Hepatic FoxO3 Supports Metabolic Resilience, Attenuates Age-Associated Obesity and Metabolic Dysfunction, and Mitigates Diet-Induced Steatohepatitis

Toshimitsu Komatsu, Seongjoon Park, Sangeun Kim, Hiroko Hayashi, Isao Shimokawa

Supplementary text, tables and figures

Methods

Genotyping

Genomic DNA was extracted from mouse tail biopsies using Cica geneus® DNA Extraction Reagent AN (08210-96, Kanto Chemical Co., Inc., Tokyo, Japan) according to the manufacturer's instructions. PCR was performed using GoTaq® G2 Hot Start Master Mix (M7422, Promega Corporation, Madison, WI, USA) with the following primers: for floxed alleles, forward 5′-CTGATACCGAAGAGCCTTGC-3′ and reverse 5′-CTTCCAAACAGAACCTGGTCC-3′; for Cre transgene, forward 5′-TGCAAACATCACATGCACAC-3′ and reverse 5′-GAAGCAGAAGCTTAGGAAGATGG-3′. PCR conditions were as follows: 95°C for 3 min; 30 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s; followed by a hold at 10°C. PCR products were resolved on 3% NuSieve® 3:1 agarose gel (#50090, Lonza, Basel, Switzerland) and visualized by staining with SYBR Gold (S1194, Thermo Fisher Scientific, Waltham, MA, USA).

Western blots

Liver tissues were homogenized and fractionated into nuclear and cytoplasmic extracts using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific) with protease and phosphatase inhibitor cocktail (#25955-11, #07575-51, Nacalai tesque, Kyoto, Japan), according to the manufacturer's instructions. Protein concentrations were determined using a BCA assay (Thermo Fisher Scientific). All samples were mixed with Laemmli's sample buffer and heated at 95 °C for 5 min. Nuclear proteins (4 μg) were separated by 12.5% SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with blocking solution (Blocking One-P, Nacalai tesque) and incubated with primary antibodies against the indicated proteins, including FoxO1 (#2880, 1:2000, Cell Signaling Technology, Denvars, MA, USA), p-FoxO1 (Ser–256, #9461, 1:1000, Cell Signaling Technology), FoxO3 (#1289, 1:2000, Cell Signaling Technology), and FoxO4 (#9472, 1:2000, Cell Signaling Technology) diluted in Can Get Signal (TOYOBO, Osaka, Japan) or TBS-T for p-FoxO1, overnight at 4°C, followed by incubation with HRP-conjugated secondary antibody (#7074, 1:10000, Cell Signaling Technology) for 1hour at room temperature. Protein bands were visualized using an enhanced chemiluminescence detection system (ECL-plus, Thermo Fisher Scientific), and quantified using a densitometer (Fusion Solo S, Vilber, Collégien, France) and MultiGauge software (Fuji Film, Tokyo, Japan). After detection of FoxO protein, membranes were stripped and re-probed with an anti-Lamin B1 antibody (ab16048, 1:2000, Abcam, Cambridge, UK) and lamin A/C (#4777, 1:2000, Cell Signaling Technology) as nuclear loading controls, following the same procedure described above.

ELIZA for plasma hepatokines

To measure plasma levels of candidate hepatokines, the following ELIZA kits were used following the manufacturer's protocols: IGFBP1 SimpleStep ELISA kit

(Abcam, ab272465, mouse), Sulf2 ELSA kit (SEH107Mu-96 tests, Cloud-Clone Corp, Katy, TX, USA), mouse Fst (Follistatin) ELISA kit (EKF60407-96T, BIOMATIK, Kitchener, Ontario, Canada), mouse TGF-beta1 Sandwich ELISA kit (#KE10005, Proteintech, Rosemont, IL, USA).

Supplementary Table 1. Primers and probes for the real-time PCR

Gene symbols	forward	reverse
p16^{Ink4a}	CCCAACGCCCCGAACT (probe: FAM-TTCGGTCGTACCCCGATTCAGGTG-TAMRA) (1)	GCAGAAGAGCTGCTACGTGAA
Fasn	AAGGCTGGGCTCTATGGATT	GGAGTGAGGCTGGGTTGATA
Fgl1	CGATCTGATGGCAGTGAGAACT	TTTGTTACCCAGCCAGTATTCG
Foxo3	CCCAGATCTACGAGTGGATGG	AGGTTGTGCCGGATGGA
Fst	TGCTGCTACTCTGCCAGTTC	TGCTGCAACACTCTTCCTTG
Gck	CTGTTAGCAGGATGGCAGCTT	TTTCCTGGAGAGATGCTGTGG
Gdf15	CCTCACCTACACTCCCCTTCA	GAAACCAAGCTCACCTTTATTAGCA
Igfbp1	GCAAAGGCTGCTGTGGTCTC	TAGGTGCTGATGGCGTTCC
Il1b	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTGCGTCAACT
Lep	TGACACCAAAACCCTCATCA	AGCCCAGGAATGAAGTCCA
Mlxipl/ Chrebp	CGACACTCACCCACCTCTTG	TTGTTCAGCCGGATCTTGTC
Pck1	TCCTTTGGAAGCGGATATGG	TTCTTGCCTTCGGGGTTAGTT
Retn	CCTTTTCCTTTTCTTCCTTGTCC	CCAGCAATTTAAGCCAATGTTC
Sepp1	ACACATCGCAGTGTACAGACAAGA	AGGAAGGAGTAAGGCAAACCAAG
Srebp1c	TGATGCTACGGGTACACACC	TTGCGATGTCTCCAGAAGTG
Serpinb1	GCTGCTACAGGAGGCATTGC	CGGATGGTCCACTGTGAATTC
Sulf2	CCTCAATGAGTACAACGGCTCA	GTTTCTCCTTCACCCCATTCC
TAKARA design primer ID*		
Acaca	MA102178	
Adipoq	MA124245	
Atp5f1	MA027956	
Foxo1	MA072856	
Foxo4	MA115957	
Rps18	MA050364	
Tnf	MA117190	

*https://www.takara-bio.co.jp/research/prt/probe/h1-2b.htm

Reference

[1] Krishnamurthy J, Torrice C, Ramsey MR, Kovalev GI, Al-Regaiey K, Su L, et al. (2004). Ink4a/Arf expression is a biomarker of aging. J Clin Invest, 114:1299 1307.

