protein interaction (s_i), 3) the number of times a protein was targeted by unique compounds (summing scores per targeted protein), 4) the degree of conservation between originally targeted mammalian proteins and their *Drosophila* orthologs (q_i).

To generate significantly targeted protein lists and account for multiple hypothesis testing, we used the standard Bonferroni correction and implemented a p-value cutoff of (0.05/d) for each set of scores. *Drosophila* protein IDs were converted to gene IDs using FlyBase[58]. We then used the Database for Annotation, Visualization, and Integrated Discover (DAVID) to identify enriched Gene Ontology and KEGG annotations[59,60]. Importantly, because our library does not target the entire proteome, this resource enables definition of the background facilitating accurate determination of pathway enrichment. Finally, REVIGO was used to reduce redundancy in ontology annotations by grouping them into terms, which are visualized in semantic similarity plots[61].

Results

Small molecule screen in ZO Fortified

From the screen, 12 and 20 unique positive hit compounds that increase cell numbers were identified for the Cl.8 and S2 cell lines, respectively, and 73 (for Cl.8) and 33 (for S2) unique negative hit compounds decreased cell numbers (Fig. 2, electronic supplementary information). Of the positive hits, three compounds were identified in both cell lines. All of the repeated positive hits were polyamines (spermine, spermidine, and putrescine), with spermine ($z_{Cl.8}$ = 10.4, z_{S2} = 17.7) and spermidine ($z_{Cl.8}$ = 10.0, z_{S2} = 8.7) representing the two strongest positive hits in both cell lines (Fig. 2E).

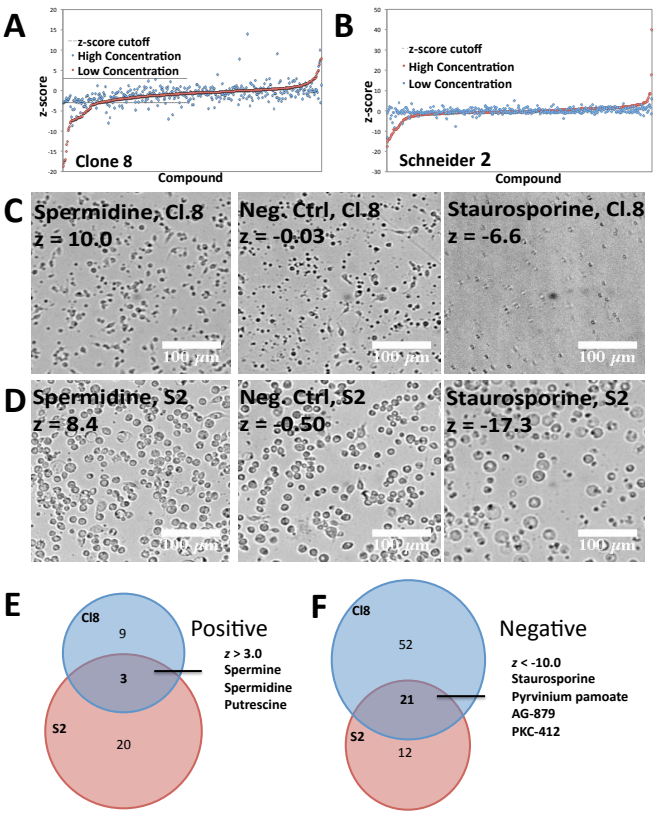

Figure 2: (A-B): Z-scores for all screened compounds on (A) Cl.8 cells and (B) S2 cells. (C-D): Representative images of hit compounds on Cl.8 (C) and S2 (D) cells. (E-F): Venn diagrams for unique positive (E) and negative (F) hit compounds.

