Stress Resistance Screen in a Human Primary Cell Line Identifies Small Molecules that Affect Aging Pathways and Extend *C. elegans'* Lifespan

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Supplemental Materials and Methods

Cell Culture & H_2O_2-resistance Screen. In our screen, to reduce the variation due to difference in the doubling levels, WI-38 cells were propagated from the initial stocks (ATCC, PDL ~23) and prepared as frozen aliquots to be used for the screen as well as follow-up characterization. Prior to experiments, frozen cells were cultured and dissociated using Accutase (Sigma) for sub-cultivation at least once. In addition, human primary dermal fibroblasts (HDFp.05, from multiple donors) were obtained from Zen-Bio and cultured in dermal fibroblast medium, following the vendor's instructions, and used for hit-validation experiments. Also, to assess variation due to seeding, non-treated control plates were prepared and analyzed for each batch as well. The mean of standard deviations for all the control plates on multiple screen days was ~11% for non-stressed cells (data not shown). The average Z' value, measuring the difference between positive and negative controls to assess the extent of variation, was 0.61 (Supplemental Figure 2), a robust value for such a screen. We discarded molecules that significantly increased the ATP signal by stimulating cell proliferation in the absence of H_2O_2.

As our controls to knock down either *AKT1* or *KEAP1*, reverse siRNA transfection of WI-38 cells was performed, following an established protocol from the Schwarzbauer lab at Princeton with slight modifications. Briefly, siRNA oligos (Qiagen Flexiplate, validated) were complexed first with RNAiMAX (Life Technologies) and applied to 384-well microtiter plates, and then 2,000 WI-38 cells of mid-PDL were dispensed into the wells. Transfection was conducted first in medium with a lower serum level (~6%), which was adjusted back to 10% final 4 hours later (50 μl, 20 nM

siRNA final concentration), and then continued for ~72 hours till the point for H_2O_2 stress and cell viability assays by measuring both ATP content and fraction of PI-positive cells. In the experiment to address *NRF2* dependency, WI-38 cells were transfected for ~90 hours with double-stranded *NRF2* siRNA oligos (5'-UCCCGUUUGUAGAUGACAA-3') (Singh et al. 2008) and then treated with Gr-4D for 24 hours before treatment with H_2O_2. Knockdown of *NRF2* was verified by RT-qPCR (normalized to the peptidylprolyl isomerase A gene *PPIA*). Primers used for qPCR analysis of *NRF2* and *PPIA* were: 5'-ACACGGTCCACAGCTCATCATG-3' (exon 1 of transcript *NRF2-201*) and 5'-TGCCTCCAAAGTATGTCAATCA-3' (exon 2 of *NRF2-201*); 5'-ATGGTCAACCCCACCGTGT-3' (exon 1 of transcript *PPIA-201*), 5'-TCTGCTGTCTTTGGGACCTTGTC-3' (exon 3/4 of *PPIA-201*).

To perform the original dose-response and cell-death imaging analysis, 209 candidate hits were picked individually from the screen library and re-analyzed at six different final concentrations (0.6 μM, 1.25 μM, 2.5 μM, 5 μM, 10 μM and 20 μM) to examine their ability to promote H_2O_2-resistance of WI-38 cells. Cells pre-treated with these molecules (at 1.25 μM and 10 μM) were also analyzed by propidium-iodide imaging to examine cell death following 3 hours of H_2O_2 treatment. For DMSO pre-treated controls (n = 30), the percentages of PI-positive cells were 23.7% ± 6.9% (average ± standard deviation, 1.25 μM assay plate) and 27.4% ± 6.5% (average ± standard deviation, 10 μM assay plate). When assayed at 1.25 μM and 10 μM, 107 hits were found to reduce the percentage of PI-positive dead cells (by 1 to 3 standard deviations from the mean) upon H_2O_2 (data not shown). Note that certain effective small molecules could have been missed in the imaging assays, as false negatives

could arise due to potential stability issues of the library compounds. Fresh small molecules were obtained from different vendors, including ChemBridge, ChemDiv, Vitascreen and MolPort, and analyzed by LC-MS for quality validation. Except for three molecules (Gr-6A, O12 and O17), molecular masses were confirmed to match with predicted values.

To determine whether hit compounds possess any structural similarity to each other and/or to chemical moieties with known properties, we used the MetaDrug chemical data-mining tool (Ekins et al. 2007) and the Similarity Ensemble Approach (SEA) statistical method (Keiser et al. 2007), which compares compounds to known therapeutic drugs.

CdCl$_2$ resistance Assays. Small molecules were tested for cadmium resistance twice in two independent assays. Each small molecule was analyzed at five different concentrations (0.25 μM to 20 μM final concentration), with technical triplicates for each dose. Data were analyzed using a global variance t-test. The experimental setups on 384-well plates and small molecule incubation times were the same as for H$_2$O$_2$ assay, and the incubation time was 12 hours for both cadmium (700 μM) and MMS (900 μM). We tested several conditions, including conditions used to assay fibroblasts from long-lived animals (Salmon et al. 2005). These conditions produced better results with the positive controls (*AKT1* and *KEAP1* siRNAs) in the MMS assay. Thus, for MMS, the cells were shifted from growth media – OptiMEM plus 10% FBS, to DMEM (Gibco) plus 2% BSA (no serum) just before adding small molecules, which occurred 24 hours before MMS addition.

DPPH Assay. 2,2-diphenyl-1-picrylhydrazyl (DPPH) is a radical-containing purple dye that can be reduced by ROS scavengers. This cell-free assay was performed as described previously (Sharma and Bhat 2009). DPPH (Sigma) stock (25 mM) was prepared in methanol and diluted to 50 μM in acetic acid-buffered methanol (0.1 M, pH 5.5). 50 μl diluted solution was dispensed into three 384-well assay plates (technical triplicates). 209 small molecule hits were picked individually from the library stock onto a stock plate, and then introduced by a pin-tool into the assay plates at 10 µM final concentration. DMSO negative control and several positive control ROS scavengers, including N-acetyl cysteine, amodiaquine dihydrochloride and 8-hydroxyquinoline quinoline (8-HQ) (all from Sigma), were introduced into separate wells. Plates were sealed and incubated in a humid chamber at 30º C in the dark. Absorbance at 519 nm was measured on a FlexStation 3 multi-mode microplate reader (Molecular Devices) 3 hours and 24 hours later.

AmplexRed Assay. This cell-free assay was performed using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Life Technologies), following the manufacturer's instructions. Briefly, small molecules (at 10 µM final concentration) were pre-incubated with 700 μM H_2O_2 in water for 3 hours in a 37ºC CO_2 incubator. Catalase (MP Biomedicals) was used as the positive control. Amplex Red reagent was mixed with horseradish peroxidase (HRP) in buffer and then incubated with 1:10 diluted small molecule/ H_2O_2 mixture for 30 minutes in dark. Fluorescence at 590 nm was measured on a FlexStation 3 multi-mode microplate reader (Molecular Devices).