Supplementary Table 2. Body and organ weights at middle age

Genotype	n

Body weight (g)	Ctrl	34.0 (0.5)	6
	h-Foxo3$^{-/-}$	43.5 (2.3)*	4
Liver (g)	Ctrl	1.25 (0.02)	6
	h-Foxo3$^{-/-}$	1.78 (0.19)*	4
Fat (g)	Ctrl	1.94 (0.10)	6
	h-Foxo3$^{-/-}$	3.42 (0.38)*	4

The data represent the mean (SE). n, numbers of mice examined.
Fat weights represent the sum of peritesticular, inguinal, and perirenal adipose tissue weights. # $p < 0.10$, * $p < 0.05$ vs. Ctrl by non-parametric Wilcoxon rank-sum test.

Supplementary Table 3A. Pathways enriched in upregulated genes by fasting in Ctrl liver.

Enrichment FDR	nGenes	Pathway Genes	Fold Enrichment	Pathway	Genes
2.5E-15	18	89	17.11	Path:mmu03320 PPAR signaling pathway	*Acadl Acadm Acaa1a Cpt1a Cpt2 Cyp4a10 Cyp4a14 Cyp8b1 Acsl1 Gk Hmgcs2 Pck1 Slc27a1 Slc27a2 Angptl4 Cyp4a31 Plin5 Ehhadh*
9.7E-11	12	52	19.02	Path:mmu00071 Fatty acid degradation	*Acadl Acadm Acaa1a Cpt1a Cpt2 Cyp4a10 Cyp4a14 Acsl1 Hadhb Eci2 Cyp4a31 Ehhadh*
2.6E-09	52	1611	2.66	Path:mmu01100 Metabolic pathways	*Cyp4a32 Cth Asl Acadl Acadm Acaa1a Mat1a Bcat2 Bhmt Cpox Cyp3a13 Cyp4a10 Cyp4a14 Cyp8b1 Ephx2 Acsl1 Fh1 Fpgs Gstp2 Gstt2 Gk Hsd17b10 Hmgcs2 Ldha Lipg Acot8 Nnmt Oat Pck1 St3gal5 Acnat2 Etnk2 Hadhb Mat2a Tat Mgll Sdsl Acot1 Ahcy Aass Dgkh Mlycd Pla2g12a Crls1 Cyp4a31 Pnpla2 Cmbl Ehhadh Acot12 Oplah Agmat Hadha*
3.1E-09	13	86	11.91	Path:mmu04146 Peroxisome	*Acaa1a Ephx2 Acsl1 Acot8 Acnat2 Eci2 Slc27a2 Ech1 Mlycd Hacl1 Pxmp4 Crot Ehhadh*
4.6E-07	9	53	14.26	Path:mmu00270 Cysteine and methionine metabolism	*Cth Mat1a Bcat2 Bhmt Ldha Mat2a Tat Sdsl Ahcy*
3.5E-05	8	62	10.31	Path:mmu01212 Fatty acid metabolism	*Acadl Acadm Acaa1a Cpt1a Cpt2 Acsl1 Hadhb Ehhadh*
1.5E-04	7	56	10.24	Path:mmu00280 Valine leucine and isoleucine degradation	*Acadm Acaa1a Bcat2 Hsd17b10 Hmgcs2 Hadhb Ehhadh*
1.6E-03	6	63	8.78	Path:mmu00561 Glycerolipid metabolism	*Gk Lipg Mgll Dgkh Pnpla2 Plpp5*
8.7E-03	6	78	6.34	Path:mmu01230 Biosynthesis of amino acids	*Cth Asl Mat1a Bcat2 Mat2a Sdsl*
2.2E-02	6	98	5.25	Path:mmu00564 Glycerophospholipid metabolism	*Lcat Etnk2 Dgkh Pla2g12a Crls1 Plpp5*
2.2E-02	3	18	14.26	Path:mmu00120 Primary bile acid biosynthesis	*Cyp8b1 Acot8 Acnat2*
3.2E-02	4	44	7.61	Path:mmu00620 Pyruvate metabolism	*Fh1 Ldha Pck1 Acot12*
3.2E-02	6	105	4.61	Path:mmu04064 NF-kappa B signaling pathway	*Il1r1 Lbp Gadd45b Myd88 Nfkbia Zap70*
3.5E-02	5	85	5.28	Path:mmu00590 Arachidonic acid metabolism	*Cyp4a10 Cyp4a14 Ephx2 Pla2g12a Cyp4a31*

3.5E-02	6	109	4.39	Path:mmu04931 Insulin resistance	*Cpt1a Nfkbia Pck1 Creb3l3 Slc27a1 Slc27a2*
3.5E-02	6	109	4.43	Path:mmu05145 Toxoplasmosis	*Hspa8 Hspa1b Lamb3 Myd88 Nfkbia Lamc3*

Enrichment FDR, false discovery rate-adjusted q value; nGenes, number of differentially regulated genes in each pathway, with individual genes listed in the Genes column; Pathway Genes, total number of genes annotated to the pathway or GO term; Fold enrichment, magnitude of enrichment; Pathway, KEGG pathway name or GO term.

Supplementary Table 3B. pathways enriched in genes downregulated by fasting in Ctrl liver.