Of the negative hits, 21 compounds were found in both cell lines (Fig. 2F, electronic supplementary information). To narrow the analysis, another threshold was applied, yielding 4 compounds with z-scores < -10 in both cell lines: pyrvinium pamoate ($z_{Cl.8}$ = -17.7, z_{S2} = -13.0), staurosporine ($z_{Cl.8}$ = -18.6, z_{S2} = -17.3), AG-879 ($z_{Cl.8}$ = -17.3, z_{S2} = -13.9), and PKC-412 ($z_{Cl.8}$ = -17.1, z_{S2} = -11.0) (Fig. 2F). Pyrvinium pamoate is an androgen receptor inhibitor and anticancer agent[62,63]. Staurosporine and PKC-412 are inhibitors of protein kinases, with staurosporine being the precursor for PKC-412 development[64,65]. AG-879 is tyrosine kinase inhibitor and suppressor of malignant transformation[66,67]. Staurosporine is a known autophagy inducer, indicating that our approach does detect cell number changes resulting from compound treatments.

Growth properties in chemically defined medium

Due to the lower cost of spermidine and our desire for an economical CDM we incorporated 1 μM spermidine into ZO Fortified, newly termed "ZB Media," and conducted growth kinetics experiments to characterize the effect of supplementing ZO Fortified with spermidine. To determine the extensibility of this medium, the growth of Kc167 cells, an isolate of the Kc167 embryonic cell line, was also investigated[24,68,69].

For all three cell lines tested, ZB Media yields higher proliferation rates than ZO Fortified, indicating that spermidine does indeed promote cell growth and attachment (Fig. 3A-C, G-I). Kc167 cells in particular proliferate in ZB Media at comparable rates to in the complete serum-containing medium. Interestingly, ZO Fortified, which we developed for Cl.8 cells, does not significantly improve growth compared to ZO unsupplemented for S2 or Kc167 cells (Fig. 3 D-F).