PARP Inhibitor Assay. The assay was performed with the HT Fluorescent Homogeneous PARP Inhibition Assay Kit (Trevigen), following the manufacturer's instructions. Briefly, nicotinamide adenine dinucleotide (NAD), human PARP1 and activated-DNA solution were distributed across a 96-well plate. Fifty-one repurchased, mass-checked small molecules were introduced at 10 µM final concentration and incubated in a humid chamber at room temperature for 30 minutes in the dark. Cycling mixture, with resazurin and cycling enzyme diaphorase, was then added and incubated further for 1 hour in dark. The reaction was terminated with stopping buffer. Fluorescence was then measured on a FlexStation 3 multi-mode microplate reader (544 nm excitation/ 590 nm emission).

Cell Confluency Analysis. WI-38 cells (2,000 per well) were seeded on 96-well plates and cultured and scanned every 2 hours to record their confluency (relative percentage of surface area in a cell-culture vessel covered by cells) in an IncuCyte Zoom Live-Cell Analysis System (Essen Bioscience) for up to 112 hours. Small molecules (10 µM final concentration, n = 3 for each small molecule) were introduced at 24 hours following the start point. Relative cell confluency (by surface area of a given well) was analyzed with the vendor's software.

DNA Damage Marker Analysis. WI-38 cells were seeded (~8,000 cells per well) on 96-well plates, cultured for 24 hours and then incubated with small molecules for another 24 hours. Doxorubicin (300 nM, 24 hours) or H_2O_2 (700 µM, 3 hours) was also

individually introduced as the positive control to damage DNA. Cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 30 minutes and then blocked with 5% normal goat serum (Cell Signaling) and 0.3% Triton X-100 for 1 hour. Primary antibody cocktail [Cell Signaling, rabbit anti-phospho-histone γH2A.X (Ser-139), 1:100; anti-phospho-TP53BP1 (Ser-1778), 1:100] was prepared in PBS with 1% BSA and 0.3% Triton X-100 and incubated overnight at 4ºC. The next day, samples were washed with PBS and further incubated with fluorophore-conjugated secondary antibody (1:1,000) for 1 hour in dark. Samples were then washed and incubated with DAPI dye (Life Technologies) (4 μg/ml final concentration) for 30 minutes. Images were collected on the INCell Analyzer 2000 (10X objective) and analyzed with the Developer Toolbox. Cells that showed immuno-staining intensity above a software-defined threshold were scored.

Microarray Analysis. For microarray analysis, WI-38 cells were treated with small molecules (10 μM final, a dose confirmed to promote H_2O_2-resistance in a paralleled experiment – see Supplemental Figure 6; n = 3 each for group-4 and group-5 molecules, plus O10) or 0.1% DMSO control (n = 15) in the absence of H_2O_2 for 24 hours and then processed for RNA isolation with the RNeasy kit (Qiagen). Reverse transcription (RT) and production of Cy5-labeled cRNA and further hybridizations were performed with the Agilent two-color microarray kit (SurePrint G3 human v3 arrays, 8x60K), following the manufacturer's instructions. Expression levels of gene probes were obtained by normalizing their absolute signals, using the Cy3-labeled Universal Human Reference cRNA (Agilent). Genes that showed significant change of expression

(normalized to DMSO control-treated samples) were obtained using Statistical Analysis of Microarray (SAM, with false discovery rate < 0.10, 1.5-fold) with the Multi Experiment Viewer (version 4.8). Normalized expression levels and FDR values for significant genes were further used for pathway analysis. Gene clustering analysis for microarray data was performed using Gene Cluster (version 3.0).

qPCR Analysis. For qPCR analysis, WI-38 cells were treated with small molecules (10 μM final concentration, n = 4) for 24 hours and processed for RNA isolation and reverse transcription with the Cells-to-Ct Kit (Life Technologies), following the manufacturer's instructions. RT products were diluted with H_2O and used for qPCR analysis on an ABI 7300 system (Life Technologies) (technical triplicates). Relative expression levels of target genes were calculated by the $\Delta\Delta$Ct method, using the reference gene beta-2-microglobulin *B2M* or the peptidylprolyl isomerase A gene *PPIA*, and relative fold changes were obtained by normalizing to negative controls and further analyzed using the Student's t-test. We analyzed four NRF2-regulated genes, including *HMOX1* (heme oxygenase (decycling) 1; an anti-oxidant), *NQO1* [NAD(P)H dehydrogenase, quinone 1; a phase 2 detoxification enzyme], *GCLC* (glutamate–cysteine ligase, catalytic; a glutathione-synthesis enzyme) and *GSTM1* (a glutathione S transferase). Fourteen molecules (3 of 4 best hits: Gr-4D, Gr-5A, Gr-5D; plus 11 with liabilities: Gr-4A, Gr-4B, Gr-4C, Gr-5B, Gr-6B, Gr-7C, Gr-9A, O6/PAIN, O13, O18 and O20) induced the expression of two or more NRF2-regulated genes at least modestly (by more than 1.5-fold). We also analyzed another five FOXO3A-regulated genes: *SOD2* (superoxide dismutase), *GADD45A* (a cell cycle regulator), *CAT* (catalase),

DDB1 (damage-specific DNA binding protein) and *TXNIP* (thioredoxin-interacting protein). Three molecules (all with liabilities: Gr-7A, Gr-7C and Gr-9B/PAIN) induced the expression of two or more NRF2-regulated genes at least modestly (by more than 1.5-fold). Sestrin 1 (*SESN1*), a gene known to be regulated by both NRF2 and FOXO3A, was also analyzed by qPCR. Nine molecules (1 of 4 best hits: Gr-4D; plus 8 with liabilities: Gr-1A, Gr-1C, Gr-1D, Gr-1F, O11, O20, O21/PAIN, O27) induced the expression of *SESN1* by more than 1.5-fold. qPCR primers used in this study included: 5'-TGCTGTCTCCATGTTTGATGTATCT-3' (exon 4 of transcript *B2M-204*) and 5'-TCTCTGCTCCCCACCTCTAAGT-3' (exon 4 of *B2M-204*); 5'-CAGTCAGGCAGAGGGTGATAG-3' (exon 3 of transcript *HMOX1-201*), 5'-CCTGCAACTCCTCAAAGAGC-3' (exon 3/4 of *HMOX1-201*); 5'-ACGCTGCCATGTATGACAAA-3' (exon 4 of transcript *NQO1-201*), 5'-GGATCCCTTGCAGAGAGTACA-3' (exon 5 of *NQO1-201*); 5'-ATGCCATGGGATTTGGAAT-3' (exon 6 of transcript *GCLC-214*), 5'-AGATATACTGCAGGCTTGGAATG-3' (exon 7 of *GCLC-201*); 5'-GGAGGAACTCCCTGAAAAGC-3' (exon 6 of transcript *GSTM1-214*), 5'-GGGCTCAAATATACGGTGGA-3' (exon 7 of *GSTM1-214*); 5'-ATCAACGCGCAGATCATGCA-3' (exon 2 of transcript *SOD2-210*), 5'-GCTGTAACATCTCCCTTGG-3' (exon 3/4 of *SOD2-210*); 5'-GAGAGCAGAAGACCGAAAGG-3' (exon 1/2 of transcript *GADD45A-202*), 5'-TGACTCAGGGCTTTGCTGA-3' (exon 3/4 of *GADD45A-202*); 5'-AGAAAGCGGTCAAGAACTTCAC-3' (exon 11/12 of transcript *CAT-201*), 5'-GCATTCTTAGGCTTCTCAGCA-3' (exon 12/13 of *CAT-201*); 5'-