Enrichment FDR	nGenes	Pathway Genes	Fold Enrichment	Pathway	Genes
1.5E-11	67	1611	2.65	Path:mmu01100 Metabolic pathways	*Acacb Gck Acly Acaca Pygl Pgd Acat2 Adh1 Pnpla3 Car3 Cs Cyp26a1 Cyp2a4 Dct Dhcr7 Hnmt Fasn Gapdh Gpam Gsta3 Gstm1 Gstm2 Gstm4 Gstm6 Hsd3b3 Elovl6 Rdh11 Me1 Nsdhl Pde4b Enpp1 Pik3c2g Pklr Scd1 Scd2 Ggt7 Synj2 Tkt Gstp3 Cyp2c70 Sc5d Dlat Papss1 Ugt2b35 Cyp2r1 Ugt1a5 Acsl5 Acsl4 Echdc1 Pla2g6 Hao2 Extl1 Acss2 9130409I23Rik Asrgl1 Gstm7 Mogat1 Pmvk Cyp2c55 Aldh1b1 Gbe1 Acsl3 Gale Fads1 Aacs Lrat Nans*
1.8E-06	12	92	8.51	Path:mmu00983 Drug metabolism-other enzymes	*Gsta3 Gstm1 Gstm2 Gstm4 Gstm6 Gstp3 Ces2c Ces2e Ugt2b35 Ugt1a5 Gstm7 Ces2g*
3.2E-06	10	62	9.98	Path:mmu01212 Fatty acid metabolism	*Acaca Acat2 Fasn Elovl6 Scd1 Scd2 Acsl5 Acsl4 Acsl3 Fads1*
3.2E-06	11	84	8.64	Path:mmu05204 Chemical carcinogenesis-DNA adducts	*Gsta3 Gstm1 Gstm2 Gstm4 Gstm6 Gstp3 Cyp2c70 Ugt2b35 Ugt1a5 Gstm7 Cyp2c55*
4.1E-06	10	71	9.50	Path:mmu00982 Drug metabolism-cytochrome P450	*Adh1 Gsta3 Gstm1 Gstm2 Gstm4 Gstm6 Gstp3 Ugt2b35 Ugt1a5 Gstm7*
6.3E-06	10	73	8.92	Path:mmu00980 Metabolism of xenobiotics by cytochrome P450	*Adh1 Gsta3 Gstm1 Gstm2 Gstm4 Gstm6 Gstp3 Ugt2b35 Ugt1a5 Gstm7*
1.1E-05	8	44	11.78	Path:mmu00620 Pyruvate metabolism	*Acaca Acat2 Adh1 Me1 Pklr Dlat Acss2 Aldh1b1*
5.0E-05	9	72	8.03	Path:mmu00480 Glutathione metabolism	*Pgd Gsta3 Gstm1 Gstm2 Gstm4 Gstm6 Ggt7 Gstp3 Gstm7*
8.0E-05	11	120	5.84	Path:mmu01200 Carbon metabolism	*Gck Pgd Acat2 Cs Gapdh Me1 Pklr Tkt Dlat Hao2 Acss2*
1.1E-04	5	19	18.40	Path:mmu00061 Fatty acid biosynthesis	*Acaca Fasn Acsl5 Acsl4 Acsl3*
1.1E-04	9	80	7.07	Path:mmu01524 Platinum drug resistance	*Birc3 Casp3 Gsta3 Gstm1 Gstm2 Gstm4 Gstm6 Gstp3 Gstm7*
2.8E-04	9	97	6.23	Path:mmu00830 Retinol metabolism	*Adh1 Cyp26a1 Cyp2a4 Rdh11 Cyp2c70 Ugt2b35 Ugt1a5 Cyp2c55 Lrat*
1.8E-03	11	173	4.02	Path:mmu05225 Hepatocellular carcinoma	*Gadd45a Fzd4 Fzd7 Gsta3 Gstm1 Gstm2 Gstm4 Gstm6 Tgfbr2 Gstp3 Gstm7*

2.7E-03	6	52	7.36	Path:mmu00071 Fatty acid degradation	*Acat2 Adh1 Acsl5 Acsl4 Aldh1b1 Acsl3*
2.7E-03	4	20	13.86	Path:mmu00100 Steroid biosynthesis	*Dhcr7 Nsdhl Sc5d Cyp2r1*
2.8E-03	9	125	4.45	Path:mmu04152 AMPK signaling pathway	*Acaca Cidea Fasn Pparg Scd1 Scd2 Slc2a4 Srebf1 Adipor2*
2.8E-03	9	139	4.45	Path:mmu04936 Alcoholic liver disease	*Acaca Adh1 Casp3 Fasn Scd1 Scd2 Srebf1 Adipor2 Aldh1b1*
4.5E-03	5	40	8.18	Path:mmu04216 Ferroptosis	*Lpcat3 Prnp Acsl5 Acsl4 Acsl3*
5.3E-03	7	89	5.15	Path:mmu03320 PPAR signaling pathway	*Me1 Pparg Scd1 Scd2 Acsl5 Acsl4 Acsl3*
5.3E-03	11	209	3.39	Path:mmu05207 Chemical carcinogenesis-receptor activation	*Chrna4 Gsta3 Gstm1 Gstm2 Gstm4 Gstm6 Jun Rps6ka1 Ugt2b35 Ugt1a5 Gstm7*
6.1E-03	7	86	4.97	Path:mmu04146 Peroxisome	*Paox Acsl5 Acsl4 Hao2 Nudt7 Pmvk Acsl3*
6.1E-03	6	66	5.79	Path:mmu00010 Glycolysis/Gluconeogenesis	*Adh1 Gapdh Pklr Dlat Acss2 Aldh1b1*
6.1E-03	9	145	3.87	Path:mmu05418 Fluid shear stress and atherosclerosis	*Gsta3 Gstm1 Gstm2 Gstm4 Gstm6 Jun Dusp1 Gstp3 Gstm7*
1.1E-02	4	33	8.72	Path:mmu00500 Starch and sucrose metabolism	*Gck Pygl Enpp1 Gbe1*
1.4E-02	4	31	8.12	Path:mmu00630 Glyoxylate and dicarboxylate metabolism	*Acat2 Cs Hao2 Acss2*
1.9E-02	4	34	7.36	Path:mmu01040 Biosynthesis of unsaturated fatty acids	*Elovl6 Scd1 Scd2 Fads1*
4.2E-02	5	71	4.60	Path:mmu04920 Adipocytokine signaling pathway	*Slc2a4 Acsl5 Acsl4 Adipor2 Acsl3*

Supplementary Table 4A. Pathways enriched in upregulated genes under the fasting condition.

Enrichment FDR	nGenes	Pathway Genes	Fold Enrichment	Pathway	Genes
1.7E-05	7	84	17.70	Path:mmu05204 Chemical carcinogenesis-DNA adducts	*Gstm2 Gstm3 Gstp3 Cyp2c70 Ugt1a5 Sult2a7 Cyp2c55*
1.5E-03	5	73	14.69	Path:mmu00980 Metabolism of xenobiotics by cytochrome P450	*Gstm2 Gstm3 Gstp3 Ugt1a5 Sult2a7*
1.5E-02	4	71	11.94	Path:mmu00982 Drug metabolism-cytochrome P450	*Gstm2 Gstm3 Gstp3 Ugt1a5*
1.6E-02	4	72	11.04	Path:mmu00480 Glutathione metabolism	*Gstm2 Gstm3 Gstp3 Gpx6*
2.6E-02	4	97	8.67	Path:mmu00830 Retinol metabolism	*Cyp2a4 Cyp2c70 Ugt1a5 Cyp2c55*
2.6E-02	4	92	8.78	Path:mmu00983 Drug metabolism-other enzymes	*Gstm2 Gstm3 Gstp3 Ugt1a5*
2.6E-02	4	100	8.28	Path:mmu04976 Bile secretion	*Aqp8 Abcb1a Ugt1a5 Sult2a7*

Supplementary Table 4B. Pathways enriched in downregulated genes under the fasting condition.