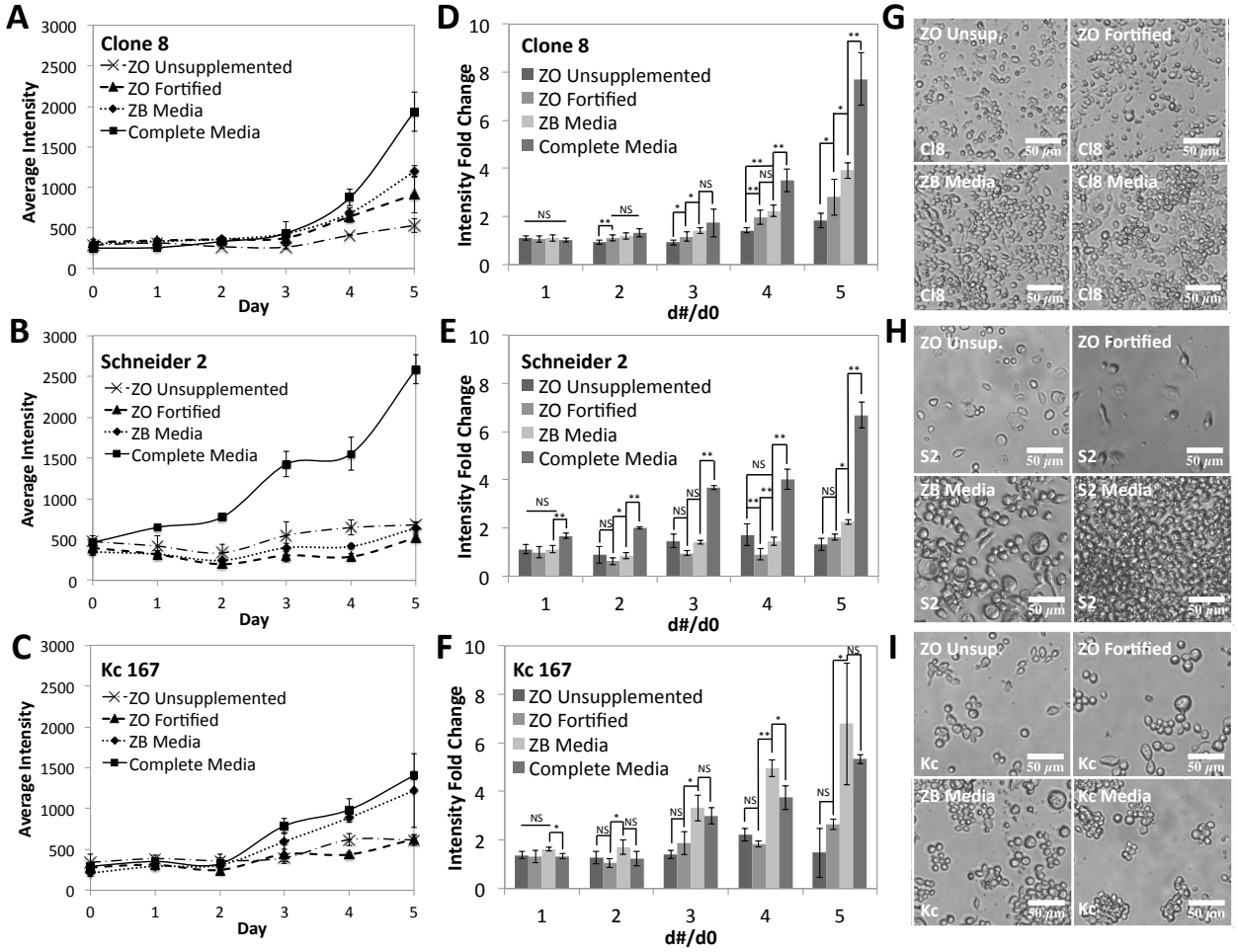

Figure 3: Growth kinetics of Clone 8, Schneider 2, and Kc167 cells in ZO unsupplemented, ZO Fortified, ZB Media, and their respective complete, serum-containing media. Error bars represent standard deviations. (A-C) Changes in averaged fluorescent intensity measurements indicate changes in DNA content from viable cells. Error bars represent standard deviations. (D-F) Fold changes in intensity from day 0 are taken to represent fold changes in cell number. Each of the four cell lines proliferate significantly better in ZB Media (with spermidine supplementation) than ZO Fortified by the fifth day. Kc167 cells in particular proliferate at comparable rates to the complete, serum-containing medium by day 5. Error bars represent standard deviations for which propagation of error is accounted. Single and double asterisks denote $p < 0.05$ and $p < 0.01$, respectively, for two-tailed t-test for unmatched pairs. Corresponding p-values can be found in the electronic supplementary information. (G-I) Cells from day five of the same experiment. The apparent low confluency of Kc167 cells (I) is due to a high number of suspension cells that cannot be seen in one plane of view.

Long-term culture in chemically defined medium

To determine ZB Media's ability to support long-term cell growth, we cultured Cl.8, S2, and Kc167 cells in ZB Media versus ZO Fortified. After 3 passages in the two chemically defined media, Cl.8 cells continue to proliferate in ZB media (ZO Fortified supplemented with spermidine) whereas those cultured in ZO Fortified (no spermidine supplementation) stall in growth (Supplementary Fig. 2). Inclusion of spermidine has enabled Cl.8 growth through 98 passages (thus far) with no signs of decreasing growth rates. Importantly, cell morphology in ZB Media is consistent with the serum containing media. Surprisingly, Cl.8 cells do not require a weaning from serum to adapt to the CDM, they adapt quickly to sustained growth in ZB Media. Adapted Cl.8 cells (passaged 10 times in ZB Media) passaged 1:2 become confluent after 1-2 days, comparable to Cl.8 cells cultured in Cl.8 medium. Adapted Cl.8 cells can also be passaged at higher dilutions (1:6) and reach confluency within 3-4 days. Further, an investigation into the growth kinetics of Cl.8 cells at various seeding densities shows that spermidine improves growth of cells in ZO Fortified even at low concentrations, and that Cl.8 cells proliferate in ZB Media even when seeded at low concentrations, albeit much more slowly (down to 250,000 cell/mL, Supplementary Fig. 3-4). To determine if there is any dependence of proliferation on potential secreted factors, we have performed spent media titrations, which fail to show any benefit on growth induced by spent media (Supplementary Fig. 5). ZB media is also capable of supporting growth of Kc167 cells through at least 14 passages without any signs of stalling in growth (so far; experiments with Kc167 having been initiated subsequent to the initial studies).