TTGTGTTTGTCGGGTCTCG-3' (exon 8 of transcript *DDB1-201*), 5'-

GGAGCCTTGTTCATTACTGTCA-3' (exon 9 of *DDB1-201*); 5'-

GGCTAAAGTGCTTTGGATGC-3' (exon 1 of transcript *TXNIP-204*), 5'-

TGATCACCATCTCATTCTCACC-3' (exon 1/2 of *TXNIP-204*); and 5'-

GAATGTATGATAGCTTCTGGAGG-3' (exon 9 of transcript *SESN1-204*), 5'-

GCAGATTAACATGAACCTTCTCAG-3' (exon 9/10 of *SESN1-204*).

qPCR primers used for the analysis of worm genes were: 5'-

GACGAGTCCAACGTACTCTCCAAC-3' (exon 10/11 of *ama-1* transcript *F36A4.7.1*)

and 5'-TACTTGGGGCTCGATGGGC-3' (exon 11 of *F36A4.7.1*); 5'-

GATTGCACTACTGATTCGTC-3' (exon 6/7 of *skn-1* transcript *T19E7.2a.1*), 5'-

GTGTATCGTGGAGATTCCGAAG-3' (exon 7 of *T19E7.2a.1*); and 5'-

GGCAGCTTGCTATCCACATA-3' (exon 4 of *sesn-1* transcript *Y74C9A.5.1*), 5'-

AGCCATCGCTACCATCATTAC-3' (exon 5 of *Y74C9A.5.1*).

mTOR Inhibition (RPS6 Phosphorylation Status) Analysis. In-Cell Western

assays were performed, following a standard immuno-staining protocol. Briefly, WI-38

cells were treated with small molecules for 24 hours, and then processed and incubated

with primary antibody cocktail (Cell Signaling, mouse anti-RPS, 1:25; rabbit-anti-pRPS6-

Ser-235/236, 1:100) overnight at 4º C. The next day, samples were processed and

incubated with fluorophore-conjugated secondary antibodies (Cell Signaling, DyLight

680-goat anti-mouse, 1:500; DyLight 800-goat anti-rabbit, 1:1,000). Images were

collected on an Odyssey Imager (LI-COR) and analyzed with the Image Studio Lite

software (version 5.0.21).

Poly(Q) Toxicity & Viability Analysis. Assessment of poly(Q) toxicity was performed as described previously (Aiken et al. 2004). PC12 cells that stably express the inducible poly(Q)103-Htt-EGFP were grown in culture for 24 hours and then subjected to the treatment with small molecules (10 μM). Ponasterone A (Life Technologies), an ecdysone analog, was introduced at 10 μM 24 hours later to induce transgene expression, and the formation of aggregation puncta was examined and confirmed using the Eclipse 200 fluorescent microscope (Nikon). Cell viability was analyzed 48 hours later following the induction, by measuring ATP content with CellTiter-Glo. The parental WT-PC12A cells, which do not express poly(Q)103-Htt-EGFP, were used as the negative control to exclude the possibility that certain small molecules may enhance ATP content even in the absence of toxic aggregates.

H_2O_2 Stress Assays in C. elegans. This stress assay was performed in liquid, following procedures similar to lifespan assays as described above. Wild-type worms were treated with the small molecules from young adulthood, and then H_2O_2 (0.5 mM final concentration) was added on day 8 (1st trial to test all the molecules repurchased) or day 6 (2nd trial to retest candidate hits from the 1st trial) of adulthood, and then animals were scored for viability every day.

Effects on pathway-activity reporters in C. elegans. Transgenic worms expressing different reporters of several longevity-related pathways were raised from the L4 stage on mini-plates that were seeded with normal OP50 bacteria, supplemented

with 20 μM FUDR (to block progeny production, and supplied with either DMSO (as the control) or different compounds. About 30 hours later, at least 12-15 adult worms per condition were imaged on a Nikon Eclipse Ti spinning disk confocal microscope (10x objective, 408nm).

RNAi gene inhibition in C. elegans. Overnight cultures of RNAi bacteria (vector control L4440, Vidal-library *skn-1* or Ahringer-library *sesn-1* RNAi clones, confirmed by DNA-sequencing) were diluted 10 times and cultured at 30ºC for 4 hours in the presence of 1.0 mM IPTG to induce the production of double-stranded RNA. Induced bacteria were then seeded onto NGM plates (containing 100 μg/ml carbenicillin, 12.5 μg/ml tetracycline and 1.0 mM IPTG), which were further supplemented with FUDR (50 μM final) and the chalcone Gr-4D at different doses. Note that RNAi bacteria were not subjected to UV irradiation, in order to avoid crosslink that might destroy dsRNA. RNAi-sensitive mutants, CF3152 [*rrf-3 (pk1426)*], were transferred on day 1 of adulthood onto these plates for lifespan assay. Parallel cultures were used for isolation of total RNA on day 4 of adulthood. Total RNA was extracted using TRIzol reagent, purified with RNeasy kit (Qiagen), and reverse-transcribed using the Sensiscript kit (Qiagen) for further qPCR analysis to confirm knockdown of *skn-1* or *sesn-1*.

Supplemental Results:

Addressing Long-term Effects of Small Molecules

We first performed viability assays to address the long-term effects of the molecules on WI-38 cells, by measuring ATP levels and scoring cell death during a course of 5 days of continuous treatment (in the absence of H_2O_2). Of 32 core-set molecules, we found at least 11 (Gr-1F, Gr-3B, Gr-3C, Gr-7A, Gr-7B, Gr-7C, O11, O27, plus PAINs Gr-6C, Gr-9B and O21) that, compared with controls, reduced ATP content by more than 30% by day 5 of treatment (Supplemental Figure 5-1), suggesting potential anti-proliferative activity and/or cell toxicity of these molecules. Consistent with this, as indicated by the examination of cell confluency, morphology and cell death-imaging, 9 of these 11 molecules were found to reduce cell number and increase the fraction of PI-positive dead cells, and most cells treated with the remaining two (Gr-7A & Gr-9B) were dead (data not shown). Conversely, rapamycin (5 μM), a potent inhibitor of mTOR that reduces growth rates, reduced the ATP content and cell confluency by ~50%, yet did not significantly increase cell death.