Enrichment FDR	nGenes	Pathway Genes	Fold Enrichment	Pathway	Genes
2E-06	12	93	8.87	Path:mmu04640 Hematopoietic cell lineage	*Cd14 Cd19 Ms4a1 Cd22 Cd24a Cd37 Cd3e Cd4 H2-DMb2 Il7r Il9r Tfrc*
2E-06	8	36	16.21	Path:mmu05340 Primary immunodeficiency	*Cd19 Cd3e Cd4 Cd79a Il2rg Il7r Lck Ptprc*
3E-04	15	292	4.17	Path:mmu04060 Cytokine-cytokine receptor interaction	*Cd4 Csf2rb Il12rb1 Il2rb Il2rg Il7r Il9r Lta Ccl21a Ccl22 Ccl5 Ccl8 Tnfrsf9 Cxcl13 Crlf2*
4E-04	9	87	6.81	Path:mmu04658 Th1 and Th2 cell differentiation	*Runx3 Cd3e Cd4 Gata3 H2-DMb2 Il12rb1 Il2rb Il2rg Lck*
9E-04	9	104	5.95	Path:mmu04659 Th17 cell differentiation	*Cd3e Cd4 Gata3 H2-DMb2 Il12rb1 Il2rb Il2rg Irf4 Lck*
2E-03	8	95	6.20	Path:mmu04061 Viral protein interaction with cytokine and cytokine receptor	*Il2rb Il2rg Lta Ccl21a Ccl22 Ccl5 Ccl8 Cxcl13*
4E-03	11	192	3.86	Path:mmu04062 Chemokine signaling pathway	*Fgr Itk Ncf1 Ccl21a Rac2 Ccl22 Ccl5 Ccl8 Vav1 Pik3r5 Cxcl13*
3E-02	6	78	5.31	Path:mmu01230 Biosynthesis of amino acids	*Cth Acy1 Asl Sds Sdsl Asns*
3E-02	8	168	3.90	Path:mmu04630 JAK-STAT signaling pathway	*Ccnd1 Csf2rb Il12rb1 Il2rb Il2rg Il7r Il9r Crlf2*

Supplementary Table 4C. Pathways enriched in upregulated genes in h-Foxo3–/– vs. Ctrl under the fed condition.

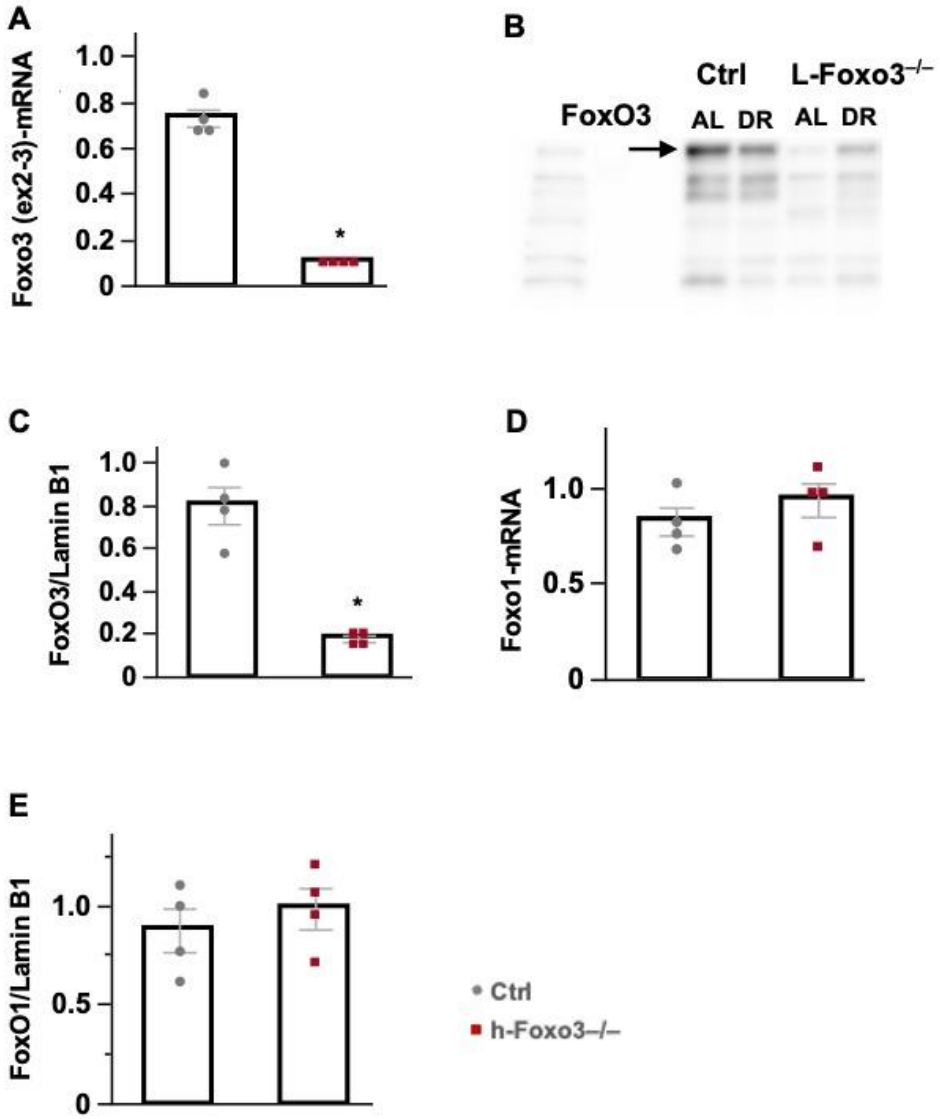
Enrichment FDR	nGenes	Pathway Genes	Fold Enrichment	Pathway	Genes
1.4E-08	11	92	15.92	Path:mmu04610 Complement and coagulation cascades	*Fgb Serping1 C3 C4b C6 C9 Cfh Cfi Fga Hc C8a*
6.6E-05	11	235	6.68	Path:mmu05171 Coronavirus disease-COVID-19	*Fgb C3 C4b C6 C9 Fga Hc Il1b Il6ra Stat3 C8a*
3.3E-03	6	107	8.49	Path:mmu05146 Amoebiasis	*C9 Il1b Il1r1 Lama3 C8a Lamc3*
3.3E-03	5	119	11.10	Path:mmu05150 Staphylococcus aureus infection	*C3 C4b Cfh Cfi Hc*
3.3E-03	6	142	8.05	Path:mmu05322 Systemic lupus erythematosus	*C3 C4b C6 C9 Hc C8a*
5.0E-03	5	76	9.47	Path:mmu05133 Pertussis	*Serping1 C3 C4b Hc Il1b*
6.4E-03	5	84	8.70	Path:mmu05204 Chemical carcinogenesis-DNA adducts	*Cyp3a13 Cyp3a16 Gstp2 Gstp1 Cyp2c70*
1.4E-02	6	145	5.68	Path:mmu05418 Fluid shear stress and atherosclerosis	*Acvr2b Cav1 Gstp2 Gstp1 Il1b Il1r1*
2.8E-02	4	71	8.05	Path:mmu04920 Adipocytokine signaling pathway	*G6pc Pck1 Stat3 Irs2*
3.9E-02	5	139	5.41	Path:mmu04936 Alcoholic liver disease	*C3 C4b Hc Il1b Lbp*
4.8E-02	8	357	3.27	Path:mmu04151 PI3K-Akt signaling pathway	*Efna1 Efna3 Fgfr1 G6pc Il6ra Lama3 Pck1 Lamc3*

Supplementary Table 4D. Pathways enriched in downregulated genes under the fed condition.

