A Coherent FOXO3-SNAI2 Feed-Forward Loop in Autophagy

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Drosophila Stocks and Genetics

Flies strains were raised on standard Drosophila media and crosses were performed at 25°C. Fly strains used in current study include: ptc-Gal4, GMR-Gal4, UAS-LacZ (3956), UAS-dFoxO\(^p\) (9575), UAS-dFoxO-GFP (44214), dFoxO\(^{94}\), UAS-dFoxO-IR (27656), UAS-sna-IR\(^b\) (28679), yw hs-Flp; act\(>\)y\(+\)>Gal4 UAS-GFP were obtained from Bloomington Drosophila stock center. UAS-sna-IR\(^v\) (6263) were obtained from Vienna Drosophila RNAi center. UAS-Sna\(^{74}\) fly was a kind gift from J. Kumar. UAS-FOXO\(^{3A}\) and Atg8a-pmcherry were kind gifts from Tor Erik Rusten and E. Hafen, respectively. Transgenic flies expressing UAS-Snail-Myc, UAS-NLS-Sna\(^{C}\)-Myc, UAS-Flag-dFoxO\(^{A+B}\) and UAS-Flag-dFoxO\(^{E}\) were generated by standard P element-mediated transformation as described (1).

RNA Sequencing

Total RNAs from HeLa cells treated by Torin1 or serum-free starvation were extracted with Trizol reagent (Beyotime, R0016, China). RNA quality was assessed by NanoDrop spectrophotometer (Thermo Scientific, USA). Sequencing libraries were generated as following procedures. First, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads and then fragmentation was performed in an Illumina proprietary fragmentation buffer via divalent cations. Random oligonucleotides and Super Script II were utilized for first strand cDNA synthesis, and DNA Polymerase I with RNase H for the second strand cDNA synthesis. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of the 3’ ends of the DNA fragments, Illumina PE adapter oligonucleotides were ligated to prepare for hybridization. To select cDNA fragments of the preferred 400-500 bp in length, the AMPure XP system (Beckman Coulter, Beverly, CA, USA) was used for the fragments purification. The DNA fragments with ligated adaptor molecules on both ends were purified and then sequenced on NovaSeq 6000 platform (Illumina) by Shanghai Hua Gene Biotechnology Cp. Ltd. Difference of genes expression normalized by FPKM was further analyzed by DESeq. In addition, we used R language heatmap package to perform bi-directional clustering analysis of all different genes of samples. Torin 1 (Selleck, S2827, USA) was used for inhibition of mTOR activity in samples whereas DMSO acted as negative control.
Data and Code availability
Raw data of RNA-seq in study has been deposited in NCBI Sequence Read Archive under accession SRA: PRJNA791826.

RNA isolation and qRT-PCR analysis
Total RNAs from *Drosophila* S2 cells or human cells were extracted with Trizol (Beyotime, R0016, China) following the manufacturer’s instructions. Nanodrop1000 spectrophotometer was used for quantification. Total RNA was reverse transcribed into cDNA with EasyScript® All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (TransGen Biotech, AE341, China). Quantitative PCR was performed with Hieff™ qPCR SYBR® Green Master Mix (Yeasen, 11202ES08, China) with the Stratagene Mx3000P system. $2^{-\Delta\Delta Ct}$ method was used for relative quantification. *rp49* and *GAPDH* served as internal controls for *Drosophila* and human cells as previously described (2), respectively. Besides, the primers for *PIK3CA* have been previously described (3). The primers used are provided as follows:

SNAI2-fwd: AGATGCATATTCCGGACCCAC
SNAI2-rev: CCTCATGTGGTGCAAGGAGA
ULK1-fwd: GCAAGGACTTTCCTGTGACAC
ULK1-rev: CCACTGCACATCAGGCTGTCTG
FOXO3-fwd: TCTACGAGTGGATGGTGCGTTG
FOXO3-rev: CTCTTGCCAGTTCCCTCATTCTG
4E-BP-fwd: CCATGATCGAGAGGTGTGA
4E-BP-rev: AGCCGCTCCTAGATAAGTTTTGT
Dp100-fwd: AATCTGCCTGTTGCCCAATG
Dp100-rev: ATAGCCCAGTGGCATCTGTTT
dFoxO-fwd: TGACCCACACAGATAACGGCCTGG
dFoxO-rev: CTCCACAAGTTTTTCGGGACGC