We also performed cell confluency-based proliferation assays, focusing on the small molecules that did not show obvious toxicity in the experiment described above. We found that of 14 small molecules analyzed, at least 5 did not show strong inhibitory effects on cell proliferation (Supplemental Figure 5-2). Consistent with the previous analysis, the other 9 small molecules (Gr-1E, Gr-1G, Gr-4A, Gr-4B, Gr-4C, Gr-6B, O13, O18, plus PAIN molecule Gr-9A) that inhibited cell proliferation, unlike rapamycin, produced cell toxicity (assessed by cell morphology). We noted that all group-7 molecules and multiple members of group 4, except for one (Gr-4D), were toxic,

suggesting that the same attributes that elicit stress resistance may cause toxicity ("on target" effects).

Analyzing Potential DNA-damaging Effects of Small Molecules

One (undesirable) way in which a small molecule could induce stress resistance is by blocking apoptosis in response to DNA damage. Therefore, we examined effects on two DNA damage-associated cellular markers, phosphorylated histone variant γH2A.X and tumor protein p53 binding protein 1 (TP53BP1) in WI-38 cells. γH2A.X is required for checkpoint-mediated cell cycle arrest and DNA repair following double-stranded DNA breaks, and phosphorylation of γH2A.X by a group of PI3K-like kinases (ATM, ATR, and DNA-PK) occurs rapidly in response to DNA damage (Perez-Cadahia et al. 2010). Likewise, in response to DNA damage, TP53BP1 is phosphorylated and translocated into the nucleus, and retention of TP53BP1 at DNA breaks requires phosphorylated γH2A.X (Panier and Boulton 2014). Among 32 core-set hits, seven small molecules (three members of group 4, two members of group 5, and two orphans) did not appear to increase the fraction of cells scoring positively for either marker in the absence of H_2O_2 stress (Supplemental Table 4). Consistent with this observation, 5 molecules (Gr-4D, Gr-5A, Gr-5D, O10 and O14) did not show strong cell toxicity in the cell-proliferation assays (Supplemental Figure 5). Conversely, the remaining 25 molecules increased the fraction of cells scoring positively for at least one marker in one experiment (Supplemental Table 4).

We also note that, of the small molecules that increased γH2A.X and/or TP53BP1 foci, 13 molecules also increased the percentage of propidium iodide-positive

death cells under normal conditions (Supplemental Table 2), suggesting that cell toxicity is due to their effects to increase DNA damage and/or DNA damage-associated markers. In other words, a number of small molecules identified in our screen, likely by inducing modest levels of cellular stress (some, by increasing DNA damage), may protect cells from H_2O_2 through a "hormesis" mechanism, or, alternatively by preventing the apoptosis that would normally occur in response to DNA damage. Increased DNA damage can significantly elevate the risk of malignant transformation when affected cells do not undergo senescence and apoptosis. However, we noted that these DNA-damaging small molecules could still be interesting, as they may act like certain cytotoxic agents (e.g., doxorubicin) and kill highly proliferative tumor cells *in vivo*.

Poly-Q toxicity attenuation

Among the efforts to characterize our screen hits, we also examined their effects on protein aggregation, a key feature of many age-related neurodegenerative diseases (Ross and Poirier 2004; Caughey and Lansbury 2003). A number of cell lines have been established to study these diseases at the cellular level (Schlachetzki et al. 2013). Among these, PC12 rat pheochromocytoma cells that stably express GFP fused to the poly(Q) tract (exon 1) of mutant human huntingtin gene *HTT* (Q103-Htt-EGFP), have been used to study Huntington's disease (Aiken et al. 2004). In this cell line, induced expression of poly(Q)103-Htt-EGFP causes the formation of aggregates and exerts substantial cell toxicity. Previously, a medium-throughput small-molecule screen led to the identification of several compounds that could protect cells from toxic Huntington's aggregates (Aiken et al. 2004), including caspase inhibitors (e.g., Z-VAD-FMK) and anti-

inflammatory cannabinoids that have been proposed as therapies in Alzheimer's and Parkinson's disease (Booz 2011; Aso and Ferrer 2014; Rohn 2010). We analyzed our repurchased molecules (at 10 μM) in these cells, and then retested several candidates at multiple doses (2.5 μM, 5 μM, 10 μM and 20 μM).

Among the non-toxic compounds, we did not find any that scored positively in this assay. Instead, we found that four toxic molecules (Gr-1A, Gr-1C, Gr-6B and Gr-7C) appeared to increase the ATP content of PC12 cells upon the induction of poly(Q)103-Htt-EGFP aggregates (Supplemental Figure 9). PubChem query indicated that Gr-3B (12 μM) and Gr-4D (5 μM) were identified by another screen and confirmed to be protective against Huntington's toxicity in the same PC12 cell line (Supplemental Table 9). The basis of this discrepancy is not clear.

"PAINS". One general concern about small-molecule screens is whether the hits could have non-specific effects in cells. In particular, reactive electrophilic "pan assay interference compounds" (PAINS), can have highly pleiotropic effects on cells (Baell and Holloway 2010; Baell and Walters 2014). A typical academic screening library could have ~5-12% of compounds that are PAINS, including catechols, rhodanines, phenolic Mannich bases, enones and others, which could behave as redox cyclers, metal complexers and covalent modifiers. Notably, different classes of PAINS have been observed to co-segregate frequently in the same biological screens. When we queried the diversity chemical library that we screened, using the known signature structures of PAINS, we found ~18,191 possible PAINS, representing 17.5% of 104,121 total molecules. We further checked our small molecules and found that 7 of 61 primary

hits (Gr-6C, Gr-9A, Gr-9B, O6, O9, O21, and including a PARP inhibitor Gr-2E) and 5 of 32 core-set hits (Gr-6C, Gr-9A, Gr-9B, O6 and O21), contained at least part of the PAINS moieties (Supplemental Table 1). Among our top non-toxic hits, one, O6, was a PAIN (phenolic Mannich base); and hence, we excluded this molecule from microarray analysis. Of the other toxic core-set hits that exhibited PAINS signatures, Gr-6C contains a rhodanine moiety and Gr-9B shows both enone and catechol structures, and they extended *C. elegans*' lifespan in multiple trials (Supplemental Table 8). Like O6, Gr-9A (enone) induced both *HMOX1* (by almost 50 folds) and *NQO1*, two NRF2-regulated genes that are involved in the xenobiotic stress response. As mentioned earlier, Gr-9A also protected worms from hydrogen peroxide. O21, another possible PAIN molecule with a rhodanine structure like Gr-6C, scored positive for DNA-damage markers (Supplemental Table 4).

Nonetheless, our screen did not seem to enrich for PAINS ($P = 0.068$, hypergeometric distribution probability, for all 61 hits). In the case of the potential PAINS, they produced many interesting phenotypes, including enhancing stress resistance, inducing expression of NRF2- and FOXO3-regulated genes, and extending lifespan of *C. elegans*. We also note that curcumin and resveratrol, two prominent PAINS (see Supplemental Table 9), extend lifespan in several species (Alavez et al. 2011; Hubbard and Sinclair 2014) and also have potential value for treating cancer, diabetes and other diseases in humans (Anand et al. 2008; Brasnyo et al. 2011). In this regard, the PAIN molecules may still produce global benefits in a specific biological or pathological context in the whole animal.