Enrichment FDR p < 0.10	nGenes	Pathway Genes	Fold Enrichment	Pathway	Genes
5.6E-09	10	89	21.1984	Path:mmu03320 PPAR signaling pathway	*Plin2 Cd36 Cyp4a10 Cyp4a14 Cyp8b1 Fabp2 Me1 Pparg Acaa1b Ehhadh*
5.9E-08	7	34	35.7061	Path:mmu01040 Biosynthesis of unsaturated fatty acids	*Elovl6 Acot2 Acot3 Acot4 Acaa1b Acot1 Elovl5*
1.5E-07	30	1611	3.26891	Path:mmu01100 Metabolic pathways	*Cyp4a32 Alpl Aldh3a2 Cyp2a4 Cyp2b13 Cyp2b9 Cyp2c38 Cyp4a10 Cyp4a14 Cyp8b1 Gstm1 Gstt2 Acod1 Elovl6 Acot2 Acot3 Acot4 Me1 Acaa1b Nt5e Acot1 Ugt1a9 Pla2g6 Gal3st1 Hao2 9130409I23Rik Mgst3 Mogat1 Elovl5 Ehhadh*
4.6E-07	6	29	36.2728	Path:mmu00062 Fatty acid elongation	*Elovl6 Acot2 Acot3 Acot4 Acot1 Elovl5*
1.6E-06	8	97	15.184	Path:mmu00830 Retinol metabolism	*Cyp2a4 Cyp2b13 Cyp2b9 Cyp2c38 Cyp4a10 Cyp4a14 Ugt1a9 Retsat*
5.7E-05	9	209	7.81409	Path:mmu05207 Chemical carcinogenesis-receptor activation	*Cyp2b13 Cyp2b9 Ephx1 Gstm1 Gstt2 Myc Ugt1a9 Mgst3 Paqr7*
1.1E-04	6	85	13.416	Path:mmu00590 Arachidonic acid metabolism	*Cyp2b13 Cyp2b9 Cyp2c38 Cyp4a10 Cyp4a14 Pla2g6*
1.1E-04	6	84	13.2347	Path:mmu05204 Chemical carcinogenesis-DNA adducts	*Cyp2c38 Ephx1 Gstm1 Gstt2 Ugt1a9 Mgst3*
1.6E-04	5	52	17.3647	Path:mmu00071 Fatty acid degradation	*Aldh3a2 Cyp4a10 Cyp4a14 Acaa1b Ehhadh*
1.9E-04	6	86	11.6591	Path:mmu04146 Peroxisome	*Crat Pex11a Acaa1b Abcd2 Hao2 Ehhadh*
6.1E-04	5	73	12.7522	Path:mmu00980 Metabolism of xenobiotics by cytochrome P450	*Ephx1 Gstm1 Gstt2 Ugt1a9 Mgst3*
2.9E-03	4	63	13.3247	Path:mmu04913 Ovarian steroidogenesis	*Acot2 Acot3 Acot4 Acot1*
5.4E-03	4	62	11.0663	Path:mmu01212 Fatty acid metabolism	*Elovl6 Acaa1b Elovl5 Ehhadh*
5.7E-03	4	71	10.7035	Path:mmu00982 Drug metabolism-cytochrome P450	*Gstm1 Gstt2 Ugt1a9 Mgst3*

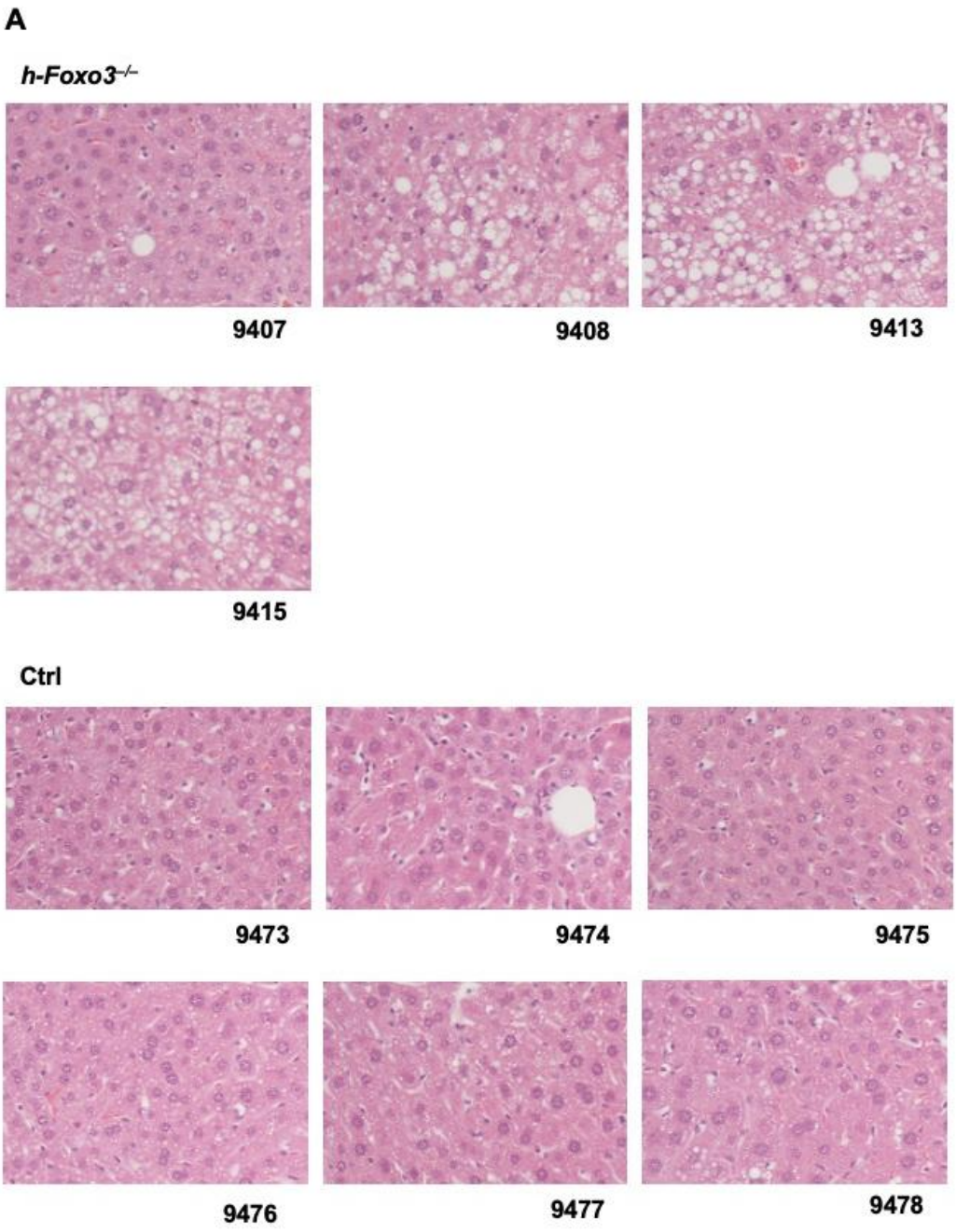
1.3E-02	4	92	8.37065	Path:mmu00140 Steroid hormone biosynthesis	*Cyp2b13 Cyp2b9 Cyp2c38 Ugt1a9*
1.4E-02	5	145	6.00102	Path:mmu05418 Fluid shear stress and atherosclerosis	*Gpc1 Gstm1 Gstt2 Sumo2 Mgst3*
1.4E-02	4	92	7.96233	Path:mmu00983 Drug metabolism-other enzymes	*Gstm1 Gstt2 Ugt1a9 Mgst3*
2.5E-02	5	173	5.13295	Path:mmu05225 Hepatocellular carcinoma	*Frat1 Gstm1 Gstt2 Myc Mgst3*
3.0E-02	3	56	9.60163	Path:mmu00280 Valine leucine and isoleucine degradation	*Aldh3a2 Acaa1b Ehhadh*
3.2E-02	2	18	20.4035	Path:mmu00120 Primary bile acid biosynthesis	*Cyp46a1 Cyp8b1*
3.6E-02	4	126	5.82956	Path:mmu04750 Inflammatory mediator regulation of TRP channels	*Cyp2c38 Cyp4a10 Cyp4a14 Pla2g6*
4.5E-02	2	25	16.3228	Path:mmu00592 alpha-Linolenic acid metabolism	*Acaa1b Pla2g6*