S2 cells grown in ZB Media reach passage 4 before growth stalls whereas those grown in ZO Fortified are unable to progress through passage 1 (Supplementary Fig. 2). Thus while ZB Media enables additional growth of S2-DSRC cells, ZB Media requires further improvement to support long term growth of S2 cells.

Therefore, we have developed a medium capable of supporting long-term growth of Cl.8 and Kc167 cells by identifying a supplement cocktail of 5 components. Cl.8 cells adapted to ZB media are able to recover after storage in liquid nitrogen in ZB Media supplemented with 0.2 M trehalose and 10% DMSO. We have found that inclusion of trehalose in freezing media significantly improves cryopreservation in multiple media/cell types, consistent with findings for mammalian cells[70–72].

Biological effect of spermidine

Short-term dose-response experiments (data not shown) failed to show strong dose-dependence of proliferation on spermidine concentration. However, when polyamine-depleted Cl.8 cells were used, a clear dependence of proliferation on spermidine concentration was observed (Fig. 4A-B). Supplementation with 1 μM spermidine caused a significant increase in proliferation ($p < 5x10^{-10}$) of Cl.8 cells compared to 0 or 0.1 μM spermidine supplementation. A higher dose, however, failed to produce any further increase in cell number after 6 days.

To more specifically show that spermidine does indeed promote proliferation, we measured DNA synthesis by 5-ethynyl-2´-deoxyuridine (EdU) incorporation experiments on Cl.8 and S2 cells. ZB Media yields a significantly higher percentage of EdU positive cells than ZO Fortified for both Cl.8 ($p < 0.005$) and S2 ($p < 0.025$) cells (Fig. 4 C-E).

