Supplementary Information for

# Graded regulation of cellular quiescence depth between proliferation and senescence by a lysosomal dimmer switch

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## SUPPLEMENTARY METHODS

### E2f-GFP, EdU, and PI readouts for cell cycle re-entry

Serum-stimulated cells were harvested at the indicated time points by trypsinization. For the E2f-GFP readout, harvested cells were fixed with 1% formaldehyde in DPBS. For the EdU assay, 1 μM EdU was included in culture medium throughout the serum-stimulation experiment; harvested cells were subjected to the Click-iT EdU reaction according to the manufacture's protocol (Invitrogen, C10418/C10340). For the PI assay, harvested cells were lysed in Nuclear Isolation Medium (0.5% bovine serum albumin, 0.1% NP-40, and 1% RNase A in DPBS) containing 5 μg/ml PI. E2f-GFP, EdU, and PI signal intensities in individual cells (~10,000 cells per sample) were measured using a BD LSRII or Invitrogen Attune Acoustic Focusing flow cytometer; the acquired data were analyzed using FlowJo software (v. 10.3).

### Assays for lysosomal mass, proteolytic activity, and mitochondrial ROS

To assess lysosomal mass, cells in serum-starvation medium were incubated with 50 nM LysoTracker Deep Red (Invitrogen, L12492) for 30 minutes. Subsequently, cells were either trypsinized and processed for flow cytometry, or washed with DMEM, placed back in serum-starvation medium, and observed under a Deltavision Elite Microscope (GE Healthcare). To count LysoTracker foci, images from a Cy5 filter were stacked across the Z-axis and binary processed to define foci. Cellular boundaries were manually determined based on images obtained from both POL and Cy5 filters. The foci number within each cell was determined using the particle analysis function in Fiji (1). To assess lysosomal proteolytic degradation, cells were incubated with 10 μg/ml DQ-Red BSA (Invitrogen, D12051) for an hour and subsequently incubated with or without lysosomal inhibitor for 5.5 hours. Cells were then stained with 2 μM CellTrace Violet (Invitrogen, C34557) for 20 minutes to stain the cell body, washed twice with DMEM, and placed back in serum-starvation medium for Deltavision imaging. To assess mitochondrial ROS level, serum-starved cells were stained with 3.25 μM MitoSox Red (Invitrogen, M36008) for 20 minutes and subsequently trypsinized and processed for flow cytometry.

### Modulation of lysosomal/autophagic function and lysosome biogenesis

To inhibit lysosomal/autophagic function, cells were treated with CQ (chloroquine; Sigma, C6628), Baf (bafilomycin A1; LC Laboratories, B-1080), nocodazole (Sigma, M1404), or vinblastine (CAYMAN, 11762) at the indicated concentrations. To inhibit lysosomal biogenesis, cells were treated with ML-SI3 or ML-SI4 (2) (a gift from Dr. Haoxing Xu) at the indicated concentrations. To enhance lysosomal/autophagic function, cells were transfected with a human MITF expression vector pEGFP-N1-MITF-A (Addgene, #38132) or control vector (pd2EGFP-N1 from Clontech, or pCMV-mCherry, a gift from Dr. Lingchong You) using Neon electroporation (Invitrogen). Briefly, approximately $10^6$ cells with 10 μg plasmid DNA were electroporated in a 100-μl Neon tip with a 20-ms pulse at 1900 V. Cells were plated in 6-well plates or 100-mm dishes at ~50% confluence and incubated in growth medium for 30 hours to allow recovery. Cells were further cultured in serum-starvation medium for 4 days before assessing the modulation of lysosomal function and quiescence depth by Mitf expression. To confirm ectopic MITF expression, cells transfected with pEGFP-N1-MITF-A or pd2EGFP-N1

were processed for immunoblot with anti-MITF antibody (abcam, ab20663), anti-beta Actin antibody (Thermo Scientific, MA5-15738), and secondary antibody (LI-COR, 926-68023, 926-32210).

**Autophagy flux assay**

Autophagy flux was measured by a LC3-II turnover assay, similar to Ref (3). Briefly, cells were incubated with or without 40 μM CQ for 3-6 hours, washed once with DPBS, snap-frozen in liquid nitrogen, and stored in -80 °C until cell lysis. Frozen cells were lysed on ice by RIPA lysis buffer (Cell Signaling, 9806S) and processed for immunoblot with anti-LC3B antibody (Sigma, L7543), anti-Tubulin alpha antibody (Thermo Scientific, RB-9281-P0), anti-GAPDH antibody (Thermo Scientific, MA1-140), and secondary antibody (LI-COR, 926-68023, 926-32210). Immunoblots were imaged using a LI-COR Odyssey Scanner and analyzed with Fiji software (1). The LC3-II Δ between CQ-treated and non-treated samples was quantified to reflect autophagy flux (3).

**β-galactosidase activity assay**

β-galactosidase activity was measured using a CellEvent Senescence Green Detection Kit (Invitrogen, C10850) according to the manufacturer's protocol. Briefly, REF cells were harvested by trypsinization, washed once with PBS, fixed with 2% formaldehyde for 10 minutes, washed further with 1%BSA PBS, and incubated with the β-galactosidase staining solution (containing a fluorescein-based substrate for β-galactosidase) for 2 hours at 37° C without $CO_2$. Cells were further washed three times with DPBS, resuspended in 1% BSA DPBS, and green fluorescence emitted from the enzyme-leaved product was measure by a BD LSRII flow cytometer.

**Cell size measurement**

Cell size was measured using a Moxi Z Mini Automated Cell Counter Kit (MXZ001, ORFLO) according to the manufacture's protocol. Briefly, cells were harvested by trypsinization, centrifuged, re-suspended in DPBS, and added to Moxi Z Cell Count Cassettes Type M (MXC001, ORFLO), which is then inserted into a Moxi Z Mini cell counter. The cell size measurement is based on the Coulter Principle: changes in electrical impedance are proportional to the volume of nonconductive particles (e.g., cells suspended in an electrolyte) passing through an aperture in the device.

**Cytotoxicity assay**

Cytotoxicity was determined by comparing the live cell counts in drug-treated samples and vehicle control samples, using a PI-fluorescence assay as described in Ref (4). Briefly, cells cultured in 96-well plates were incubated with 50 μg/ml of PI (propidium iodide, Biotium, 40016) for 10 min in the dark at room temperature. PI signal in each well was measured by a BioTek Synergy2 plate reader, and the signal intensity indicated the count of dead cells (that became permeable and incorporated PI). Cells were subsequently subjected to freezing and thawing; PI signal was measured again, and the signal intensity then indicated the total cell count (as all cells became permeable after freezing/thawing and incorporated PI). The difference of PI signal before and after freezing/thawing indicated the live cell count.

**cDNA library preparation, RNA-seq, and data preprocessing**

Total RNA was isolated with a Quick-RNA kit (Zymo Research, R1050). The quality of the RNA (RQN score ≥ 7.5) was confirmed using the Fragment Analyzer platform (Advanced Analytical Technologies). Libraries were prepared using the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB, E7490S) and NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, E7530L) according to the manufacturer's instructions. The final quality-ensured libraries were pooled and sequenced on an Illumina HiSeq 2500 for 100 bp paired-end sequencing. Paired-end cleaned reads were aligned to the rat reference genome rn6 (UCSC) using TopHat (v2.1.1) with default parameters (5). Transcript annotation and normalization to FPKM were handled using Cufflinks (v2.2.1) (5). Differentially expressed genes between two time points were identified based on fold difference > 2 in FPKM, after filtering out low (FPKM < 8) or inconsistent expression (fold difference > 2 between replicates). Raw data of RNA-seq can be accessed in the Gene Expression Omnibus (GEO) under the accession number GSE124109.