SUPPLEMENTARY DATA

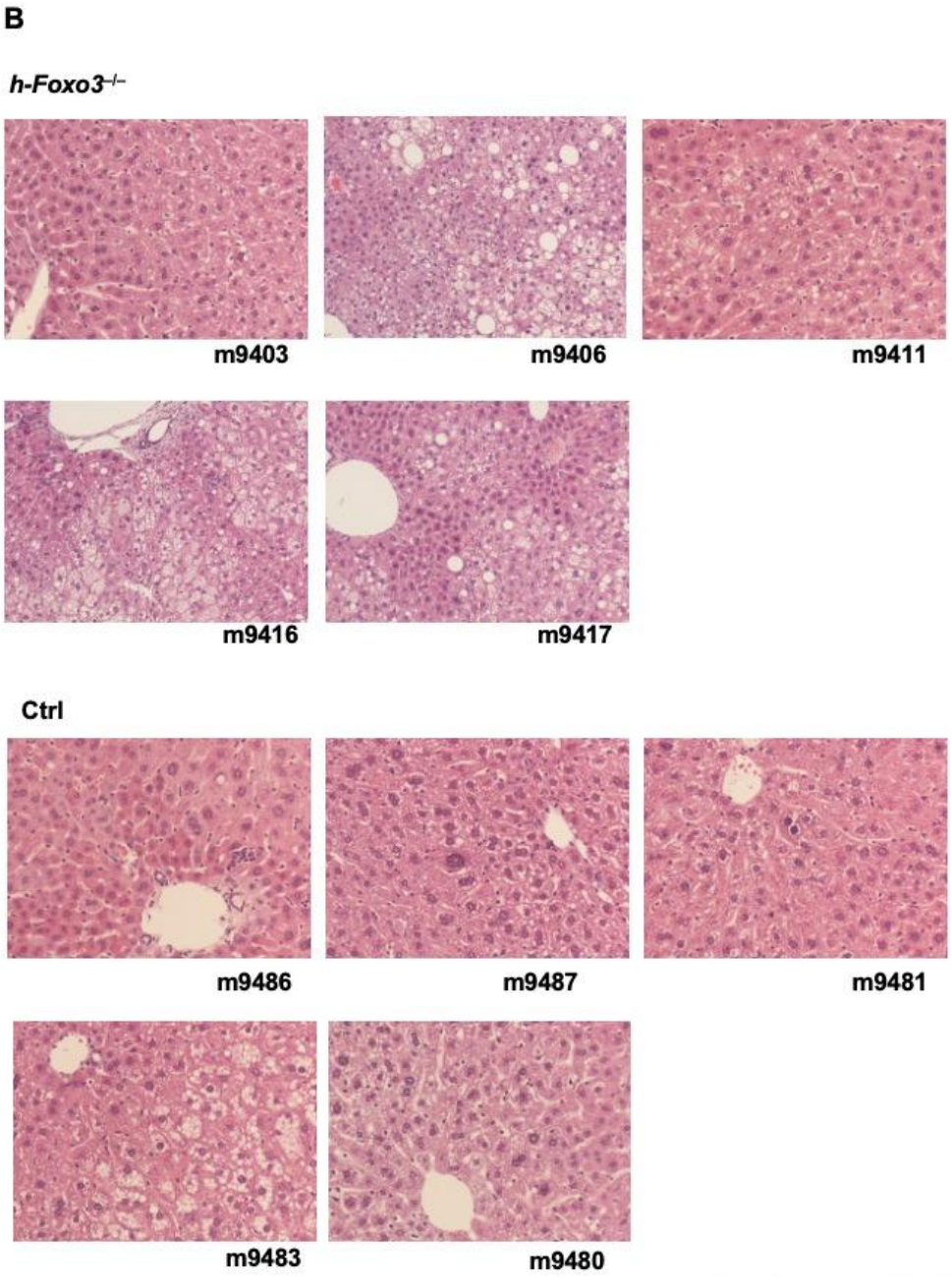


Supplementary Figure 1. *Foxo3*- and *Foxo1*-mRNA and protein levels data in *h-Foxo3*$^{-/-}$ liver. A) mRNA expression levels of *Foxo3* (ex2-3) in the liver at 31 weeks of age (wk). B) A representative image of Western blot for FoxO3 protein in the nuclear fraction of livers at 94-104 wk. AL and DR represent the ad libitum (AL) and 30% dietary-restricted feeding (DR) groups. The DR group data, which are not included in the present study, are shown for reference. C) FoxO3 protein levels in the AL groups normalized by lamin B1 in the nuclear fraction of livers. D) mRNA expression levels of *Foxo1* in the liver at 31 wk. E) FoxO1 protein levels normalized by lamin B1 in the nuclear fraction of livers at 94-104 wk. In panels A, and C-E, the bars and lines indicate the means and SE (n = 4 mice for each group). Gray circles represent Ctrl mice; red squares represent *h-Foxo3*$^{-/-}$ mice. Each point shows a measurement from an individual mouse. Statistical symbol; *, $p < 0.05$ by the Wilcoxon's rank-sum test.