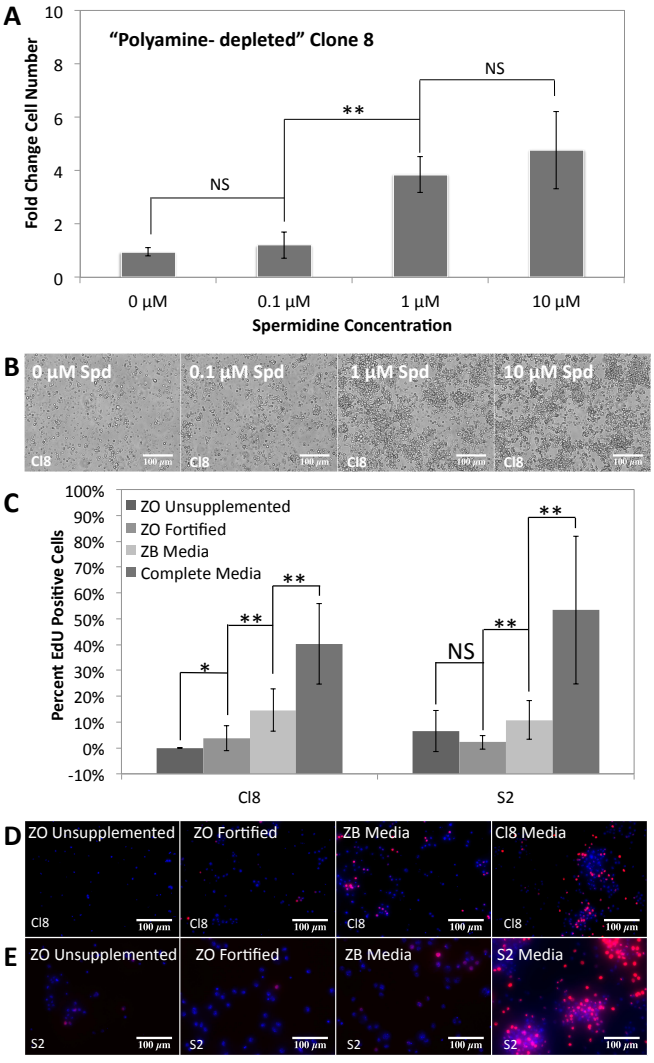

Figure 4: (A-B) Proliferation of polyamine-depleted Cl.8 cells increases in a dose-dependent manner with spermidine supplementation. Error bars represent standard deviations with for which propagation of error is accounted. Single and double asterisks denote $p < 0.05$ and $p < 0.01$, respectively, for two-tailed t-test for unmatched pairs. (C) Spermidine supplementation (ZB Media) results in increased EdU incorporation in both Cl.8 and S2 cells. Error bars represent standard deviations. Single and double asterisks denote $p < 0.05$ and $p < 0.01$, respectively, for two-tailed t-test for unmatched pairs. (D-E) Representative images of EdU (magenta) and DAPI (blue) stained Cl.8 (D) and S2 (E) cells in each culture medium tested.

Because spermidine was previously found to stimulate phosphorylation of tyrosine kinases and ERK1/2 in the

Ras/MAPK signaling cascade, we also investigated MAPK activity of Cl.8 and S2 cells in our spermidine supplemented medium[73,74]. Western blots indicate that spermidine supplementation does in fact increase ERK double phosphorylation for Cl.8 cells ($p < 0.012$), but not for S2 cells ($p > 0.75$) (Fig. 5A-B). Total ERK levels are not significantly influenced by spermidine supplementation for either cell line ($p > 0.45$), although there does seem to be a trend of increasing ERK levels across the iterating improvements to Wyss' original ZO Media (Fig. 5C-D, p-values in electronic supplementary information).