**Gene expression and pathway enrichment analysis of RNA-seq data**

To visualize the sequential transition of transcriptome, the expression matrix of differentially expressed genes was $\log_2$ transformed and subjected to Principal Component Analysis using the R function "prcomp", with the result visualized using the R package rgl. For gene expression clustering analysis, FPKM was $\log_2$ transformed and mean-centered on each gene. K-means clustering was performed by Cluster 3.0 (6) and the optimal cluster number was decided by silhouette width. Hierarchical clustering was performed by Cluster 3.0 using the average linkage method. Clustering results were visualized as heat maps using Java Treeview (7).

Pathways enriched in K-means clusters were analyzed with the DAVID functional annotation tool (8). Significantly enriched KEGG pathways (p.adj < 0.05) were determined in the KEGG over-representation test using the R package clusterProfiler (9). Gene Set Enrichment Analysis (GSEA) (10) was performed to identify gene sets significantly correlated with quiescence depth, run in the "continuous phenotype" mode using the gene set "c2.all.v6.0.symbols.gmt.geneset" from MSigDB (10) with the sample label corresponding to serum-starvation days (e.g., 2 for 2-day serum starvation). Genes were ranked by Pearson correlation. Identified significant gene sets (FDR ≤ 0.1) were visualized by NetworkX (11) and Gephi (12) in a network to resolve gene sets redundancy; two gene sets with a Jaccard index > 0.5 were connected by an edge, and node size was set to reflect the normalized enrichment score (NES).

**TF-target and lysosomal co-expression network construction**

To construct a TF-target network, TF-target interactions were downloaded from RegNetwork (13) and PAZAR (14) (mouse interactions were used as rat data were unavailable), based on which differentially expressed genes were connected into a directional graph using the Python package NeworkX (11) and visualized using Gephi (12) with the Force Atlas mode. The size and color of a node were determined by its betweenness centrality and expression level, respectively.

To construct a lysosomal co-expression network, differentially expressed genes in the form of a $\log_2$-transformed expression matrix were clustered into co-expression modules using the blockwiseModules function in the R package WGCNA (15), with the soft-thresholding power and mergeCutHeight set to 20 and 0.25 respectively. The co-expression module containing the largest number of lysosomal genes up-regulated with quiescence deepening was chosen as the lysosomal co-expression network. Genes in the network were connected based on their co-expression degree (i.e., pairwise correlation) with an adjacency threshold of 0.25. Lysosomal genes and TFs were identified using KEGG (16) and the DBD transcription factor database (17), respectively. The network was visualized using Cytoscape (18).

**Quiescence-depth signature model**

To identify a gene expression signature reflecting quiescence depth, linear regression with an elastic net penalty was performed on the time-course RNA-seq data (2- to 16-day serum starvation) using the R package penalized (19), with the sample label set to indicate serum-starvation days (e.g., 2 for 2-day serum starvation). The optimal tuning parameters for L1 and L2 penalties were determined by maximizing the cross-validated log-likelihood across the $\lambda_1$ and $\lambda_2$ combinations ($0.01 \leq \lambda_1 \leq 200$, $1 \leq \lambda_2 \leq 100{,}000$). A gene signature reflecting quiescence depth was identified in the resultant regression model. When applied to analyze a given RNA-seq dataset, this regression model generates a corresponding "quiescence depth score" (QDS). See the section below for detailed procedures and scripts.

**Calculate QDS**

*a)* The RNA-seq data corresponding to quiescence deepening of REF cells (Qui-REF data) was converted to FPKM $\log_2$ transformed mean-centered expression matrix. See below for the first 5 genes by alphabetical order in the matrix, with a sample label row (G0_depth corresponding to the days of serum starvation) added on the top of the gene rows. GA_n, growing cell sample replicate n; XNA_n, quiescent cell sample replicate n under serum starvation for N days. Note that only quiescent cell samples (X2As to X16As) but not growing cell samples (GAs) were used to build the linear regression quiescence-depth model and calculate QDS values in the subsequent steps.

```
> head(data1)
              GA_1        GA_0        GA_2       X2A_0       X2A_1       X2A_2       X3A_2       X3A_0       X3A_1       X4A_0       X4A_2
G0_depth  0.00000000  0.00000000  0.00000000  2.00000000  2.00000000  2.00000000  3.00000000  3.00000000  3.00000000  4.00000000  4.00000000
A1bg     -0.00149915 -0.00149915 -0.00149915 -0.00149915 -0.00149915 -0.00149915 -0.00149915 -0.00149915 -0.00149915 -0.00149915 -0.00149915
A1cf      0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000
A2m       0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000
A3galt2  -0.16290932 -0.40189887 -0.32475935  0.40733677  0.67489912  0.45937372  0.61453994  0.57310582  0.56383070  0.44782204  0.67642411
A4galt    0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000
              X4A_1       X6A_2       X6A_1       X6A_0       X8A_2       X8A_1       X8A_0      X10A_0      X10A_1      X10A_2      X12A_0
G0_depth  4.00000000  6.00000000  6.00000000  6.00000000  8.00000000  8.00000000  8.00000000 10.00000000 10.00000000 10.00000000 12.00000000
A1bg     -0.00149915 -0.00149915 -0.00149915 -0.00149915 -0.00149915 -0.00149915 -0.00149150 -0.00149915 -0.00149915 -0.00149915  0.04347536
A1cf      0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000
A2m       0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000
A3galt2   0.52860738  0.19940421  0.23507016  0.13073103 -0.02971416  0.03552771 -0.00695858 -0.23682835 -0.60220172 -0.22618502 -0.59459933
A4galt    0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000
              X12A_1      X12A_2      X14A_0      X14A_1      X14A_2      X16A_0      X16A_1      X16A_2
G0_depth 12.00000000 12.00000000 14.00000000 14.00000000 14.00000000 16.00000000 16.00000000 16.00000000
A1bg     -0.00149915 -0.00149915 -0.00149915 -0.00149915 -0.00149915 -0.00149915 -0.00149915 -0.00149915
A1cf      0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000
A2m       0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000
A3galt2  -0.21111061 -0.44560809 -0.48060965 -0.20121781 -0.34247248 -0.51898619 -0.35367676 -0.40693644
A4galt    0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000  0.00000000
```

*b)* The Penalized package (https://cran.r-project.org/web/packages/penalized) and expression matrix in *a* were loaded in R (see #1 and #2 respectively in the script below), transposed (#3), and converted into a dataframe (#4). See the R package manual and vignette for detailed descriptions of the used functions in the script.

```
> library(penalized)        #1 load the penalized package
Loading required package: survival
Welcome to penalized. For extended examples, see vignette("penalized").
> setwd("the path to your preferred directory")
> data1 = read.table(file="QuiREFdata.txt",header=T,row.names=1,sep="\t")          #2 load Qui-REF data
> data2 <- t(data1)                  #3 transpose the data
> ncol(data2)
[1] 17350
> nrow(data2)
[1] 30
> data2 <- as.data.frame(data2)    #4 convert it into dataframe
> is.data.frame(data2)
[1] TRUE
```