SUPPLEMENTARY DATA

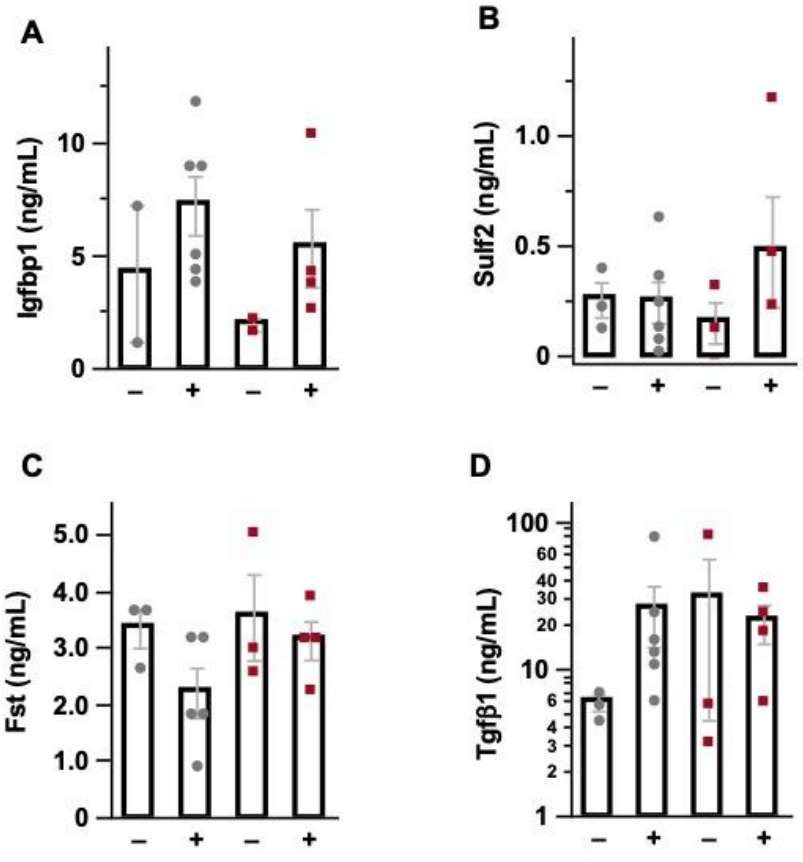


SUPPLEMENTARY DATA



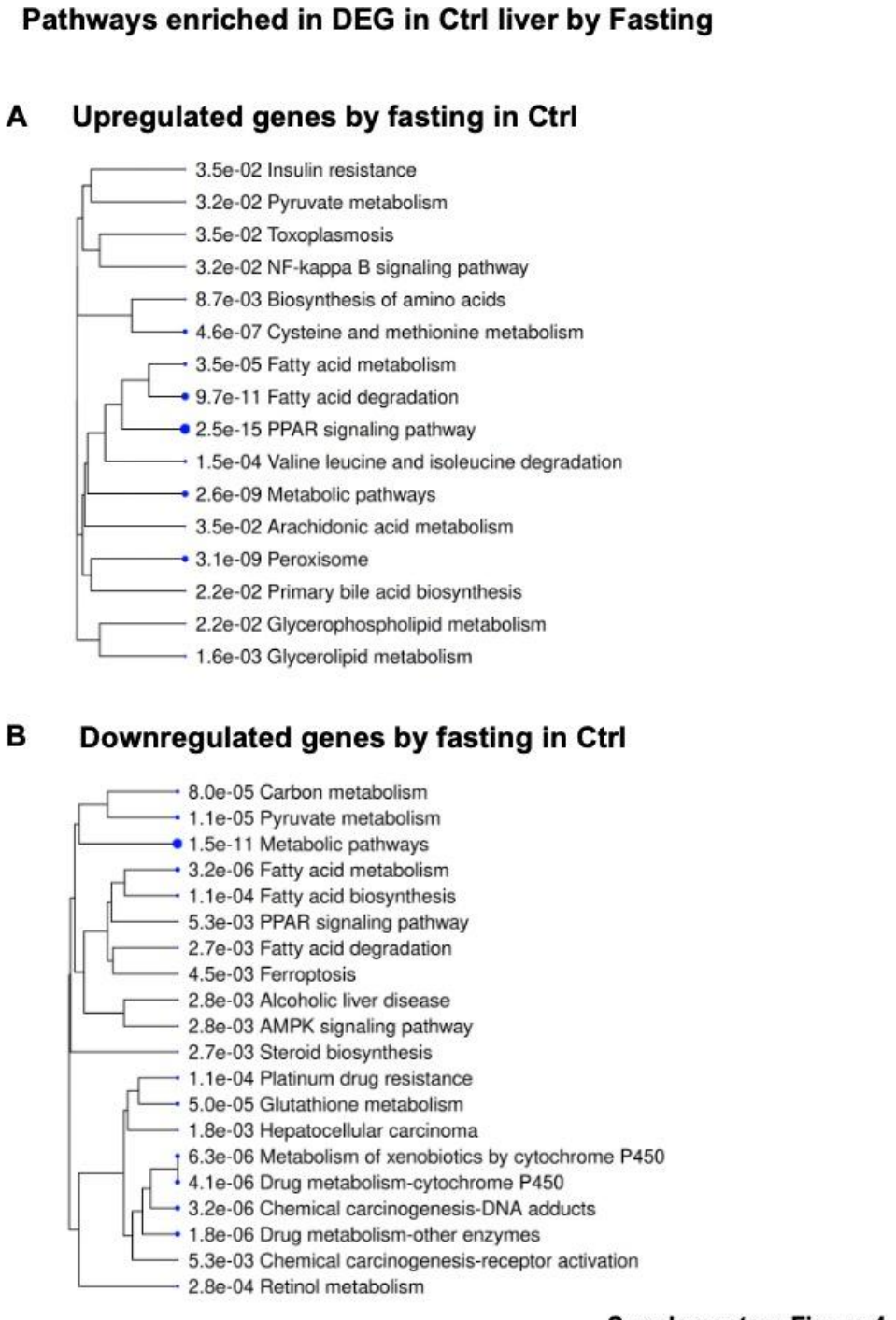
Supplementary Figure 2. Histology of the liver at middle age (74 wk) and old age (96-110 wk). A) Microscopic images of the liver from middle-aged mice. B) Microscopic images of the liver from old mice. The numbers indicate the ID of individual mice.