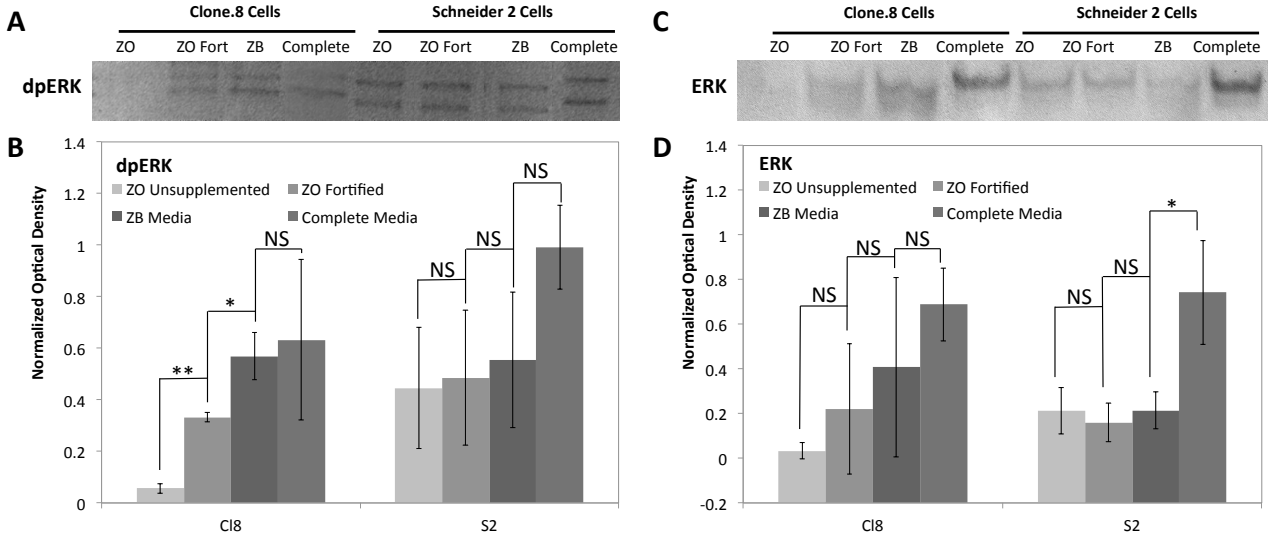

Figure 5: Spermidine supplementation (ZB Media) results in increases in ERK phosphorylation (dpERK) for Cl.8 cells ($p < 0.012$), but not for S2 cells ($p > 0.75$). Although ERK expression does not change significantly with spermidine supplementation for either Cl.8 ($p > 0.55$) or S2 ($p < 0.45$) cells, there is a trend of increasing ERK expression across ZO Media (unsupplemented) improvements. Error bars represent standard deviations across three replicates. Single and double asterisks denote $p < 0.05$ and $p < 0.01$, respectively, for two-tailed t-test for unmatched pairs. Corresponding p-values can be found in the electronic supplementary information.

Compound target analysis

Target analysis on compounds with positive average *z*-scores yielded 111 gene product candidates significantly targeted for the Cl.8 cell line and 53 gene product candidates significantly targeted for the S2 cell line. All of the gene products that were targeted in S2 were also targeted in Cl.8. Some of the strongest genes targeted by positive-scoring compounds for both cell lines were glycogen phosphorylase (GlyP), ornithine decarboxylase 1 (ODC1), S-adenosylmethionine decarboxylase (SamDC), casein kinase IIα (CkIIα), and ornithine aminotransferase precursor (Oat). These findings are consistent with the importance of polyamines, with ODC1 and SamDC both being central upstream enzymes in the polyamine biosynthesis pathway[75–77]. Ontology enrichment on scored protein lists yielded multiple annotations that were visualized using REVIGO scatter plots, which group similar annotations into broader "terms" and plot them on semantic axis where similar terms are closer together; points on scatter plots are sized according to the number of ontology annotations per term and are colored based on their p-values (Fig. 6). Among enriched ontology terms for targets of positive z-score compounds, polyamine metabolism, ornithine metabolism, and cellular modified amino acid biosynthesis pathways emerged for both cell lines.

KEGG pathway enrichment was also conducted for targets of positive-scoring compounds, with glutathione metabolism, arginine and proline metabolism, and cysteine and methionine metabolism all significantly enriched terms for Cl.8 cells, and arginine/proline metabolism and cysteine/methionine metabolism being significantly enriched for S2 cells as well. This is consistent with the importance of polyamine metabolism for cells cultured in ZO Fortified, as polyamines are synthesized from both arginine and methionine. The first step in polyamine metabolism is the production of ornithine from arginine; at the same time, L-methionine is used to create decarboxylated S-adenosyl-L-methionine (DcAdoMet) which acts as an aminopropyl group donor to either putrescine or spermidine to produce either spermidine or spermine, respectively[75,78].

Target analysis on compounds with negative average *z*-scores yielded 266 gene product candidates significantly targeted for the Cl.8 cell line and 166 gene product candidates significantly targeted for S2 cell line. Some of the strongest genes targeted by negative-scoring compounds in both cell lines were phosphorylase kinase γ (PhKγ), calmodulin (Cam), and downstream of raf1 (Dsor1). Among enriched ontology terms, protein amino acid phosphorylation, protein kinase activity, phosphorus metabolic process, and ATP binding emerged. The target of rapamycin (TOR) pathway and progesterone-mediated oocyte maturation were found as significantly enriched KEGG pathways for Cl.8 cells, with ribosome as well as again the

progesterone-mediated oocyte maturation being enriched for S2 cells. The TOR pathway is strongly involved in the regulation of cell growth, proliferation, and survival, especially in the context of coupling growth with nutrition[35,40–42].