*c)* The optimal lambda1 (λ1) was searched to perform linear regression. First, we sparsely searched a broad range (0.01 to 200, r1) of λ1 values in log scale (#5-6 in the R script below). We then decide a λ1 range (r2) that gave greater cross-validated log likelihoods (cvl) than did the other parts of r1 (#7-8).

```
> lseqBy <- function(from=1, to=100000, by=1, length.out=log10(to/from)+1) {
+   tmp <- exp(seq(log(from), log(to), length.out = length.out))
+   tmp[seq(1, length(tmp), by)]
+ }                                                    #5: create a sequence of numbers in log2 scale
> set.seed(1)
> foldid <- sample(rep(seq(9),length=27))
> L1list <- lseqBy(0.01,200,by=1)                    #6: create a sequence of λ1
> L1list
[1]   0.01000000   0.07247797   0.52530556   3.80730788  27.59459323 200.00000000
> elasticnet <- lapply(L1list, function(a){
+          profL2(data2[(nrow(data2)-26):nrow(data2),1], data2[(nrow(data2)-26):nrow(data2),2:ncol(data2)], fold=foldid, minl = 1,
  maxl = 100000, log=TRUE, standardize=TRUE, lambda1 = a, steps=10)
+ })                                                   #7: calculate cvl for each fixed λ1. The Standardize parameter is set True.
lambda= 1e+05  cvl= -70.76947
lambda= 27825.59         cvl= -61.01386
lambda= 7742.637         cvl= -73.51967
lambda= 2154.435         cvl= -290.543
lambda= 599.4843         cvl= -2523.886
……
lambda= 1         cvl= -81.74782
>
> for (i in 1:length(L1list)) {print(max(elasticnet[[i]]$cvl))}          #8: print out cvls for the list of λ1, and decide the range r2 that
  gives the greater cvl values.
[1] -61.01386              #when λ1 = 0.01000000
[1] -61.03069              #when λ1 = 0.07247797
[1] -61.11649              #when λ1 = 0.52530556
[1] -59.93934              #when λ1 = 3.80730788
[1] -59.80203              #when λ1 = 27.59459323
[1] -81.74782              #when λ1 = 200.00000000
```

*d)* Next, we searched for the optimal λ1 within the decided r2 in *c* ($3.80730788 < \lambda 1 < 50$) in a finer linear scale. Briefly, a sequence of λ1 was created in linear scale (#9 in the R script below); cvl was calculated for each λ1 value (#10); the λ1 that gave the maximum cvl was determined (#11).

```
> set.seed(1)
> foldid <- sample(rep(seq(9),length=27))
```

```
> L1list <- seq(3.80730788,50,by=1)        #9: create a sequence of λ1 (3.80730788 < λ1 < 50)
> L1list
 [1]  3.807308  4.807308  5.807308  6.807308  7.807308  8.807308  9.807308 10.807308 11.807308 12.807308 13.807308 14.807308
  15.807308 16.807308
[15] 17.807308 18.807308 19.807308 20.807308 21.807308 22.807308 23.807308 24.807308 25.807308 26.807308 27.807308
  28.807308 29.807308 30.807308
[29] 31.807308 32.807308 33.807308 34.807308 35.807308 36.807308 37.807308 38.807308 39.807308 40.807308 41.807308
  42.807308 43.807308 44.807308
[43] 45.807308 46.807308 47.807308 48.807308 49.807308
> elasticnet <- lapply(L1list, function(a){
+          profL2(data2[(nrow(data2)-26):nrow(data2),1], data2[(nrow(data2)-26):nrow(data2),2:ncol(data2)], fold=foldid, minl = 1,
  maxl = 100000, log=TRUE, standardize=TRUE, lambda1 = a, steps=10)
+ })                                          #10: calculate cvl with each fixed value of λ1
lambda= 1e+05  cvl= -71.46391
lambda= 27825.59         cvl= -61.80161
lambda= 7742.637         cvl= -59.93934
lambda= 2154.435         cvl= -73.48978
lambda= 599.4843         cvl= -94.69094
……
lambda= 1        cvl= -71.00383
>
> for (i in 1:length(L1list)) {print(max(elasticnet[[i]]$cvl))}
[1] -59.93934
[1] -58.67292
[1] -57.88228
[1] -57.4384
[1] -57.04895
[1] -55.94346
[1] -55.31691
[1] -55.02455
[1] -54.96632     #11: the maximum cvl with λ1 = 11.807308
[1] -55.08132
……
 [1] -67.61579
[1] -67.94743
```

*e)* The optimal lambda2 (λ2) was determined given the optimal λ1 in *d* (#12-13 below).

```
> opt2 <- optL2(data2[(nrow(data2)-26):nrow(data2),1], data2[(nrow(data2)-26):nrow(data2),2:ncol(data2)], fold=foldid, lambda1 =
11.807308, standardize=TRUE)                #12: determine the optimal λ2. Note that the standardize parameter is set True.
lambda= Inf              cvl= -81.74782
lambda= 1                cvl= -103.5782
……
lambda= 2079.757     cvl= -54.96309
> opt2$lambda
[1] 2079.644             #13: print out the optimal λ2 (λ2 = 2079.644)
> opt2$cvl
[1] -54.96309
```

*f)* Build a gene expression signature to indicate quiescence depth, using the optimal lambdas decided above (λ1 = 11.807308, λ2 = 2079.644). QDS was calculated for each quiescent cell sample (#14-15 below) and shown in the "mu" column of the output file (#16). 3,157 out of 17,349 genes in the Qui-REF data were included in the developed quiescence depth signature.

```
> pen <- penalized(G0_depth, penalized = data2[(nrow(data2)-26):nrow(data2),2:ncol(data2)], data = data2[(nrow(data2)-
  26):nrow(data2),], lambda1 = 11.807308, lambda2 = opt2$lambda, standardize=TRUE)        #14: again, the GA growing cell
  samples are omitted from building a quiescence depth signature.
# nonzero coefficients: 3157
> result <- predict(pen, data2[1:nrow(data2),2:ncol(data2)], data = data2[1:nrow(data2),])       #15: predict QDS for input samples
> write.table(result, file="L1L2_cooptimized_centered_penalized.txt",sep="\t")          #16: write the result to a table
> show(pen)
Penalized linear regression object
17350 regression coefficients of which 3157 are non-zero
```

```
Loglikelihood =  -33.28762
L1 penalty =       57.40157        at lambda1 =  11.80731
L2 penalty =       22.05085        at lambda2 =  2079.644
> result
            mu    sigma2
GA_1    1.675209 0.6892662
GA_0    2.346847 0.6892662
GA_2    1.766918 0.6892662
……
X16A_0 14.249556 0.6892662
X16A_1 14.578862 0.6892662
X16A_2 14.437392 0.6892662
```

*g)* The quiescence-depth regression model above was then applied to calculate QDS in other datasets (e.g., quiescence (20), senescence (21, 22), and aging (23, 24)), by executing #14-16 in *f* with the new input samples (FPKM normalized with the R package edgeR (25) if not previously normalized, $\log_2$ transformed, mean-centered as in *a*). In cases that not all of the 3,157-genes in the signature in *f* were included in the new dataset, common genes between the Qui-REF data and new dataset were identified; steps *a-e* above were repeated with this common gene set in training samples (X2As to X16As in *a*); the accordingly updated quiescence-depth signature was applied to the new dataset. If the new dataset was from another species (not rat), gene symbols were converted using NCBI HomoloGene. The resultant QDS reflects the relative "quiescence depth" of the cell type or tissue studied in the new dataset.