Supplemental Discussion:

Properties of Hit Molecules

PARP Inhibitors

Poly ADP-ribose polymerase mediates cellular responses to many types of stress, including oxidative, genotoxic, inflammatory and metabolic stress (Luo and Kraus 2012). PARP inhibition has been reported to increase cells' resistance to DNA-damaging agents like hydrogen peroxide (Zhang et al. 2007). We recovered two compounds (group 8) that are highly similar to the PARP inhibitor 4-amino-1,8-naphthalimide (Banasik et al. 1992), an experimental cancer drug (Costantino et al. 2001). This prompted us to analyze all of our 51 repurchased and validated molecules in an *in vitro* PARP assay. In addition to these two group-8 molecules, 8 small molecules (including 4 members of the structural group 2) also substantially inhibit human PARP1 activity (Supplemental Figure 8). Of these, Gr-2E is a non-specific PAIN molecule. However, we noted that, despite their ability to preserve ATP content, only 4 of these molecules modestly reduced the percentage of PI-positive cells upon H_2O_2 stress (Supplemental Table 2). Thus, PARP inhibitors from our screen did not behave as agents that protect cells from oxidative stress, which is why we excluded these molecules from most of our analyses.

On the other hand, several recent studies suggest that PARP inhibition may promote health and longevity. First, deletion of *Parp1* has been reported to increase mitochondrial metabolism through NAD^+ preservation and SIRT1 activation, and to protect animals from metabolic disease (Bai et al. 2011). Second, long-term treatment with PARP inhibitors has been found to enhance mitochondrial function and improve

skeletal-muscle in mice, and also reverse mitochondrial defects in primary myotubes of obese humans and attenuate metabolism defects in *NDUFS1* (a mitochondrial NADH dehydrogenase and oxidoreductase that transfers electrons from NADH to the respiratory chain) mutant fibroblasts (Pirinen et al. 2014). Third, disruption of the *C. elegans* ortholog *pme-1/parp-1*, as well as treatment with PARP inhibitors, induced the mitochondrial unfolded-protein response (mtUPR) and extended lifespan in a manner that required both *sir-2.1* and *daf-16/foxo* in *C. elegans* (Mouchiroud et al. 2013). Consistent with the previous reports, we also found that some of our PARP inhibitors extended *C. elegans'* lifespan (specifically, Gr-8A, ~10% to 34%, 4 of 4 trials; O14, ~6% to 14%, 3 of 3 trials) (Supplemental Table 1).

PARP inhibitors can kill certain types of tumors because of their effects on DNA repair pathways (Mason et al. 2014; Chan et al. 2010; Rouleau et al. 2010); specifically, they have been shown to be effective in clinical trials among cancer patients carrying *BRCA1/2* mutations (Fong et al. 2009). The PARP inhibitors we identified could be interesting in this regard.

Reported Properties of Hit Molecules

Inhibition of SUMOylation

Among our 32 core-set hits, two molecules (Gr-1F and O13, both toxic) have been identified in several screens and confirmed to inhibit multiple SUMO/sentrin-specific peptidases (SENPs) (~50-90% inhibition at 5-20 μM). Small ubiquitin-related modifiers (SUMOs) are ubiquitin-like proteins that can be attached covalently to a variety of target proteins (Geiss-Friedlander and Melchior 2007). SUMOylation

modification can by removed by SENPs, a class of SUMO-specific proteases, which affect many cellular processes, including apoptosis, DNA damage repair, ribosome maturation, and transcription (Yeh 2009; Hickey et al. 2012). SUMOylation of many proteins increases remarkably in response to heat shock and hydrogen peroxide (Zhou et al. 2004), which may explain, at least in part, the mechanism by which our molecules promote stress resistance. Our discovery of potential SENP inhibitors could be interesting, as these molecules have the potential in treating certain diseases including cancer, atherosclerosis and heart diseases, where perturbed SUMOylation balance has been observed (Kumar and Zhang 2015).

References

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Supplemental Figure Legends

Supplemental Figure 1 (related to Figure 1). Increased oxidative-stress resistance upon *AKT1* or *KEAP1* knockdown.

A) To define the optimal assay conditions for our screen, WI-38 cells were incubated with different doses of H_2O_2 for different periods of time in 384-well plates and then assayed for viability by measuring the ATP content. Shown here are representative data, indicating that high doses (above 600 μM) of H_2O_2 substantially depleted the ATP content after 3 hours of incubation (luminescence values are shown by the side). Control, n = 16; for all other H_2O_2 test samples, n = 11-16 (***, P <0.0001 for 500 μM H_2O_2 and above, Student's t-test). Shown in all the figures, unless otherwise specified, were mean ± SD.

B) WI-38 cells were transfected with 20 nM AllStars negative control or *AKT1* or *KEAP1* siRNA oligos (Qiagen) and then assayed for viability 72 hours post-transfection upon treatment with H_2O_2. Representative data indicated that *AKT1* or *KEAP1* knockdown significantly increased cell viability, as measured by ATP content following 3 hours incubation in 700 μM H_2O_2. One-way ANOVA was performed for statistics (P <0.001), plus post-comparison with control (***, P <0.001). Control, n = 24; *AKT1* knockdown, n = 18; *KEAP1* knockdown, n = 13. Viability was also measured in the same experiment for cells not subjected to H_2O_2 treatment. One-way ANOVA (p<0.001), plus post-comparison with control (**, P <0.01; n.s., not significant). Control, n = 16; *AKT1* knockdown, n = 12; *KEAP1* knockdown, n = 12.

C) Propidium iodide staining was performed in parallel to analyze the fraction of dead/dying cells upon H_2O_2 treatment. Consistent with the ATP assay results, *AKT1* or

KEAP1 knockdown significantly reduced the fraction of PI-positive cells. One-way ANOVA (*P* <0.001), plus post-comparison with control (***, *P* <0.001). Control, n = 8; *AKT1* knockdown, n = 6; *KEAP1* knockdown, n = 6.

Supplemental Figure 2 (related to Figure 1). Z' scores for the ATP assay across the screen. Shown are the Z' scores for the 327 plates carrying a total of 104,121 library compounds screened. Z' score, defined as 1 – [(3X standard deviation for positive controls + 3X standard deviation for negative controls)/(mean value for positive controls - mean value for negative controls)], is typically used to access the assay quality in a high-throughput screen, and assay robustness is indicated by a Z' score greater than 0.5. The positive control calyculin (EMD Biosciences), a potent serine/threonine protein phosphatase inhibitor, significantly increased the levels of ATP in H_2O_2-treated WI-38 cells, relative to the DMSO negative control. Average Z' score is 0.61±0.13 (mean ± SD) in our primary screen.