A complete list of compounds, average z-scores, target proteins, as well as lists of target proteins and their associated p-values and enriched ontology annotations are provided in the electronic supplementary information. Validation of candidates and whether the targeting is definitively antagonistic or agonistic remains to be determined in future investigations and is outside the scope of the present study.

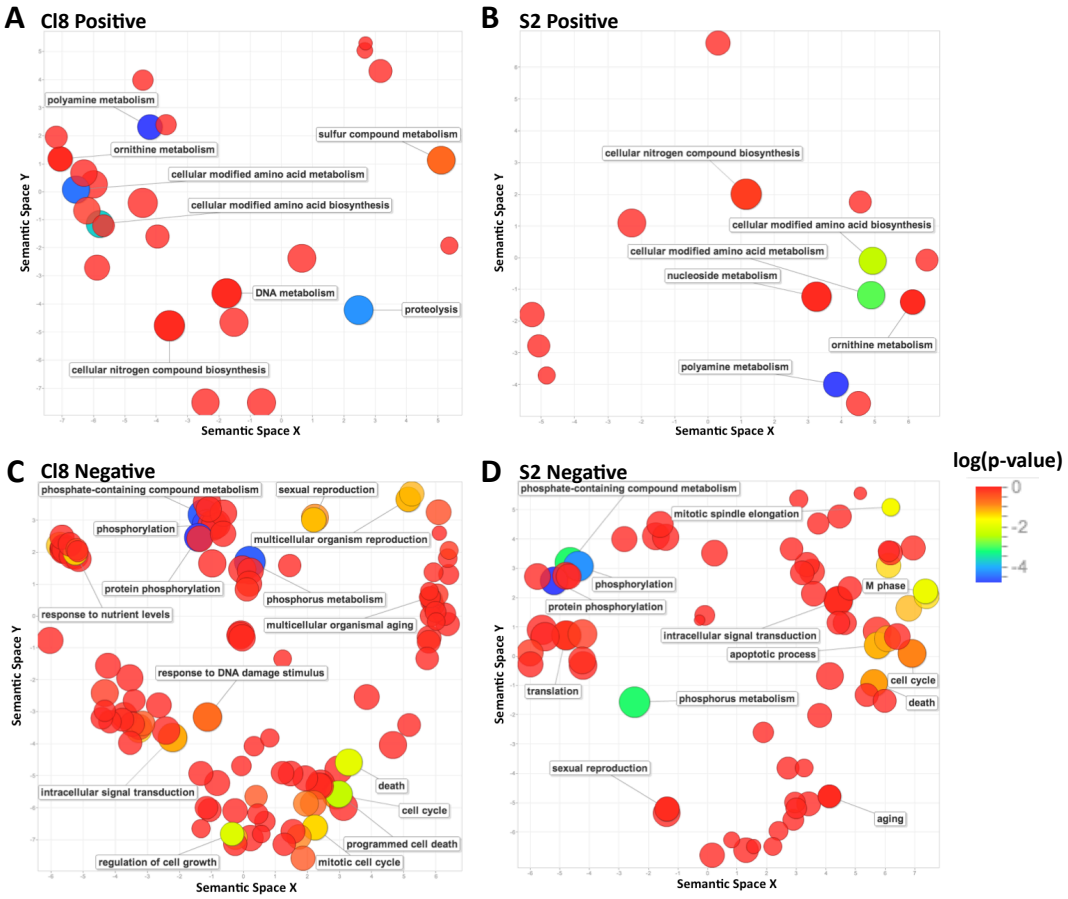

Figure 6: REVIGO scatter plots of enriched ontology terms for targets of (A) positive scoring compounds for Cl.8 cells, (B) positive scoring compound for S2 cells, (C) negative scoring compounds for Cl.8 cells, and (D) negative scoring compounds for S2 cells. Enriched ontology annotations are grouped into terms by semantic similarity and plotted on semantic axes, where similar terms are clustered closely on the plot. Circles representing terms are colour-coded according to p-value and sized according to the number of ontology annotations per term.