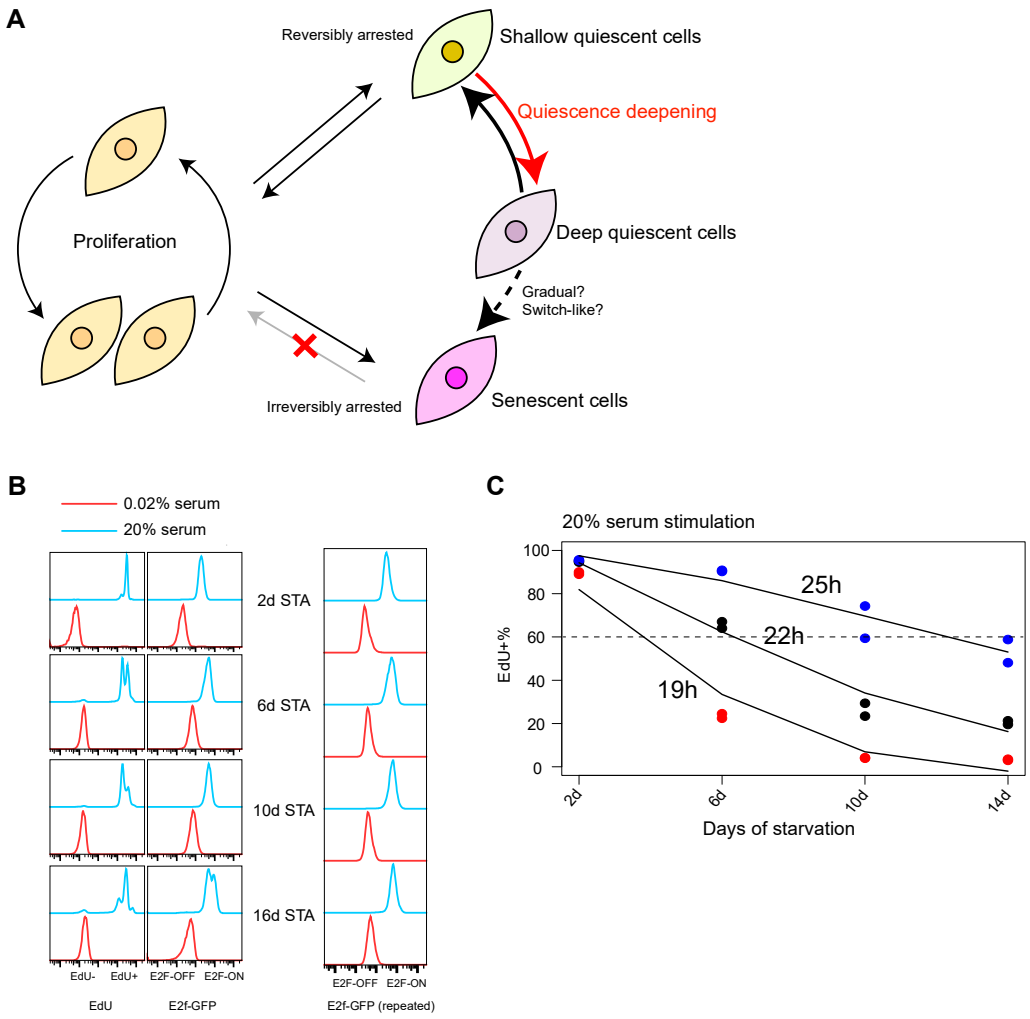


**Fig. S1. Deep quiescence model as a reversibly arrested cellular state.** (*A*) Model of quiescence deepening. The potential sequential transition of cells from shallow to deep quiescence, and eventually into senescence, is discussed later in the paper. (*B*) REF cells were serum starved from 2 to 16 days and then either kept in starvation medium (0.02% serum) or stimulated with 20% serum. Cells were subsequently harvested after 41 hours for E2f-GFP or EdU-incorporation profiling (~10,000 cells per sample, with the highest frequency set to 100% at the y-axis of each histogram). A repeated experiment performed at different time with the E2f-GFP readout is also shown. The EdU negative fraction upon serum stimulation (20%, 41 hours) was 2.2%, 4.9%, 3.1%, and 7.4% in cells previously under serum starvation for 2, 6, 10, and 16 days, respectively. (*C*) A repeated experiment of Fig. 1*A*. REF cells were serum starved from 2 to 14 days, stimulated with 20% serum for 19, 22, or 25 hours, and assayed for EdU incorporation (n = 2). Lines were fitted using the smooth.spline function in R.