Supplemental Figure 3 (related to Figure 1). Cell-free ROS-scavenging assay of known ROS scavengers and our primary screen hits. The primary screen hits were analyzed together with several known ROS scavengers, including N-acetyl cysteine (NAC), amodiaquine dihydrochloride (AmD) and 8-hydroxyquinoline quinoline (8-HQ), in the absence of cells. Of 209 screen hits assayed (at 10 μM), 56 molecules (including 40 that share a core structure of 8-HQ) were found to reduce the absorbance by 10% or more 24 hour-post incubation and were classified as putative ROS scavengers. The majority of these reduced the absorbance by ~30%. Across 3 assay plates: DMSO

(0.4%) negative control, n = 417; each scavenger control of a single dose, n = 12; each library compound, n = 3. Mean of absorbance normalized to DMSO control for each group was shown above.

Supplemental Figure 4 (related to Figure 2). No *in vitro* H_2O_2-quenching effects by small molecules. Catalase (0.02 units and 0.04 units), the positive control, substantially reduced the fluorescence in the Amplex Red assay that measures H_2O_2 concentration. By contrast, none of the 51 repurchased molecules appeared to reduce the absorbance. Across 4 assay plates: DMSO (0.2%) negative control, n = 8; catalase positive control or each repurchased small molecule, n = 4.

Supplemental Figure 5 (related to Figure 3). 5-1) Long-term effects of small molecules on ATP levels of cultured WI-38 cells. Of 32 core-set hits, 28 molecules were analyzed in multiple 384-well plates in replicates in two batches, (A) 26 and (B) 2, to assess their effects on ATP levels upon prolonged incubation in the absence of H_2O_2 for up to 5 days (10 μM, n = 6 for each molecule, average standard deviation across the whole assay is ~5.9%). Four molecules (Gr-3A, Gr-4B, Gr-4D and O6) were not included in this experiment, since likely due to compromised stability, these four of this specific batch did not retest for H_2O_2-resistance on day 2 of treatment. However, Gr-4B and Gr-4D from a new batch purchased were analyzed in the IncuCyte analysis of cell confluency (see Supplemental Figure 5-2). Green mark on the y-axis indicates the start-point ATP level measured for 1,000 cells 24 hours post-seeding, before adding any small molecules. Note that compared with DMSO control (0.1%) (black thick line) and

H$_2$O control (aqua thick line), 5 μM rapamycin (yellow thick line), which is known to reduce cell proliferation and cell size, reduced ATP level by ~50% on day 5 of treatment. Dashed lines: of 28 analyzed, at least 11 small molecules (Gr-1F, Gr-3B, Gr-3C, Gr-7A, Gr-7B, Gr-7C, O11, O27, plus PAINs Gr-6C, Gr-9B and O21) reduced the ATP level by more than 30% by day 5 (see Supplemental Table 4 for details). These molecules also were examined in the same experiment for their effects on cell morphology and cell death by PI-imaging on day 2 and day 5 of treatment. Unlike rapamycin, which also reduced ATP levels substantially, 9 of these 11 molecules (Gr-3B, Gr-6C, Gr-7A, Gr-7B, Gr-7C, Gr-9B, O11, O21 & O27) also produced cell toxicity (by the examination of cell morphology, data not shown). The other two, Gr-1F and Gr-3C, caused DNA damages, like the others (see Supplemental Table 4).

5-2) Effects of prolonged small-molecule incubation on WI-38 cell confluency. Shown is a representative plot of cell confluency for each treatment condition. Confluency of WI-38 cells was monitored in an IncuCyte Zoom Live-Cell Analysis System for 112 hours. Small molecules (10 μM final, n = 3 each) were introduced at 24 hours (indicated by arrows) following the start point. Fourteen different small molecules that did not produce obvious long-term cell toxicity (see Supplemental Figure 5-1) were retested (vendors are listed at the bottom). A) As a positive control, the TOR inhibitor rapamycin (2.5 μM) reduced cell confluency significantly. B) 5 molecules did not show strong inhibitory effects on cell confluency, and C) 9 molecules significantly reduced cell confluency.

Supplemental Figure 6 (related to Figure 3). Increased cell viability upon H_2O_2 by small molecules in the RNA-seq and microarray experiment, as well as *NRF2* non-dependency for Gr-4D. A) RT-qPCR analysis showing knockdown of *NRF2* expression by more than 95% in WI-38 cells transfected with siRNA oligos (normalized to *PPIA*, n = 8 each; one-way ANOVA, followed by Dunnett's multiple comparison, $P < 0.0001$). B) WI-38 cells were transfected with control or *NRF2* siRNA and then treated with Gr-4D (from two different vendors) at 3 different doses (2.5 μM, 3.75 μM and 5 μM), before subject to H_2O_2 or no H_2O_2 treatment, and scored for propidium iodide staining. Note that Gr-4D (from two different vendors) significantly increased the percentage of PI-positive cells, particularly, in *NRF2*-deficient WI-38 cells at higher doses in the absence of H_2O_2. Hence, we treated cells with a lower dose of H_2O_2 (500 μM instead of 700 μM) to prevent ceiling of death for *NRF2*-deficient cells. Gr-4D also protected *NRF2(-)* cells from H_2O_2, just like what it did in *NRF2(+)* cells. This experiment also served as the control for our RNA-seq analysis of Gr-4D-treated WI-38 cells to show that Gr-4D protected cells against H_2O_2. C) In parallel to our microarray analysis of WI-38 treated with other small molecules (in the absence of H_2O_2), cells were incubated with these molecules (10 μM, n = 3 for microarrays) or DMSO control (0.1%, n = 6-8) for 24 hours in 96-well plates and then analyzed for cell viability upon 3 hours of H_2O_2 treatment. Shown is the percentage of cells that scored positively for propidium iodide staining following treatment with DMSO control or small molecules (named according to the source of vendors: CB, ChemBridge; CD, ChemDiv; MP, MolPort; and V, Vitascreen). Student's t-test, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s. not significant; normalized to respective controls (color-coded).

Supplemental Figure 7 (related to Figure 3). Pearson's correlation between global transcriptional profiles for cells treated with small molecules. Shown is the normalized expression of the 18,683 probe sets detected in every array, grouped by unsupervised clustering using Pearson correlation coefficient as a distance metric. Note that most DMSO (0.1%) controls were clustered together on the tree, as were the six rapamycin (2.5 μM)-treated samples. The molecules were named according to the source of vendors (CB, ChemBridge; CD, ChemDiv; V, Vitascreen), and Gr-4A molecules were obtained from ChemDiv and Asinex.

Supplemental Figure 8 (related to Figure 2). Inhibitory effects of certain small molecules on PARP. Two known PARP inhibitors, PJ-34 (Tocris) (IC_{50}: ~20 nM) and MK-4827 (IC_{50}: ~3.8 nM), were shown to inhibit human PARP1 (at 200 nM and 40 nM, respectively), as indicated by a substantial increase of normalized fluorescence (n = 2). 10 of the 51 repurchased molecules also inhibited PARP at 10 μM. Note that all the molecules from group 2 assayed (one was not available for repurchasing), plus two group-8 molecules (analogs of the 4-amino-1,8-naphthalimide PARP inhibitor), were confirmed to be PARP inhibitors in this assay.