Immunoblotting and Subcellular Fractionation
Cells were harvested and washed by ice-cold PBS, then lysed with RIPA lysis buffer (WELLBIO, WB0101, China) supplemented with protease inhibitor cocktails (Yeasen, 20124ES03, China) on ice for 30 min. Cell lysates were then cleared by centrifugation at 15,000 rpm for 10 min at 4°C. Proteins were separated by SDS-PAGE following standard procedures. To detect the nucleocytoplasmic distribution of protein, Nuclear and Cytoplasmic Protein Extraction Kit (BIOTECH WELL, WB0107, China) was
employed according to the instruction. LAMIN B1 and β-actin are markers for nuclear and cytoplasm, respectively.

The primary antibodies were used as follows: rabbit anti-Flag (Cell Signaling Technology, CST, 2368, 1:2000), rabbit anti-Myc (CST, 2276, 1:2000), mouse anti-β actin (Abways, AB0061, 1:3000), rabbit anti-α-Tubulin (CST, 2125, 1:2500), rabbit anti-GAPDH (Novus Biologicals, NB100-56875, 1:3000), rabbit anti-GFP (CST, 2956, 1:3000), rabbit anti-HA (CST, 5017, 1:2000), rabbit anti-FOXO3 (CST, 2497, 1:2500), rabbit anti-LC3B (CST, 14452, 1:3000), rabbit anti-Lamin B1 (CST, 13435, 1:2000), rabbit anti-SNAI2 (CST, 9585, 1:2000), rabbit anti-pan-14-3-3 (CST, 8312, 1:2500), rabbit anti-14-3-3 α/β (Abways, CY5916, 1:3000), rabbit anti-14-3-3 γ (Abways, CY5852, 1:3000), rabbit anti-14-3-3 ε (Abways, CY5919, 1:3000), rabbit anti-14-3-3 θ (Abways, CY5921, 1:3000). Immunoblot experiments were repeated at least three times, and densitometric analysis was performed with the ImageJ program (http://rsbweb.nih.gov/ij/download.html) and the quantification results were normalized to the internal loading control.

**Luciferase reporter assay**
For luciferase assay in HeLa and 293T cells, 4x FOXO3 binding sites responsive luciferase reporter (4X FOXO-luc, Yeasen, 11527ES03, China) was transiently transfected into cells planted in 12-well together with CMV-Renilla to normalize for transfection efficiency. Reporter measurements were performed in triplicate using Double luciferase reporter assay system (Promega, E1910, USA) following the instruction as previously described (4).

**ChIP assay**
Chromatin immunoprecipitation (ChIP) assay was conducted with ChIP-IT Express Enzymatic Shearing Kit (Active Motif, 53009, Carlsbad, CA, USA) following the instructions as previously described (4).

**GST-pull down Assay**
GST-pull down experiments were performed with PierceTM GST Protein Interaction Pull-Down Kit (Thermo Scientific, 21516, USA). In brief, GST-FOXO3 or GST-dFoxO was purified as bait protein in E. coli expression system and incubated with lysates containing Flag-SNAI2 or Myc-Snail from mammalian or insect cells. Protein
interactions were visualized by SDS-PAGE following the instructions as previously described (5).

**Clones induction by Heat Shock**

Adult wing margin bristles clones using the *yw hs-Flp; act>y+>Gal4 UAS-GFP* strain were produced by heat shock at 37 °C for 1h, recovering at 25 °C for 4 days.

**Statistical analysis**

Statistical analysis was performed with GraphPad Prism 8 software. The data were analyzed by one-way ANOVA followed by multiple comparisons test or two-tailed unpaired student’s t-test to calculate statistical significance in all experiments (*P < 0.05; **P < 0.01; ***P < 0.001, ****P<0.0001; ns, no significant difference). The experiments were repeated at least three times.