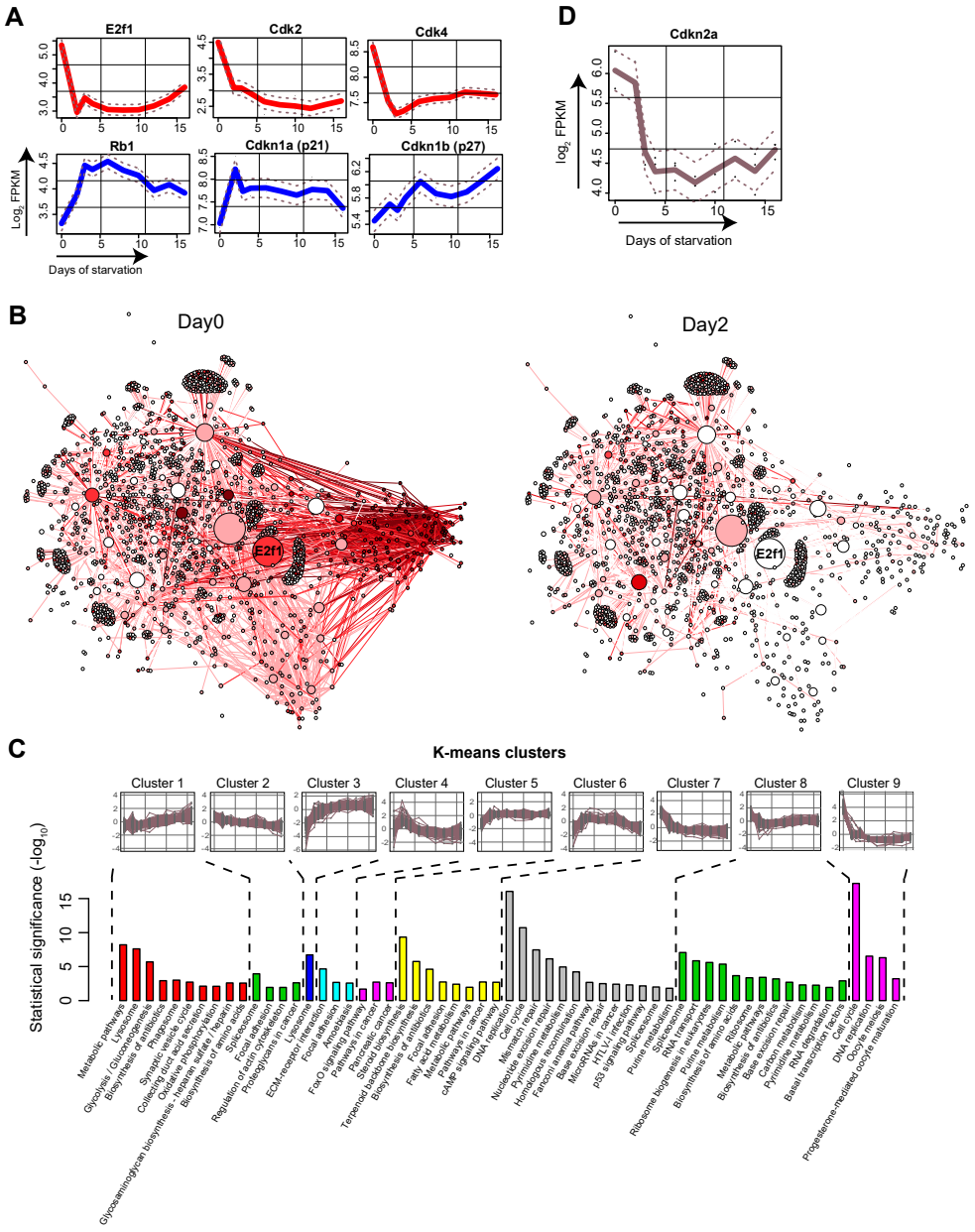


**Fig. S2. Pro- and anti-proliferative genes are down- and up-regulated, respectively, in serum-starved quiescent cells.** (*A*) Time-course expression of E2f1, Cdk2, Cdk4, Rb1, Cdkn1a, and Cdkn1b in RNA-Seq analysis (0- to 16-day serum-starved cells). Dashed line, s.e.m of fitted line. *(B)* Putative transcription factor (TF)-target network of proliferating (day0) and 2-day serum-starved (day2) REF cells. Node color intensity indicates relative gene expression (white to dark red, lowest to highest expression level). Node size indicates the betweenness centrality of the given node in the network. Color of an edge (TF → target) is the same as the color of the target node. (*C*) Pathways significantly enriched in K-means clusters from DAVID functional annotation analysis (8). Gene expression dynamics in each cluster (same as Fig. 2 *B*) is shown at the top. Y-axis, adjusted p-value calculated by Benjamini-Hochberg correction. (*D*) Time-course expression of Cdkn2a ($p16^{INK4A}$) in RNA-Seq analysis (0- to 16-day serum-starved cells). Dash lines, same as in *A*.

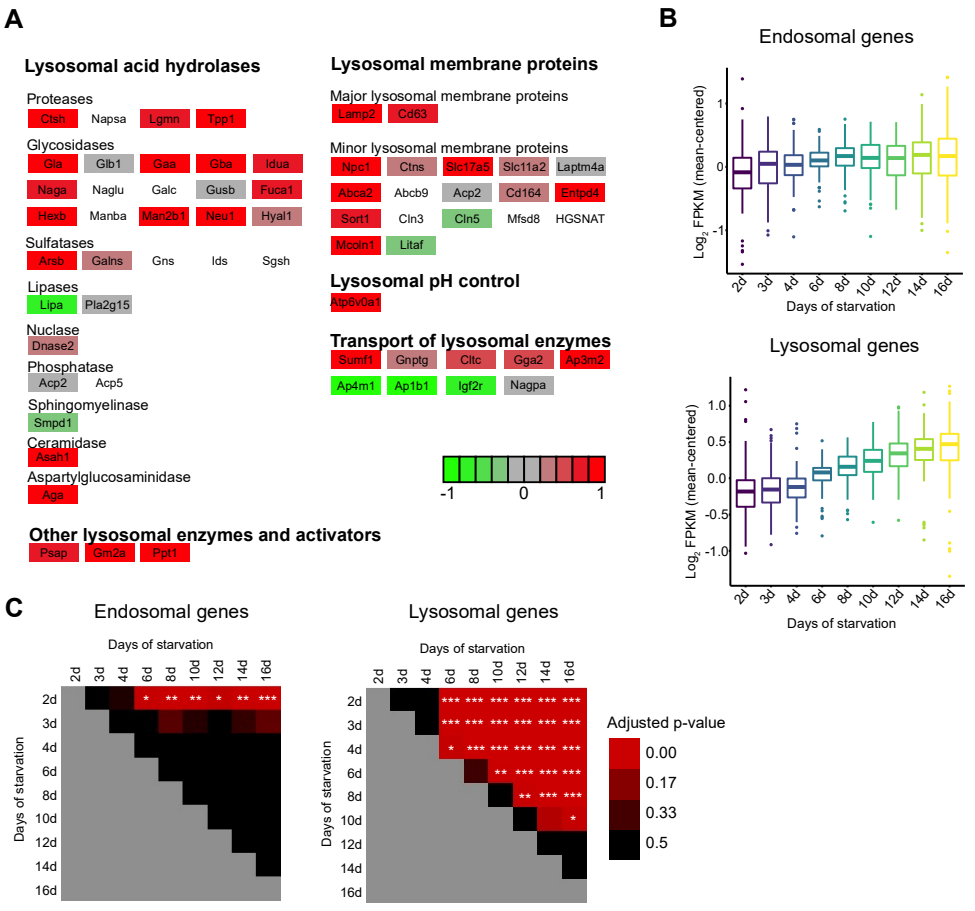


**Fig. S3. Lysosomal genes are up-regulated in deep quiescence.** (*A*) Up- or down-regulation of various categories of lysosomal genes in 16-day serum-starved cells compared to 2-day serum-starved cells are shown in red or green, respectively, with the degree of changes (log transformed) indicated by the color gradient bar. (*B*) Differentially expressed endosomal and lysosomal genes in RNA-Seq analysis (2- to 16-day serum-starved cells). (*C*) Multiple pairwise comparison (Tukey Honest Significance Differences, Tukey HSD) of gene expression means at different time points. *, **, and *** indicate adjusted p-value < 0.05, 0.01, and 0.001 in Tukey HSD, respectively. Endosomal and lysosomal genes in *A-C* are defined by MGD database (26).