Supplemental Figure 9 (related to Table 1). Protective effects of certain small molecules against poly-Q toxicity. 51 repurchased molecules were introduced initially at 10 μM to neuron-like PC12 cells that express poly(Q)-tagged GFP (Q103-Htt-EGFP), and candidates showing protective effects were further retested at multiple doses (2.5

μM, 5 μM, 10 μM and 20 μM) to analyze their effects on ATP content upon the induction of toxic poly(Q)103-Htt-EGFP aggregates. The parental PC12 cells (WT) that do not express poly(Q) were used as the control to demonstrate the specificity of protective effects. Note that 48 hours induction of poly(Q)103-Htt-EGFP reduced ATP content substantially (right, bottom panel), and four small molecules produced modest yet significant effects to enhance ATP content. However, except for Gr-6B of low doses (which might cause DNA damages), under non-induced conditions, these small molecules actually exerted cell toxicity and reduced ATP content in 72 hours (n = 6. Student's t-test, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Supplemental Figure 10 (related to Figure 4). Effects of Gr-4D on the expression of reporters for several pathways known to influence longevity in *C. elegans*. A to C) L4 animals expressing different *gfp* fusions were raised on plates containing DMSO (as the control) or different compounds as indicated for ~30 hours and then imaged for analysis. At least 12-15 worms were analyzed and shown are representative images. Note that Gr-4D (from two different suppliers), at a dose that extended lifespan, caused modest induction of the *gst-4* reporter, but not the others. No obvious effects of Gr-4D were observed on the number of LGG-1::GFP puncta, which was usually used as the readout of perturbed autophagy (data not shown). D) In an experiment to address *sesn-1* dependency, day-1 RNAi-sensitive mutant adults were fed with vector-control or RNAi bacteria expressing double-stranded RNA of *sesn-1* and treated with the chalcone Gr-4D (from two different vendors) at multiple doses. [Note: the *sesn-1* mutant and *skn-1(RNAi)* data were presented in Figure 4]. RT-qPCR analysis indicated that *sesn-1*

mRNA level was reduced by ~30% (normalized to *ama-1*, n = 4 each; one-way ANOVA, followed by Dunnett's multiple comparison, P < 0.0001). Here, Gr-4D did not extend lifespan of *sesn-1(RNAi)*-treated animals, though a high dose of this molecule (60 μM, Gr-4D-MP) produced a non-significant life-extending trend (n.s., log-rank test). See Supplemental Table 8 for details of lifespan data.

Supplemental Table Legends

Supplemental Table 1 (related to Table 1). Summary of 61 primary hits that promote resistance to H_2O_2. 1st tab: 209 primary screen hits; 2nd tab: 61 primary hits; 3rd tab: 32 "core-set" hits. Potential "pan-assay interference compounds" (PAINS) are indicated. Dose-response curves and derived EC_{50} values are shown for the library compounds identified. Note that significant reduction of performance (fold change < 1.50, highlighted in red) relative to the initial score, likely due to compromised stability, was observed in certain cases. This table also shows data for repurchased small molecules in the follow-up characterizations, including validation retests and other cell-based assays, as well as multiple independent lifespan assays performed in *C. elegans*. For lifespan assays using wild type, FUDR (100 µM) was applied to block progeny production, or temperature-sensitive *fer-15; fem-1* sterile mutants were used instead (see Supplemental Table 8 for details).

Supplemental Table 2 (related to Table 1). 1st tab: Cell death-imaging analysis of 32 core-set small molecules. Consistent with ATP assay results to assess cell viability, the 32 core-set small molecules also reduced the fraction of propidium iodide-positive dying/dead WI-38 cells upon H_2O_2 (n = 6 for each molecule, Student's t-test, three consecutive time points – 3, 4 and 5 hours of H_2O_2 incubation) [Compare the percentage of dead cells in the presence of the small molecules (green, decreased; red, increased) with the reference percentage in the DMSO control (shown at the top)]. Shown are representative data for at least three independent experiments. Note that besides the 32 core-set molecules, three PARP inhibitors (*, as reference) only

rendered WI-38 cells very modest protection. 2nd tab: In parallel to assays of WI-38 cells, 22 small molecules also protected primary, non-transformed human dermal fibroblasts (HDFs, from multiple donors) from H$_2$O$_2$ (in 2 of 2 trials). Fold change less than 1.5 is highlighted in red.

Supplemental Table 3 (related to Table 1). Protection of WI-38 cells from the heavy metal CdCl$_2$ by small molecules. Among molecules retested, at least 18 increased the resistance of WI-38 cells to both H$_2$O$_2$ and cadmium (in 2 of 2 trials, not including the one producing a little effects). Fold changes less than 1.5 are highlighted in red. In a few cases (Gr-7A, Gr-7B, Gr-9A and Gr-9B), we did not observe H$_2$O$_2$ resistance, potentially due to chemical instability (not shown). Thus, we were unable to judge these molecules for cadmium resistance.

Supplemental Table 4 (related to Table 1). Long-term effects of small molecules on ATP levels and DNA damage-associated markers in the absence of H$_2$O$_2$. 1st tab: included are the data shown in Supplemental Figure 5, which addressed long-term effects of small molecules on ATP levels. Of 32 core-set hits, 28 molecules were analyzed in multiple 384-well plates in replicates in two batches. 11 small molecules that decreased the ATP content by 50% or more on day 5 of treatment, due to their effects reducing cell growth and/or cell viability, are highlighted. Unlike rapamycin, which also reduced ATP levels substantially, 9 of these 11 molecules (Gr-3B, Gr-6C, Gr-7A, Gr-7B, Gr-7C, Gr-9B, O11, O21 & O27) also produced cell toxicity (by the examination of cell morphology, data not shown). The other two, Gr-1F and Gr-3C,

caused DNA damages, like the others. 2nd tab: WI-38 cells that had been treated with small molecules (10 μM, 24 hours) were analyzed in two independent experiments by immuno-staining for two DNA damage-associated markers, phosphorylated-γH2A.X and phosphorylated-TP53BP1. Small molecules that induced both markers (in the absence of H_2O_2) were scored positively for DNA-damaging. Normalized values for marker-positive cell fractions are shown (n = 3, Student' t-test).

In experiment 2 to analyze DNA damage-associated markers, H_2O_2 was also introduced during the last 3 hours of incubation for one set of plates. For the other set that was not exposed to H_2O_2, several wells of cells were treated with the controls, including DMSO (0.1%) and doxorubicin (300 nM) for 24 hours or for H_2O_2 (700 μM) 3 hours. The fractions of DNA damage marker-positive cells were: for γH2A.X-P – DMSO negative control, ~1.7±0.4% (n = 6); doxorubicin positive control, ~16.9±2.1% (n = 6); and H_2O_2 positive control, ~50.9±4.2% (n = 3). For TP53BP1-P – DMSO negative control, ~1.0±0.2% (n = 6); doxorubicin positive control, ~15.1±1.4% (n = 6); and H_2O_2 positive control, ~45.8±5.1% (n = 3). Green, decreased; red, increased. In certain cases, we observed a strong trend of increasing DNA damage-associated markers, while the p values did not reach statistical significance, possibly due to a rather large variation of the samples. Note that besides the 32 core-set molecules, as expected, three PARP inhibitors (*) increased significantly the fraction of cells positive for DNA damage-associated markers upon H_2O_2 treatment.