**Data and materials availability:** All study data are included in the article and supporting information.

**Detailed Fly Genotypes for All Figures**

**Figure 4**

(A) *ptc-Gal4 UAS-GFP/+*

(B) *ptc-Gal4 UAS-GFP/UAS-Sna*

(C) *ptc-Gal4 UAS-GFP/UAS-Sna; dFoxOΔ94/+*

(D) *ptc-Gal4 UAS-GFP/UAS-Sna; dFoxO-IR/+*

(E) *ptc-Gal4 UAS-GFP/UAS-FOXO3Δ; UAS-LacZ/+*

(F) *ptc-Gal4 UAS-GFP/UAS-FOXO3Δ; UAS-sna-IRβ/+*

(G) *ptc-Gal4 UAS-GFP/UAS-FOXO3Δ; UAS-sna-IRβ/+*

(H) *Atg8a-pmCherry/+; ptc-Gal4 UAS-GFP/+*
(I) $\text{Atg8a-pmCherry}^+; \text{ptc-Gal4} UAS\text{-GFP}/UAS\text{-Sna}$

(J) $\text{Atg8a-pmCherry}^+; \text{ptc-Gal4} UAS\text{-GFP}/UAS\text{-Sna}; \text{dFoxO}^{194}/^+$

(K) $\text{Atg8a-pmCherry}^+; \text{ptc-Gal4} UAS\text{-GFP}/UAS\text{-Sna}; \text{dFoxO-IR}^+/^+$

(L) $\text{Atg8a-pmCherry}^+; \text{ptc-Gal4} UAS\text{-GFP}/UAS\text{-FOXO}^{3A}; UAS\text{-LacZ}^+/^+$

(M) $\text{Atg8a-pmCherry}^+; \text{ptc-Gal4} UAS\text{-GFP}/UAS\text{-FOXO}^{3A}; UAS\text{-sna-IR}^V^+/^+$

(N) $\text{Atg8a-pmCherry}^+; \text{ptc-Gal4} UAS\text{-GFP}/UAS\text{-FOXO}^{3A}; UAS\text{-sna-IR}^8^+/^+$

**Figure 5**

(B) $\text{GMR-Gal4}/UAS\text{-dFoxO-GFP}$

\[ \text{GMR-Gal4}^+; UAS\text{-Sna-Myc}^+/^+ \]

\[ \text{GMR-Gal4}/UAS\text{-dFoxO-GFP}; UAS\text{-Sna-Myc}^+/^+ \]

(J) $\text{GMR-Gal4}/UAS\text{-FOXO}^{3A}$

\[ \text{GMR-Gal4}/UAS\text{-FOXO}^{3A}; UAS\text{-sna-IR}^V^+/^+ \]

**Figure 7**

(J) $\text{ptc-Gal4} UAS\text{-GFP}^+/^+$

\[ \text{ptc-Gal4} UAS\text{-GFP}/UAS\text{-dFoxO}^P \]

\[ \text{ptc-Gal4} UAS\text{-GFP}/UAS\text{-FOXO}^{3A} \]

**Figure S2**

(A) $\text{ptc-Gal4} UAS\text{-GFP}/UAS\text{-dFoxO}^P; UAS\text{-LacZ}^+/^+$

(B) $\text{ptc-Gal4} UAS\text{-GFP}/UAS\text{-dFoxO}^P; UAS\text{-sna-IR}^V^+/^+$

(C) $\text{ptc-Gal4} UAS\text{-GFP}/UAS\text{-dFoxO}^P; UAS\text{-sna-IR}^8^+/^+$

(D) $\text{ptc-Gal4} UAS\text{-GFP}^+/^+$

\[ \text{ptc-Gal4} UAS\text{-GFP}^+; d\text{FoxO-IR}^+/^+ \]
ptc-Gal4 UAS-GFP/+; dFoxO<sup>594/+</sup>

(F) yw hs-Flp/+; act>y+>Gal4 UAS-GFP/+ 

(G) yw hs-Flp/+; act>y+>Gal4 UAS-GFP/UAS-Sna

(H) yw hs-Flp/+; act>y+>Gal4 UAS-GFP/UAS-Sna; dFoxO<sup>594/+</sup>

(I) yw hs-Flp/+; act>y+>Gal4 UAS-GFP/UAS-dFoxO<sup>P</sup>; UAS-LacZ/+ 

(J) yw hs-Flp/+; act>y+>Gal4 UAS-GFP/UAS-dFoxO<sup>P</sup>; UAS-sna-IR<sup>V</sup>/+