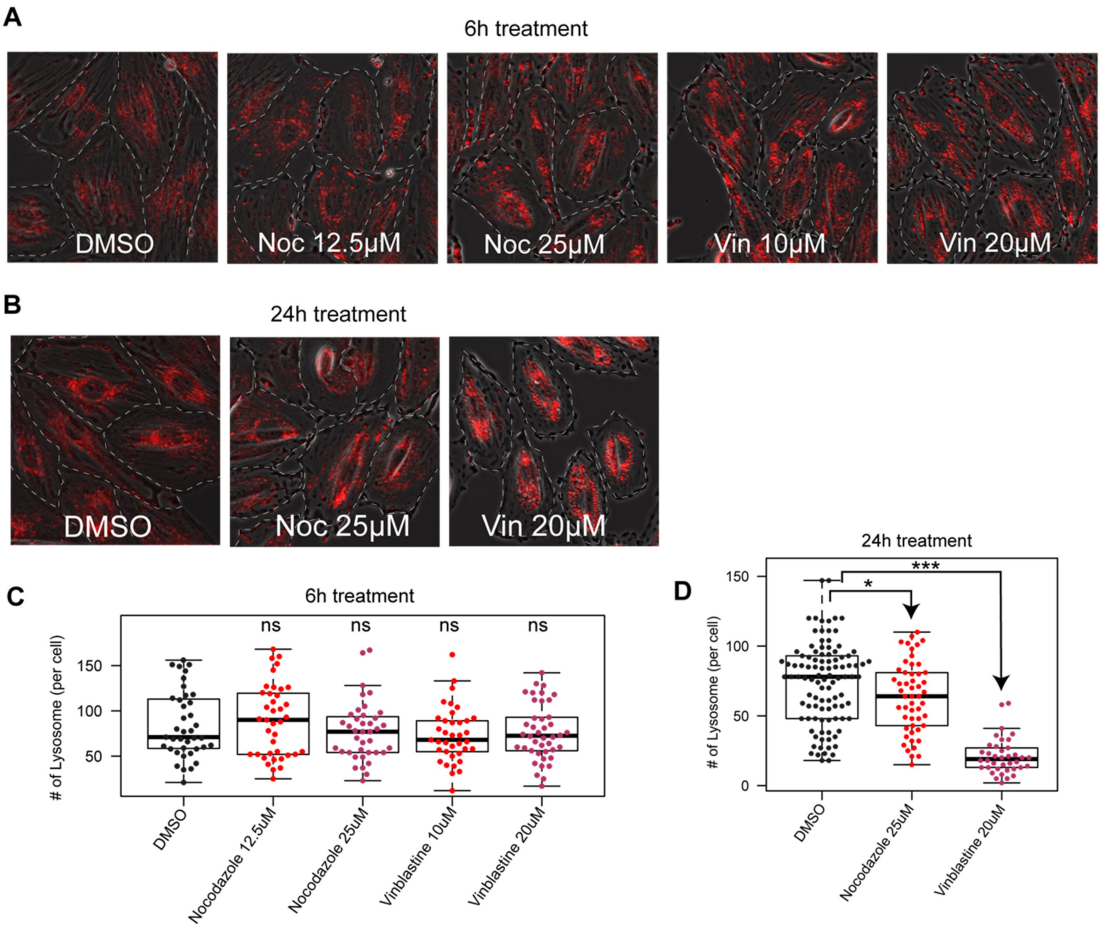


**Fig. S4. Inhibiting autophagy does not increase lysosomal number.** Cells were serum starved for 2 days and treated with nocodazole (Noc) and vinblastine (Vin) at the indicated concentrations for 6 hours (*A, C*) or 24 hours (*B, D*). Following LysoTracker staining, cells were imaged and quantified for the number of lysosomes (LysoTracker foci) per cell. (*A, B*) Representative microscopy images. (*C, D*) Quantifications of lysosomal number per cell (~40 cells per sample). ns, p-value > 0.05 (compared to the DMSO control, two-tailed *t*-test); * and ***, p-value < 0.05, and  < 0.001 (one-tailed *t*-test), respectively. Note that with 24-hour vinblastine treatment, quantified lysosomal number may be an underestimate due to lysosomes concentrating in the perinuclear region.

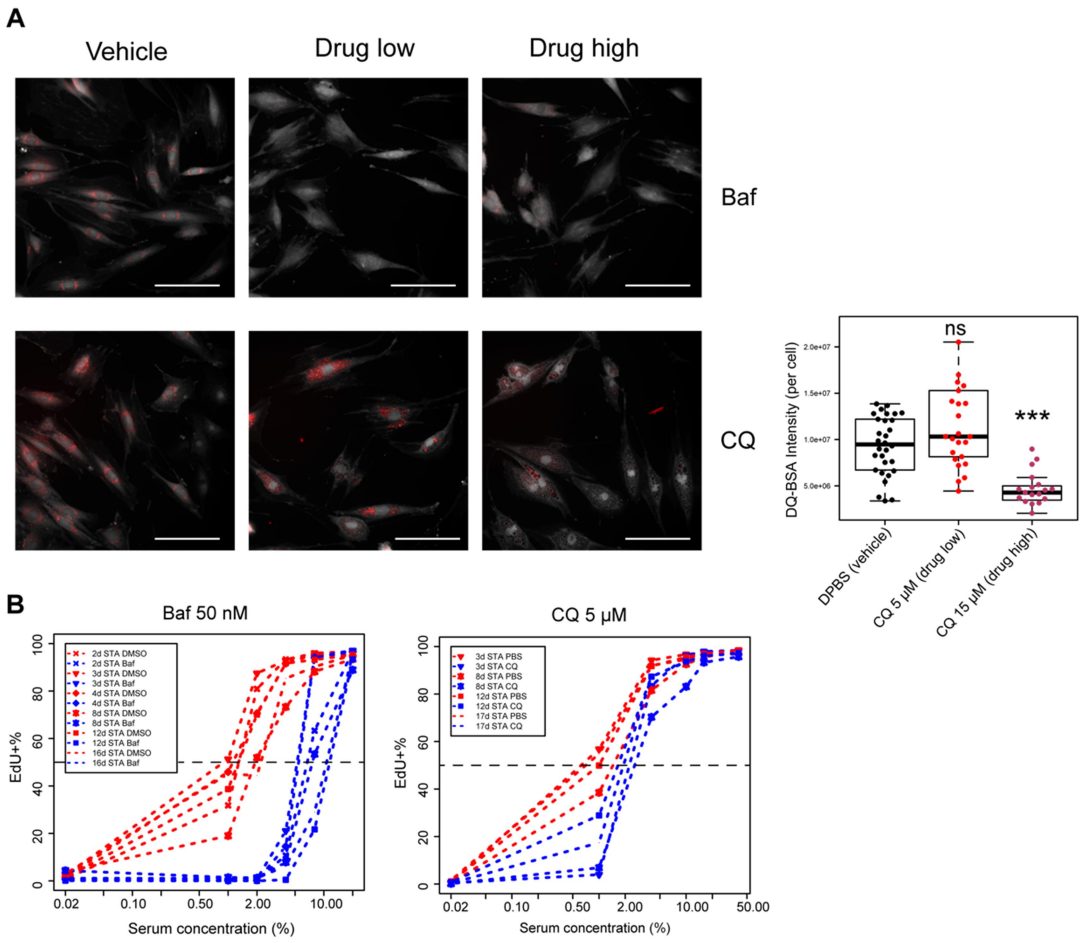


**Fig. S5. Inhibiting lysosomal/autophagic function deepens quiescence.** (*A*) The effects of CQ and Baf on proteolytic degradation. Drug low and Drug high: for CQ, 5 μM and 15 μM, respectively; for Baf, 10 nM and 50 nM, respectively. The degree of proteolytic degradation was indicated by DQ-BSA signal intensity (red puncta); cells were co-stained with CellTrace (gray background stain, see Methods for details). Quantifications of DQ-BSA signal intensity per cell in CQ-treated cells are shown on the right; ns and ***, $p > 0.05$ and $< 0.001$ (two-tailed *t*-test), respectively, compared to the DPBS vehicle control. (*B*) Cells serum-starved for indicated durations were treated with Baf or CQ as in Fig. 4*A*. Cells were stimulated with serum at indicated concentrations for 40-42 hours and subjected to EdU assay.