Discussion

In this study, we have identified five compound additives that enable Wyss' ZO medium to support long-term growth and maintenance of *Drosophila* Cl.8 and Kc167 cells[25,27]. In particular, the polyamine spermidine was found through an inverse drug screen to be the critical component missing from ZO Fortified, our supplemented version of ZO medium that enables this long-term growth. The other two central polyamines, spermine and putrescine, were also identified from our compound screen to be significant growth promoters for both Cl.8 and S2 cell lines. Consistent with this finding, polyamines were found to be required in a chemically defined medium formulation for the flesh fly, *Boettcherisca peregrina*, potentially generalizing the need for polyamine supplementation in chemically defined media for invertebrate cell culture[79]. While many of the supplements used in culture media are undefined, it is known that polyamines are typically present in high levels in fermented foods such as yeast extract, which is routinely included in many *Drosophila* culture media[80]. It is possible that *Drosophila* cells may be missing upstream components for polyamine synthesis, or lack the signals to synthesize polyamines, and thus required exogenous polyamine supplementation.

Although polyamines are known to be essential for proliferation, the exact biological functionalities for these molecules are both pleiotropic and incompletely defined[49,75]. Here we show that spermidine supplementation significantly

increases phosphorylation levels and, potentially, expression levels of ERK. This is consistent with findings that spermidine specifically stimulates the phosphorylation of tyrosine kinases and ERK1/2 in the Ras/MAPK activated signaling cascade[73,74].

Importantly, the magnitude of the effects of spermidine on proliferation is relatively small, at least as measured in assays of short duration (4 days or less), suggesting that polyamines become limiting only after significant cellular depletion. This assertion is also supported by the finding that dose-dependence of proliferation on spermidine concentration is stronger for cells that have been depleted of polyamines compared to normal cells. It appears that *Drosophila* cells may be unable to synthesize sufficient polyamines from the available nitrogen sources in ZO Fortified; therefore, polyamine supplementation is required and transport is likely crucial. This hypothesis is supported by the gene target analysis and ontology enrichment implicating the importance of arginine and methionine metabolism, both of which are required upstream for polyamine synthesis. In normal rat kidney cells, pharmacological knockout of polyamine synthesis required several days before polyamine levels were significantly reduced, potentially explaining why exogenous polyamine supplementation would have small effects in the short term but be required in the long term[81]. Due to the suboptimal growth conditions of the screening media (ZO Fortified), use of two cell lines to increase screening resolution, and our protein-target analysis, we were able to detect the relatively small short-term effects of polyamine supplementation.

Our media development pipeline can be expanded for the rational design of media to improve conditions for industrial production of recombinant proteins from insect cells, increase success rates for creating new cell lines from primary cultures, or define the minimal essential factors required for the proliferation of other types of insect cells. However, one drawback of our pilot screen was the low genome coverage; STITCH target analysis indicated that only 22% of the proteome was targeted. This is due to our screening of a limited number of targeted compound libraries. This library selection and coverage issue is another promising application of our target analysis approach, which could be extended to develop rationally designed compound libraries that target the maximum possible genome coverage based on known interactions cataloged in STITCH. However, it is important to note that the target analysis approach is heavily dependent on the quality and quantity of data cataloged by interaction databases like STITCH.

One drawback of our method is the single-factor basis of our screening pipeline, which does not specifically identify compound synergies promoting proliferation. Pooling strategies specifically used for synergy identification, however, are not compatible with our target scoring approach[82]. As an alternative method to identify synergies, we propose iterative expansion of the screen, where validated factors would be incorporated into the media background before compound rescreening. This methodology could elucidate compound synergies with previously identified compounds, and our biological target identification technique coupled with this iterative approach could potentially lead to important clues about novel biological mechanisms and crosstalk between pathways and proteins important for growth. Our cumulative gene target scoring approach is an improvement over traditional screens that only consider biological targets of individual hit compounds, and can be applied to query a wide range of biological processes in other model systems.

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Notes and references

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