**SUPPLEMENTARY FIGURES AND TABLES CAPTIONS**

**Supplementary Figure S1.** A) Agarose gel electrophoresis of single and multiplex dU-containing hetero-duplex 3Cs DNA. Single strand (ss); double strand (ds); wild type (WT); mCherry (mCh); kilobase (kb). B) Distributions of sequencing depths of single and combinatorial GFP, mCherry, and GFP+mCherry 3Cs libraries. The median and quartiles are shown per library as red straight and black dotted lines, respectively. C) Distribution skew (skew) and completeness (compl.) per library, based on read counts derived from B).

**Supplementary Figure S2**. A) Distributions of sequencing depths of randomized and non-randomized 3Cs multiplex libraries with increasing diversity (1-4N, 0.91M). The median and quartiles are shown per library as red straight and black dotted lines, respectively. B) Distribution skew (skew) and completeness (compl.) per library, based on read counts derived from A). C) Distributions of sequencing depths of artificially skewed 3Cs multiplex libraries (1:1, 1:10, 1:100). The median and quartiles are shown per library as red straight and black dotted lines, respectively. D) Distribution skew (skew) and completeness (compl.) per artificially skewed 3Cs multiplex library, based on read counts derived from C). E-F) Comparison of single and double gene log2 fold changes for the artificially skewed 3Cs multiplex library 1:1 screened with 20-fold (E) and 200-fold (F) coverages. Left panel: single phenotypes per cassette (h7SK vs hU6, r=0.84). Middle panel: single h7SK phenotype vs the double phenotype (h7SK vs double, r=0.94). Right panel: single U6 phenotype vs the double phenotypes (double vs hU6, r=0.96). Regression line in blue.

**Supplementary Figure S3.** A-B) Hierarchically clustered pairwise sample correlation (Pearson’s correlation coefficient, r) for all replicates (n1-n3) of the artificially skewed libraries (1:1, 1:10, 1:100) and coverages (20x, 200x). The values in individual cells indicate r of the normalized read counts. The color code visualizes no correlation (black) to high correlation (white), at gRNA (A) and gene (B) level.

**Supplementary Figure S4.** A-B) Distribution skew (skew) and completeness (compl.) for single (A) and multiplex (mpx) (B) autophagy libraries. C) Cumulative distributions of the single (orange) and multiplex (blue) autophagy libraries. A uniformly distributed library (ideal) is shown in grey. Area under the curve values are indicated next to each library identifier. D) The fluorescent GFP-LC3-RFP reporter is cleaved into equimolar amounts of GFP-LC3 and RFP by endogenous ATG4 family proteases. While GFP-LC3 is degraded by autophagy, RFP remains in the cytosol and serves as an internal control to determine the ratio of GFP/RFP to estimate autophagic flux (adapted from Kaizuka et al., 2016). E) FACS-based autophagy flux analysis of the monoclonal RPE1 reporter cell line. The reporter cell line shows low levels of basal autophagy (6.83%). Torin1 induced autophagy (99.6%) is blocked by Bafilomycin A1 (4.21%). F) Correlation of the post-FACS autophagy multiplex-inherent and dedicated single screening data on gRNA (r=0.97) and gene level (r=0.97). Core essential genes, according to Hart et al., 2017, are highlighted in red, NHTs are highlighted in blue.

**Supplementary Figure S5.** Distributions of sequencing depths of all autophagy libraries and screen replicates.

**Supplementary Figure S6.** A-D) Hierarchically clustered pairwise sample correlation (Pearson’s correlation coefficient, r) on gRNA and gene levels. The values in individual cells indicate r of normalized read counts. Libraries are indicated with a blue bar on the left side of each heatmap. A) The color code visualizes no correlation (black) to high correlation (white). B) Pairwise correlation of the autophagy single library and single post-FACS replicates. C) Pairwise correlation of the autophagy multiplex (mpx) library and pre- and post-FACS replicates. D) Pairwise correlation of the autophagy mpx library and the post-FACS highgate replicates. E) Relative abundance of reads containing an *ATG4B*-targeting gRNA per post-FACS highgate replicate.

**Supplementary Figure S7.** MAGeCK derived significance of enriched genes post-FACS in the dedicated single (left) and multiplex (mpx)-inherent single (right) screens. Genes with a p-value<0.05 are highlighted in red. Significant hit genes unique to the respective analysis are highlighted in blue.False-discovery-rate (FDR).

**Supplementary Figure S8.** A) Comparison of single and double gene log2 fold changes for core essential genes (according to Hart et al., 2017) in the autophagy proliferation screen. Left panel: single phenotypes per cassette (h7SK vs hU6, r=0.59). Middle panel: single h7SK phenotype vs the double phenotype (h7SK vs double, r=0.81). Right panel: single U6 phenotype vs the double phenotypes (double vs hU6, r=0.91). Regression line in blue. B) Comparison of single and double gene log2 fold changes for core essential autophagy genes in the autophagy flux screen. Left panel: single phenotypes per cassette (h7SK vs U6, r=0.79). Middle panel: single h7SK phenotype vs the double phenotype (h7SK vs double, r=0.88). Right panel: double phenotypes versus single U6 phenotype (double vs U6, r=0.97). Regression line in blue. C) Autophagy flux validation of single essential autophagy genes when targeted with one (yellow) or two (blue) gRNAs. Error bars represent standard error of mean (SEM) over three biological replicates (n=3).

**Supplementary Table S1.** Oligonucleotide sequences used for 3Cs reactions, cloning, and sequencing library preparation.

**Supplementary Table S2.** Read counts for GFP-mCh library.

**Supplementary Table S3.** Read counts of randomized and 0.91M libraries.

**Supplementary Table S4.** Read counts of artificially skewed libraries and screens.

**Supplementary Table S5.** Read counts of autophagy single and multiplex libraries, and screens.

**Supplementary Table S6.** GI model summary.

**Supplementary Table S7.** hTERT-RPE1(Cas) RNA-seq data.