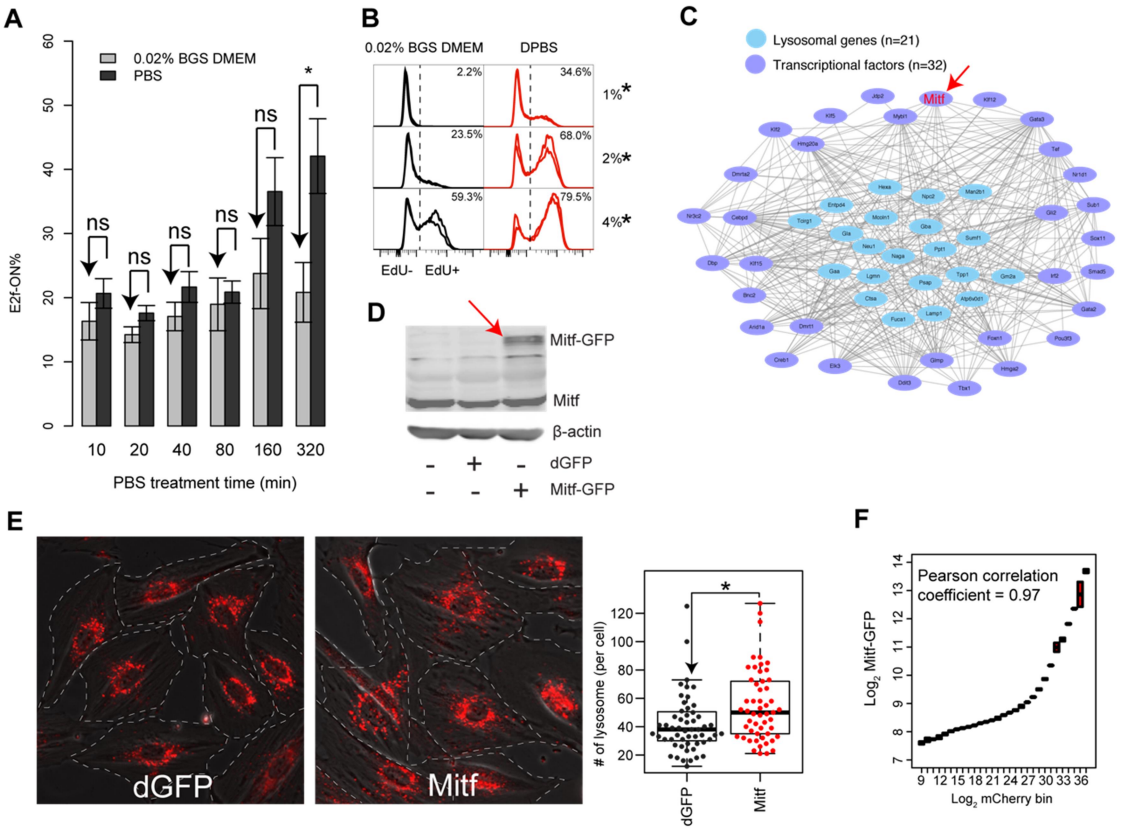


**Fig. S6. Enhancing lysosomal function pushes cells toward shallower quiescence.** (*A*) REF cells were serum starved for 4 days and further cultured in serum starvation medium (DMEM containing 0.02% BGS) or nutrient and serum starvation condition (DPBS) for the indicated durations (x-axis). Cells were stimulated with 1% serum (in DMEM) for 24 hours and measured for E2f-GFP activity (y-axis). Error bar, s.e.m from triplicates.; ns and *, p-value > 0.05 and < 0.05 (one-tailed *t*-test), respectively. (*B*) Cells were treated as in *A*, except that the nutrient/serum starvation duration = 320 min and serum stimulation concentration = 1%, 2%, and 4% as indicated, followed by EdU assay (n = 2). *, p-value < 0.05 (one-tailed *t*-test). (*C*) Lysosomal co-expression network associated with deep quiescence. Highly co-expressed lysosomal genes (blue) and TFs (purple) are connected based on the degree of co-expression. See Methods for details. Mitf is highlighted by a red arrow. (*D*) Immunoblot of ectopic Mitf expression. Mitf-GFP- or dGFP-transfected or mock-transfected cells were subjected to Western Blot using anti-Mitf and anti-β actin. Red arrow indicates ectopic Mitf-GFP band. (*E*) LysoTracker staining and lysosomal number quantification. Mitf-GFP- or dGFP-transfected cells were serum starved for 2-days and stained with LysoTracker. Representative microscopy images are shown to the left and quantifications of lysosomal number are shown to the right (~50 cells per sample). *, p-value < 0.05 (one-tailed *t*-test). (*F*) mCherry intensity indicates the expression level of co-transfected Mitf-GFP. REF cells were co-transfected with Mitf-GFP and mCherry expression vectors and subsequently serum starved for 4 days. mCherry intensity (x-axis) was found positively correlated with GFP intensity (y-axis) and thus ectopic Mitf expression (Pearson correlation coefficient = 0.97). Cells were grouped according to their mCherry intensity (log transformed) into 29 even-width bins (bins with cell number < 10 were filtered out).

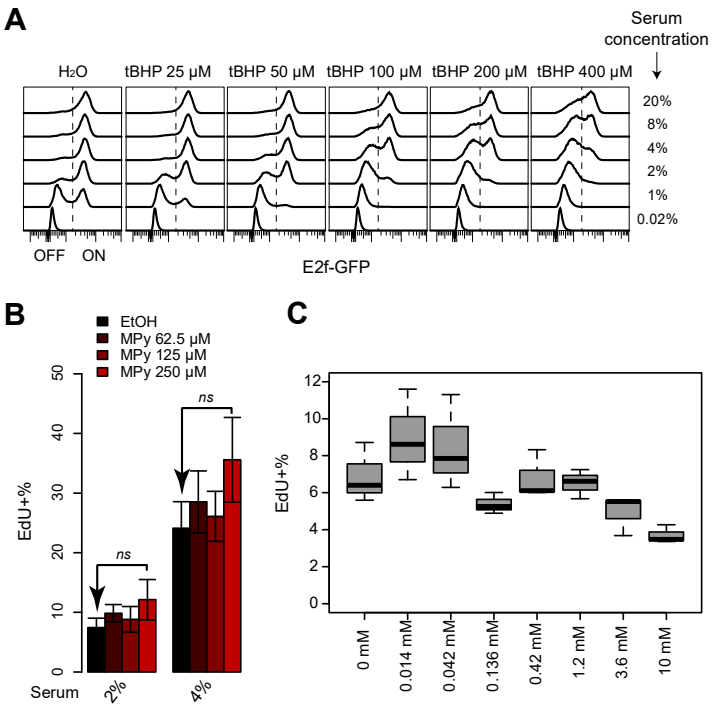


**Fig. S7. Effects of tBHP and MPy on quiescence depth.** (*A*) 2-day serum-starved cells were treated with tBHP at indicated concentrations for 1 hour and stimulated with serum at indicated concentrations for 24 hours, followed by E2f-GFP assay. (*B, C*) Effects of MPy supplement. 2-day serum-starved cells were further serum starved with daily supplementation of MPy at indicated concentrations (0-0.25 mM, *B*; 0-10 mM, *C*) for 4 days (*B*) or 2 days (*C*). Cells were stimulated with 2% or 4% serum (*B*) or 1% serum (*C*) for 24 hours and subjected to EdU assay (triplicates). Error bar in *B*, s.e.m. Box plot in *C*, same as in Fig. 2*H*.

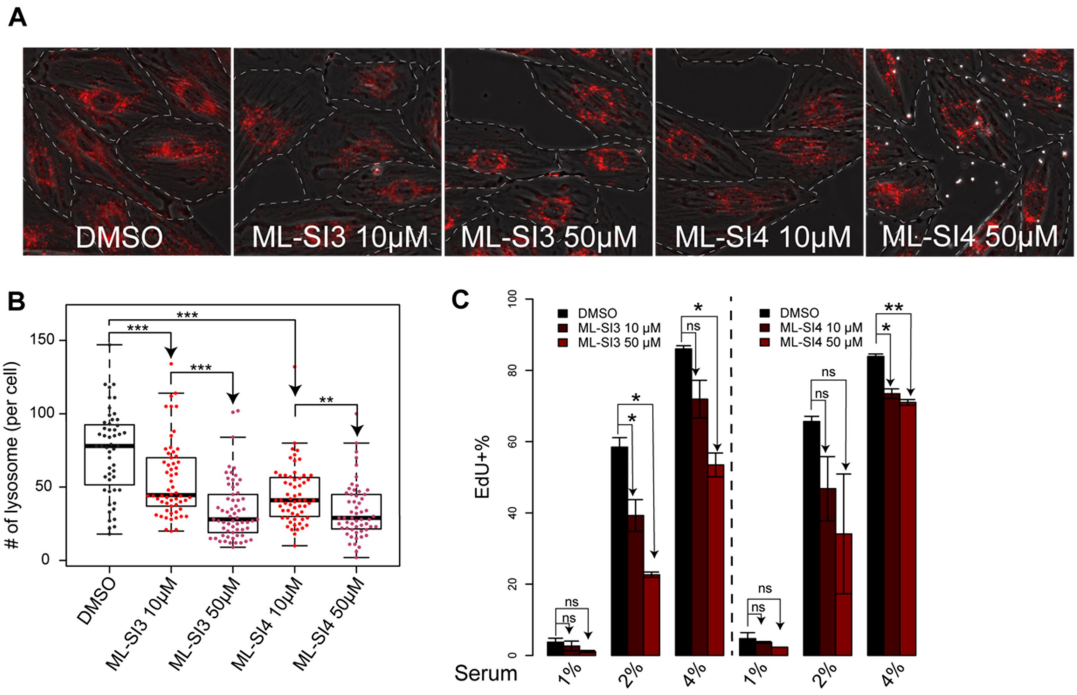


**Fig. S8. Inhibiting lysosome biogenesis deepens quiescence.** Cells were serum starved for 2-days, treated with Mcoln1 inhibitor ML-SI3 or ML-SI4 for 24 hours at indicated concentrations. Cell were then stained with LysoTracker (*A, B*), or stimulated with serum at indicated concentrations for 24 hours and subjected to EdU assay (*C*). (*A*) Representative microscopy images of LysoTracker staining. (*B*) Quantifications of lysosomal number per cell (~40 cells per sample). (*B, C*) ns, *, **, and ***, p-value > 0.05, < 0.05, < 0.01, and < 0.001 (one-tailed *t*-test), respectively. Error bar in *C*, s.e.m (n = 2).