Supplemental Table 5 (related to Figure 3). A) List of significant genes identified in RNA-seq analysis for cells treated with Gr-4D. WI-38 cells were treated with Gr-4D

(2.5 μM, n = 6; from two different vendors – CB, ChemBridge; MP, MolPort) for 24 hours (in the absence of H_2O_2) and then analyzed by RNA-seq to address Gr-4D's effects on gene expression. Genes whose expression levels are either up- or down-regulated significantly (FDR-adjusted q value < 0.05) are shown in red or blue, respectively (unlogged effect size > 1.5-fold, or > 1.25-fold, plus significant genes identified in samples treated with both Gr-4Ds). Total number of significant genes is indicated on the top. B) List of significant genes identified by SAM analysis of microarray for cells treated with other small molecules. WI-38 cells were treated with small molecules (10 μM, n = 3) for 24 hours (in the absence of H_2O_2) and then analyzed by microarray analysis to address small molecules' effects on gene expression. Besides the 0.1% DMSO negative controls (n = 15), rapamycin (2.5 μM, n = 6) was included for comparison as well. For each small molecule treatment (group-4 and group-5 molecules from different vendors: CB, ChemBridge; CD, ChemDiv; and V, Vitascreen), genes whose expression levels are either up- or down-regulated significantly are shown in red or blue, respectively. Total number of significant genes is indicated on the top, with unlogged fold change ($\geq$ 1.5) and FDR-adjusted q value [< 0.10 (10%)] shown. Overlap between significant genes affected by the same molecules (from different vendors) or molecules of the same structural group were shown as well.

Supplemental Table 6 (related to Figure 3). A) Summary of pathway analysis of Gr-4D RNA-seq data. 1st tab: analysis of 550 significant genes (normalized fold change $\geq$ 1.25) using Enrichr or PANTHER GO analysis tool. Shown were top pathways: for Enrichr-KEGG analysis, with a high combined score ($\geq$ 10), which combines both FDR-

adjusted P value and Z score and represents the best rankings of enrichment (Chen et al. 2013); for PANTHER GO analysis, ranked by FDR-adjusted significance (< 0.05) for each GO term category (pathways, molecular function, biological process, etc). Significant genes (up- or down-regulated, in red or blue) were shown also for several top pathways of interest. 2nd tab: comparison of significant genes identified in WI-38 cells treated with our small molecules with other perturbation conditions (gene knockdown or small-molecule treatment). B) Summary of pathway analysis results for other small molecules analyzed by microarray. One tab showed only top pathways (with FDR-adjusted significance < 0.05), plus genes of the pathway indicated by certain analysis for each molecule, and another tab showed the iLINCS similarity analysis results. Note that no significant similarity was suggested using significant genes for Gr-5D.

Supplemental Table 7 (related to Table 1). Potential mTOR inhibition by certain small molecules. Small molecules were examined initially at 4 doses (2.5 μM, 5 μM, 10 μM and 20 μM, n = 4 for each dose) and then retested twice by In-Cell Western analyses for their effects on the phosphorylation status of ribosomal protein S6, a readout of mTOR activity. Small molecules that caused more than 30% reduction in the p-RPS6/RPS6 ratio in at least 2 of 3 independent experiments were regarded as candidate mTOR inhibitors. Note that rapamycin, as the positive control, reduced the normalized ratio of p-RPS6/RPS6 substantially. Unlike rapamycin, these putative upstream inhibitors were toxic to cells at the doses examined.

Supplemental Table 8 (related to Figure 4). Effects of small molecules on *C. elegans'* lifespan and H_2O_2-resistance. Multiple independent trials were conducted to analyze the effects of small molecules on lifespan. Trials 1 and 2 were conducted in liquid (1.0X10^9 bacteria/ml and 2.5X10^9 bacteria/ml, respectively), using wild-type animals in the presence of 100 μM FUDR to block progeny production; and small molecules were administered at the final concentration of 60 μM (containing 0.3% DMSO in liquid). Trials 3 and 4 were conducted on plates (2.0X10^10 bacteria/ml and 1.0X10^11 bacteria/ml, respectively; 100 μl per plate, treated with UV and kanamycin), using temperature-sensitive sterile mutant animals in the absence of FUDR; and small molecules were provided at 60 μM (containing 0.2% DMSO on plates). Two confirmative trials 5 and 6 were also conducted, using the same conditions as trials 3 and 4.

For the lifespan assay to address the requirement of *skn-1* and/or *sesn-1*, RNAi was initiated from day 1 of adulthood using RNAi-sensitive *rrf-3* mutants. We also analyzed *sesn-1(ok3157)* mutants, which carry a 535-bp genomic lesion that results in 410-bp deletion of coding sequence from exon 3, and presumably, premature stop codons that might disrupt the messenger RNA. These mutants were outcrossed to the Kenyon lab wild-type strain (N2E) twice and genotyped, using primers suggested by the CGC. To analyze effects of Gr-4D on wild type or *sesn-1(ok3157)* mutants, animals were raised on plates containing DMSO or Gr-4D at different doses beginning on day 1 of adulthood. In this experiment, a high fraction of animals showed protruded vulva phenotype, likely due to early exposure to FUDR during development, and were censored. Also, Gr-4D at the highest dose appeared to exert toxicity in this experiment

and did not extend lifespan of wild type (see Supplemental Table 8). The reason for this

discrepancy was unclear. Nonetheless, Gr-4D at two lower doses was able to extend

wild-type's lifespan.

For the H_2O_2-stress resistance assays, wild-type animals were treated first with

small molecules (60 μM) and then subjected to 0.5 mM H_2O_2 in the presence of small

molecules (1st trial, 8-day-old; 2nd trial, 6-day-old. Note that the mean survival times of

controls are different between these two trials). Shown are the normalized changes of

mean survival time (mean ± SEM; in days for lifespan assay, and in hours for H_2O_2

stress assay) and observed/total numbers of animals (log-rank test, $P < 0.05$ high-

lighted). Green, lifespan increased; red, lifespan decreased.

Supplemental Table 9. Potential targets for small molecules identified in other

assays. Shown are potential (direct or indirect) human protein targets for 9 small

molecules that were identified and reported previously in other screens. 9 of our small-

molecule hits scored positively (from PubChem,

https://www.ncbi.nlm.nih.gov/pccompound). 1st tab: list of potential (direct or indirect)

human protein targets of each small molecule (see URLs below), based on these

screens.