**Figure S5**

(A) GMR-Gal4/UAS-Flag-dFoxO<sup>A+B</sup>

(B) GMR-Gal4/+; UAS-NLS-Sna<sup>C</sup>-Myc/+ 

(C) GMR-Gal4/UAS-Flag-dFoxO<sup>A+B</sup>; UAS-NLS-Sna<sup>C</sup>-Myc/+ 

(D) GMR-Gal4/UAS-Flag-dFoxO<sup>E</sup>

(E) GMR-Gal4/+; UAS-NLS-Sna<sup>C</sup>-Myc/+ 

(F) GMR-Gal4/UAS-Flag-dFoxO<sup>E</sup>; UAS-NLS-Sna<sup>C</sup>-Myc/+ 

Supplementary Figures
Fig. S1. SNAI2 positively regulates autophagy. (A) RT-qPCR analysis of serum-free starvation in HeLa cells (mean ± SD, n=3). Cells were incubated with serum-free medium for 4h before harvest. (B) Validation of two independent siRNA-SNAI2 used in current study. siRNA-treated 293T cells for 72h were subject to RT-qPCR (mean ± SD, n=3). (C) Immunoblot analysis of the effect of SNAI2 knockdown on autophagy induced by Torin 1 with or without Baf-A1 treatment in HeLa cells. (D) Immunoblot
analysis of the effect of SNAI2 depletion on Torin 1-induced autophagy in HeLa cells (mean ± SD, n=3). DMSO, the Torin 1 vehicle, has been previously identified as an autophagy inducer (6). (E) Analysis of SNAI2 knockdown on basal autophagy in HeLa cells (mean ± SD, n=4). (F, I) Analysis of SNAI2 overexpression on Rapamycin-induced autophagy in 293T cells treated by Baf-A1 or CQ, respectively (mean ± SD, n=3). (G, H) Immunoblot analysis of SNAI2 alone on basal autophagy in 293T and HeLa cells (mean ± SD, n=4). Baf-A1 or CQ was used for inhibiting lysosome inhibition, while DMSO acts as negative control for Baf-A1 treatment. For statistics, one-way ANOVA followed by multiple comparisons test (B, D, E and F) or two-tailed unpaired student’s t-test (A, G and H) was applied. ****p<0.0001, ***P < 0.001, **P < 0.01, * P < 0.05. ns, no significant difference.

Fig. S2. Snail generally regulates dFoxO in Drosophila. (A-C) Fluorescence micrographs of Drosophila wing imaginal discs are shown. ptc>dFoxO<sup>P</sup>-induced Lysotracker staining (A) was abrogated by knockdown of Sna (B, C). (D) RT-qPCR analysis of dFoxO mRNA level in fly wing imaginal discs of ptc>dFoxO.RNAi or
heterozygous mutants $d\text{FoxO}^{\Delta 94}$ (mean ± SD, n=3). (E) 4E-BP promoter-luciferase activity was measured in S2 cells (mean ± SD, n=3). (F-J) Light micrographs of Drosophila adult wing margin bristles are shown. (K) Quantification of bristle size in clones shown in F-J (F, n=10; G, n=9; H, n=7; I, n=10; J, n=10). (L) RT-qPCR analysis of 4E-BP and Dp100 mRNA level was shown. HA-dFoxO was transfected into S2 cells with or without expressing Myc-Sna for 48h before harvest (mean ± SD, n=6). For statistics, one-way ANOVA followed by multiple comparisons test. ****p<0.0001, ***p < 0.001, **p < 0.01, *p < 0.05. Scale bars, 50μm (A-C) and 100μm (F-J).