**Table S1. Lysosomal genes in Figure 2*E*.**

| | |
|---|---|
| Cluster A | Ap1m1, Ap4m1, Ap3m1, Ap1b1, Ap1s1, Ctsz, Ap4b1, Ap4s1, Clta, Cd63, Arsb, Ctsl, Ap3s2, Gusb, Ctsb, Dnase2 |
| Cluster B | Ap3b1, Cltc, Ap1g1, Atp6v0a2, Ap4e1, Igf2r, Gga3, Litaf, Ap1s2, Cln5, Lipa, Laptm4a, Smpd1, Pla2g15 |
| Cluster C | Galns, Arsg, Acp2, Nagpa, Ap3m2, Gnptg, Atp6v0b, Gga1, Ap3d1, Ctns, Atp6v0a1, Cltb, Arsa, Ctsf, Glb1, Atp6v1h, Ap3s1, Aga, Sumf1, Ppt1, Fuca1, Lgmn, Naga, Abca2, Idua, Ctsd, Atp6v0d1, Ctsa, Psap, Gba, Lamp1, Npc2, Asah1, Tpp1, Lamp2, Slc17a5, Atp6ap1, Man2b1, Atp6v0c, Gla, Entpd4, Gaa, Hexb, Hexa, Neu1, Npc1, Mcoln1, Ctsh, Gm2a, Scarb2, Sort1, Hyal1, Gga2, Tcirg1, Cd164, Ppt2, Laptm4b, Mcpt8l2, Slc11a2 |

Note: genes are listed according to their order (top to bottom) in each cluster of Figure 2*E*.

**Table S2. Gene sets enriched in down-regulated lysosomal genes in deep quiescence by GSEA.**

| Gene set | MSigDB | FDR |
|---|---|---|
| GO_TRANS_GOLGI_NETWORK | C5 | <0.0001 |
| GO_COATED_MEMBRANE | C5 | <0.0001 |
| GO_ORGANELLE_SUBCOMPARTMENT | C5 | <0.0001 |
| GO_GOLGI_MEMBRANE | C5 | 0.0003 |
| GO_GOLGI_APPARATUS_PART | C5 | 0.0004 |
| REACTOME_MEMBRANE_TRAFFICKING | C2 | 0.0005 |
| GO_CLATHRIN_COAT | C5 | 0.0006 |
| REACTOME_TRANS_GOLGI_NETWORK_VESICLE_BUDDING | C2 | 0.0006 |
| GO_GOLGI_APPARATUS | C5 | 0.0013 |
| GO_AP_TYPE_MEMBRANE_COAT_ADAPTOR_COMPLEX | C5 | 0.0034 |
| GO_INTRACELLULAR_PROTEIN_TRANSPORT | C5 | 0.0084 |
| GO_CELLULAR_MACROMOLECULE_LOCALIZATION | C5 | 0.0131 |
| GO_MEMBRANE_PROTEIN_COMPLEX | C5 | 0.0160 |
| GO_IMMUNE_SYSTEM_PROCESS | C5 | 0.0193 |
| GO_VESICLE_MEDIATED_TRANSPORT | C5 | 0.0209 |
| GO_VACUOLAR_TRANSPORT | C5 | 0.0565 |
| GO_ENDOCYTIC_VESICLE | C5 | 0.0793 |

Note: the MSigDB collection from which the gene sets were derived are indicated in the middle column. Gene sets with FDR < 0.1 are shown.

**Table S3. Cell viability and irreversibly arrested cell fraction under treatment with lysosomal modulator, oxidant, and anti-oxidant.**

| | | Cell viability | | Irreversible arrest (EdU-%) | |
|---|---|---|---|---|---|
| Batch | condition | Median | sd | Median | sd |
| Baf | 0nM | 1.00 | 0.07 | 3.13 | 0.18 |
| | 10nM | 0.98 | 0.06 | 3.15 | 0.15 |
| | 50nM | 0.93 | 0.05 | 3.21 | 0.15 |
| CQ | 0μM | 1.00 | 0.05 | 3.07 | 0.01 |
| | 5μM | 0.93 | 0.07 | 3.92 | 0.06 |
| | 20μM | 0.91 | 0.04 | 7.94 | 0.29 |
| tBHP | 0μM | 1.00 | 0.07 | 3.00 | 0.13 |
| | 100μM | 0.96 | 0.05 | 5.27 | 0.38 |
| | 200μM | 0.91 | 0.04 | 9.45 | 1.07 |
| | 400μM | 0.85 | 0.03 | 15.2 | 0.07 |
| βME | 0μM | 1.00 | 0.06 | 2.65 | 0.18 |
| | 25μM | 0.97 | 0.10 | 2.76 | 0.40 |

Note: 1) Cell viability value of each treatment is normalized to vehicle control, which is set to 1. See Methods for details. 2) Cells that did not incorporate EdU (EdU-%) after 2-day 20% serum stimulation were considered irreversibly arrested. Sd, standard deviation.

**Table S4**. **GSEA result for KEGG_lysosome gene set in aged and senescent cells (Figure 7*B, D*).**

| Cell/Tissue type | Processes | Lysosomal correlation | FDR q-val |
|---|---|---|---|
| Adrenal gland | Aging | positive | 9.6E-04 |
| Brain | Aging | positive | 5.3E-03 |
| Heart | Aging | positive | 8.4E-05 |
| Kidney | Aging | positive | 6.1E-03 |
| Liver | Aging | positive | 2.6E-04 |
| Lung | Aging | positive | 1.2E-05 |
| Muscle | Aging | negative | 5.4E-02 |
| Spleen | Aging | positive | 0.0E+00 |
| Thymus | Aging | positive | 2.6E-03 |
| Uterus | Aging | positive | 1.4E-02 |
| Testis | Aging | positive | 0.0E+00 |
| Fibroblasts (BJ) | Replicative senescence | positive | 1.1E-02 |
| Fibroblasts (IMR-90) | Replicative senescence | positive | 8.0E-02 |
| Fibroblasts (WI-38) | Replicative senescence | positive | 3.4E-03 |
| Fibroblasts (HFF) | Replicative senescence | positive | 9.0E-04 |
| Fibroblasts (MRC-5) | Replicative senescence | positive | 7.2E-02 |
| Fibroblasts (MDAH041) | Replicative senescence | none | 9.9E-01 |
| Fibroblasts (MDAH041) | Stress-induced senescence (5-aza) | none | 1.0E+00 |
| Fibroblasts (MDAH041) | Stress-induced senescence ($H_2O_2$) | none | 7.4E-01 |
| Fibroblasts (MDAH041) | Stress-induced senescence (Adriamycin) | none | 8.2E-01 |

Note: FDR < 0.1 regarded as statistical significance.

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