Supplemental Material for Strohecker et al. "Identification of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB4) as a Novel Autophagy Regulator by High Content shRNA Screening"

Supplemental Methods

Arrayed shRNA screening 11,415 hairpins representing 1383 unique genes encompassing the murine protein kinome, GTPases, and components of the vesicle trafficking machinery, listed in Supplementary Table S1, were screened to identify autophagy substrate modulators following metabolic stress and recovery.

Cells were plated into black barcoded flat bottom 384 well plates (Corning 8793BC) at a density of 700 cells/well by the Biotek microfill (Winooski, VT) and allowed to attach overnight. Infection and media changes for plates were achieved by use of two robotic liquid handlers at the Broad Institute, the Perkin Elmer Janus and EP3 (Akron, OH). Viral plates were populated with 20 control hairpins (targeting either RFP, luciferase, or EGFP) in addition to wells containing no virus. An additional two hairpins targeting p62 were manually spiked into each plate at the time of infection to ensure that positive and negative controls were present on all plates. Immediately prior to infection, media was changed with the Janus robot to DMEM containing 8 μg/mL polybrene. The EP3 robot was used to add 6 μl of virus to each well. Cells were spin infected (2250 rpm 30 mins, 30°C.) in the presence of 6 μl of virus and 8 μg/ml of polybrene before returning to the 37°C incubator. Virus and polybrene containing media was removed 4 hours post infection and cells were incubated with normal growth media overnight (DMEM high glucose, 10% FBS, 1% penicillin streptomycin). Viral infections were done in quadruplicate. 24 hours post infection, media was changed with the Janus to DMEM containing 3 μg/mL puromycin (for the three puro plus plates) or DMEM alone (for the puro minus replicate). Following 72 hour puromycin selection, media containing DMEM high glucose was removed, and cells were washed twice in ischemia media (DMEM

containing no glucose, 10% FBS, 1% PS) to remove residual glucose in wells, and subsequently transferred to a hypoxia incubator set to 1% oxygen. At the conclusion of the 7.5 hour metabolic stress, cells were allowed to recover in normal growth media (DMEM high glucose 10% FBS, 1% PS) overnight at 37°C. 18 hours post recovery, media was removed from plates and the cells were washed twice in PBS prior to fixation in a 4% paraformaldehyde/PBS solution for 10 minutes. Nuclei were visualized by inclusion of Hoechst 33342 at a dilution of 1:10,000 in the fixation solution. Plates were washed 3 times with the ELx405 automated plate washer (Biotek, Winooski, VT). 80 μl of filtered PBS was left in each well at the end of washing to allow for evaporation during imaging.

Image acquisition and p62 aggregation algorithm Plates were imaged on the Arrayscan VTI (Thermo Scientific) housed within the Genome Technology Core of the Whitehead Institute using a modified version of the Cellomics Compartmental Analysis BioApplication. Nuclei were visualized in channel 1. EGFP-p62 aggregates were visualized in channel 2. Nine images per channel were captured for each field, with an autofocus field interval of 3. MEAN valid object count channel 1 represents the mean nuclear count within the field. MEAN ring spot average integrated intensity channel 2 represents the mean intensity of the p62 aggregates in the field. To properly identify the p62 aggregates the following settings were employed: Spot kernel radius: 10, ring distance from nucleus: 0, ring width: 10 pixels. A p62 aggregate score equal to Mean Ring Spot total intensity/Mean nuclei was calculated for each well. Viral infections were done in quadruplicate, with three plates receiving puromycin, one not. Values from the three ‘yes puro’ plates were averaged, and used to calculate a robust z-score for each hairpin, a standard metric for high throughput assays (91). A comparison of the nuclei counts from the puro+/puro- plates allowed calculation of the infection efficiency of each

hairpin. Hairpins with less than 1500 nuclei per well (which may represent autofocus issues or toxicity) or those that had an infection efficiency less than 25% were omitted from subsequent analysis. Data was exported to Excel for further analysis.