Fig. S3. Snail directly interacts with FoxOs. (A, B) Analysis of DNase I treatment on dFoxO-Snail interaction judged by Co-IP assay. (C, D) Direct interaction of Snail/SNAI2 and dFoxO/FOXO3 by GST-pull down analysis. GST-tagged FOXO3 or dFoxO was purified as bait protein, and Flag-SNAI2 or Myc-Snail acts as prey protein. Cell lysates were treated by 20 U/mL DNase I with 1 M MgCl$_2$ at 16 ºC for 8h.
Fig. S4. Snail regulates dFoxO localization in vitro. (A-F) Immunofluorescence staining in S2 cells. Compared with SnailC (A), recombinant SnailC with a nuclear localized signal (NLS) was mostly located in nucleus (B). Flag-dFoxO<sup>A+B</sup> alone was mainly located in cytoplasm (C), but accumulated in nuclear upon NLS-Snail<sup>C</sup> overexpression (D). Flag-dFoxO<sup>D</sup> was equally distributed between the cytoplasm and nucleus (E), which was not altered upon overexpression of NLS-Snail<sup>C</sup> (F). Scale bar, 5μm (A-F).
Fig. S5. Snail regulates dFoxO localization in vivo. (A-F) Fluorescence micrographs of Drosophila eye imaginal discs. GMR-Gal4 was used as a driver. Flag-dFoxO<sup>A+B</sup> was mainly located in cytoplasm (A), while NLS-Snail<sup>C</sup> predominantly resided in nucleus (B). Flag-dFoxO<sup>A+B</sup> was translocated into nucleus by ectopically expressed NLS-Snail<sup>C</sup> (C). The truncated Flag-dFoxO<sup>E</sup> shifted from cytoplasm to nucleus when Sna<sup>C</sup>-Myc was co-expressed (D-F). Scale bar, 10μm (A-F).
Fig. S6. **Snail has no effect on 14-3-3 expression.** (A) The effect of SNAI1 or SNAI3 on FOXO3 cellular localization was measured by Nucleocytoplasmic separation assay (mean ± SD, n=3). β-actin and LAMIN B1 served as internal control of cytoplasm and nucleus, respectively. (B, C) siSNAI2-1/2 or siFOXO3 was first transfected into 293T or HeLa cells and incubated for 24h, and then 4X FOXO-luc plasmid together with CMV-Renilla was transiently transfected for another 48h before harvest and then double luciferase activity was measured (mean ± SD, n=3). (D, E) Immunoblot analysis of the effect of knockdown or overexpression of SNAI2 on 14-3-3 protein level. (F) Immunoblot analysis of individual 14-3-3 proteins by SNAI2 overexpression in a dose-dependent manner. For statistics, one-way ANOVA followed by multiple comparisons test was applied. ****p<0.0001. ns, no significant difference.
**Fig. S7.** SNAI2 disrupts FOXO3/CRM1 interaction via enhancing FOXO3 binding to responsive DNA. (A) Immunoblot analysis of the effect of FOXO-luc containing 4X FOXO3 binding motifs on the interaction between exogenous FOXO3<sup>3A</sup> and CRM1 in 293T cells. (B) Immunoblot analysis of the effect of FOXO-RES on the interaction between endogenous FOXO3 and CRM1 in 293T cells. Ctrl-luc or Ctrl-RES acts as a negative control.

**Fig. S8.** FOXO3 mediates energy stress-induced SNAI2 expression. (A) siRNA-FOXO3 was first transfected into 293T cells and incubated for 72h, and then 1μM Rapamycin was used to treat FOXO3-knockdown cells for another 4h before immunoblot analysis of SNAI2 protein level (mean ± SD, n=3). (B) Double luciferase
activity of generated E1 or E2 reporter in HeLa cells transfected with empty vector or Flag-FOXO3<sup>3A</sup> (mean ± SD, n=3). (C) RT-qPCR analysis of relative A region enrichment in ChIP experiment (mean ± SD, n=3). One-way ANOVA followed by multiple comparisons test (A) and two-tailed unpaired student’s t-test (B, C) was applied. ****p<0.0001, ***P < 0.001, **P < 0.01, *P < 0.05.

**Fig. S9. SNAI2 induces autophagy in HSkCM cells.** (A) FOXO3 expression in different cell lines from The Human Protein Atlas Database. Arrows indicate HSkCM and HeLa cells, respectively. (B, C) Validation of FOXO3 expression by RT-qPCR (B) and immunoblot analysis (C) in HSkCM and HeLa cells (mean ± SD, n=3). (D) Immunoblot analysis of autophagy induced by SNAI2 expression in HSkCM cells (mean ± SD, n=3). For statistics, two-tailed unpaired student’s t-test was applied. ****p<0.0001, **P < 0.01, *P < 0.05.
Fig. S10. SNAI2 is specifically regulated by FOXO3. (A) Co-IP analysis of FOXOs-SNAI2 interaction in 293T and HeLa cells. (B) Validation of plasmids encoding mFoxo1, FOXO4 or FOXO6 in 293T cells. (C, D) RT-qPCR analysis of SNAI2 mRNA level upon mFoxo1, FOXO4 or FOXO6 expression in 293T and HeLa cells (mean ± SD, n=3). For statistics, one-way ANOVA followed by multiple comparisons test was applied. **P < 0.01. ns, no significant difference.

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