Screen quality control and candidate selections Data quality (batch-to-batch variation, similarity of replicates, control distribution) was examined with Spotfire decision analysis software (TIBCO, Palo Alto, CA) and RNAeyes. A subset of viral plates was re-screened to ensure reproducibility of the assay and analyses.

The in-house Gene-E software ranked genes at both a hairpin and a gene level (65) using three distinct ranking tools: the preferred 'Second best' analysis (ranking based on performance of second highest scoring hairpin), weighted sum analysis (in which 75% of the score is based on the robust z-score of the second best hairpin for a given gene, while the other 25% of the score is based on the rank of the robust z-score of the best hairpin), or the Kolmogorov-Smirov Statistic that was basis of the original ranking tool of the TRC. Complete Screen data is contained in Supplemental Table S1.

RNA Interference Lentiviral shRNA clones targeting the murine protein kinome, GTPase and vesicle trafficking libraries were obtained from the RNAi Consortium (TRC) of the Broad Institute of MIT and Harvard (www.broadinstitute.org/rnai/trc/library), publically available through Sigma-Aldrich. Genes within these libraries are targeted by between five and ten unique hairpins. Hairpins for Fig.3 are listed in order of appearance in the manuscript (A,B): *Mtor (*TRCN0000232387, TRCN0000054979*), Atg7 (*TRCN0000092163), *Becn1 (*TRCN0000087288, TRCN0000087290*), Ulk1 (*TRCN0000028768, TRCN0000028755), *Prkaa1 (*TRCN0000220671, TRCN0000220672) *, Ikbkb (*TRCN0000026913, TRCN0000026945*), Hrs (*TRCN0000088688, TRCN0000088689*), Etnk1 (*TRCN0000024554, TRCN0000024557)

*Rab7 (*TRCN0000100881, TRCN0000100883*),* The following control hairpins were used to silence *RFP* expression *(*TRCN0000072208, TRCN0000072212*).*

For transient silencing studies, cells were transfected with 50nM of Dharmacon siGENOME smartpools targeting mouse or human PFKFB4 (#M-054640-01-0010 or #M-006764-01-0010) or non-targeting siRNA pool (#D-001206-14-20) using Dharmafect1 per the manufacturer's instructions (Dharmacon Reasearch Inc.).

Generation of stable cell lines *Beclin1*$^{+/-}$; iBMKs (without EGFP-p62 reporter), K-Ras-TDCLs and PC3 cells stably expressing ptf-LC3 were generated using Lipofectamine 2000 (Invitrogen) followed by neomycin selection (500μg/ml for iBMKs and K-Ras-TDCLs, and 750μg/ml for PC3 cells). Single cell colonies were picked and expanded for use in subsequent experiments.

Production of a PFKFB4 antibody The cDNA clones for PFKFB4 (Clone ID: 6810667) was obtained from Open Biosytems. A 48-nucleotide trunctation at N-terminus of the gene was corrected to obtain the complete wild-type gene sequence, which was cloned into the *pet22b* overexpression vector, and protein was purified from *E. coli*. inclusion bodies under denaturing conditions. The purified protein was run on SDS-PAGE and the gel band containing the purified protein was cut, crushed and sent to Cocalico Biological Inc. for immunizations. Test sera after different booster doses were analyzed for reactivity with the antigen and the endogenous protein in cell lysates. Antibody specificity was confirmed by western blot of lysates in which PFKFB4 expression had been silenced via siRNA (data not shown). Antibodies against PFKFB4 were further purified using Protein-G columns.

Statistical Analyses Statistical analyses were carried out with GraphPad Prism version 5.0. Mean ± standard deviation (S.D.) are presented. Data shown are the result of three

independent experiments. Statistical significance was calculated by two way ANOVA with Bonferroni post-test.

Supplemental Figure Legends

Supplemental Figure 1. Validation of Selected Screen Hits

Complete validation western blots shown in Figure 3. Lanes included in Figure 3 are marked A and B as in the main body of the text.