SUPPLEMENTARY DATA



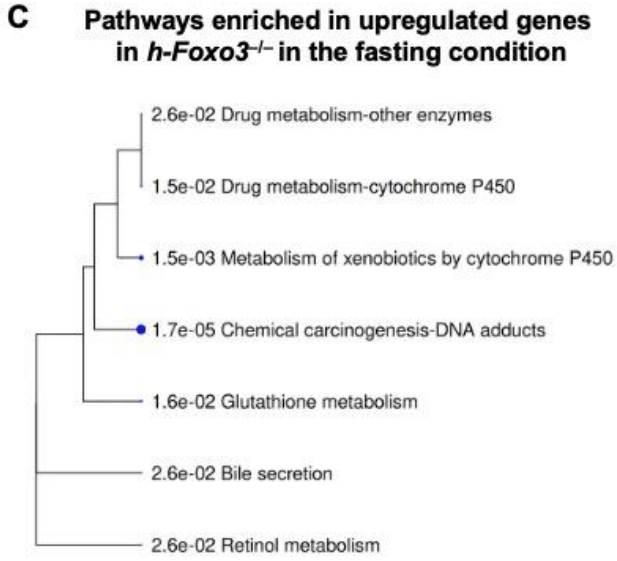
Supplementary Figure 3. The plasma levels of hepatokines. A) Igf-binding protein 1 (Igfbp1, ng/mL). B) Sulfatase 2 (Sulf2, ng/mL). C) Follistatin (Fst, ng/mL). D) Transforming growth factor beta 1 (Tgfß1, ng/mL). The bars and lines indicate the means and SE (n = 2,3, 4, or 6 mice for each group). Gray circles represent Ctrl mice; red squares represent *h-Foxo3*$^{-/-}$ mice. Each point shows a measurement from an individual mouse.

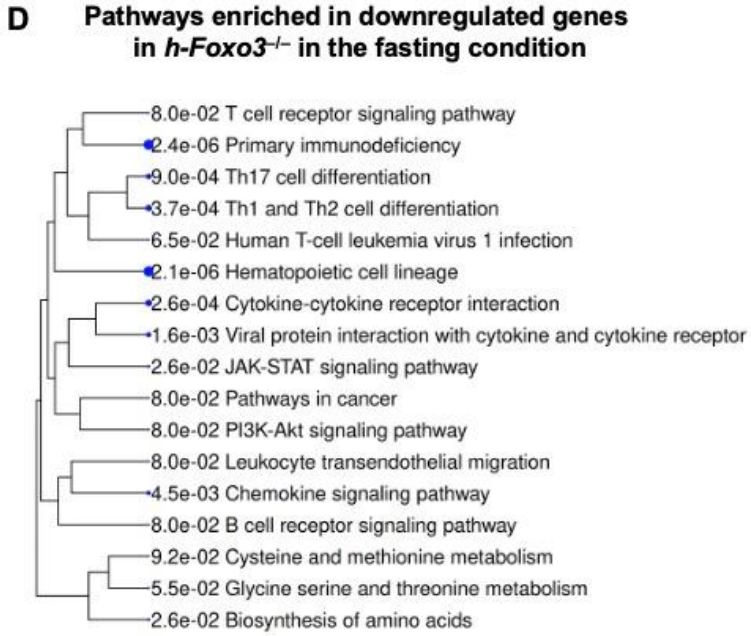
SUPPLEMENTARY DATA



Supplementary Figure 4

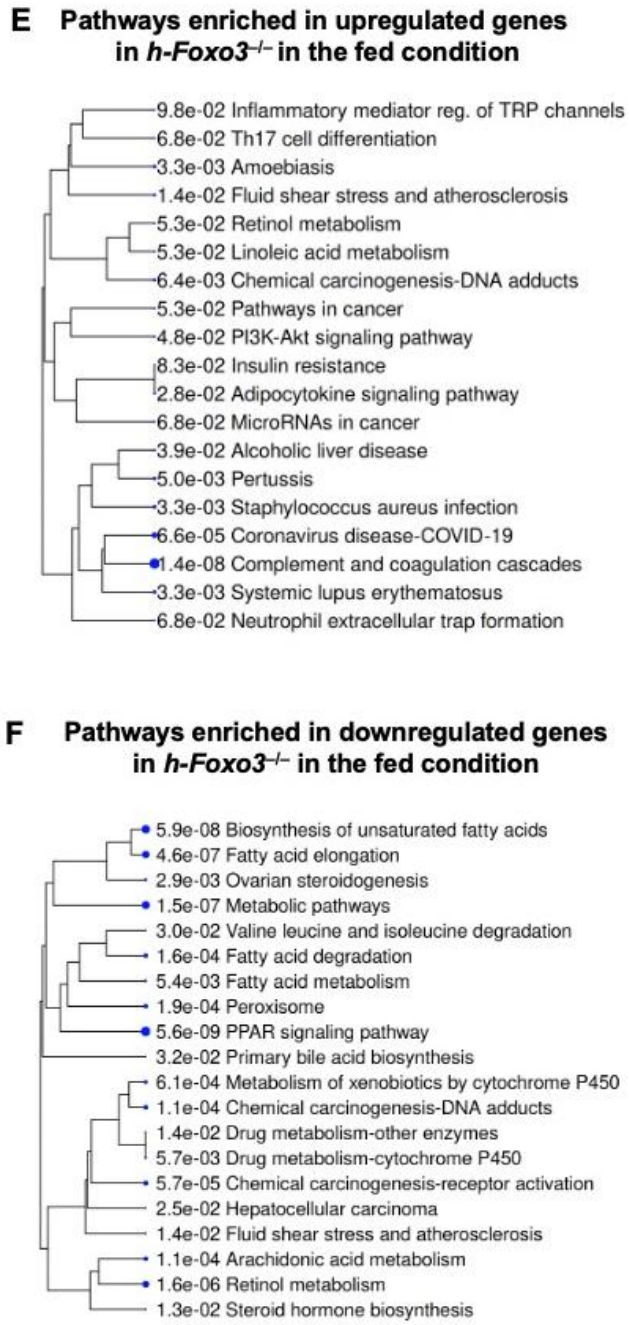
SUPPLEMENTARY DATA





Supplimentary Figure 4C,D

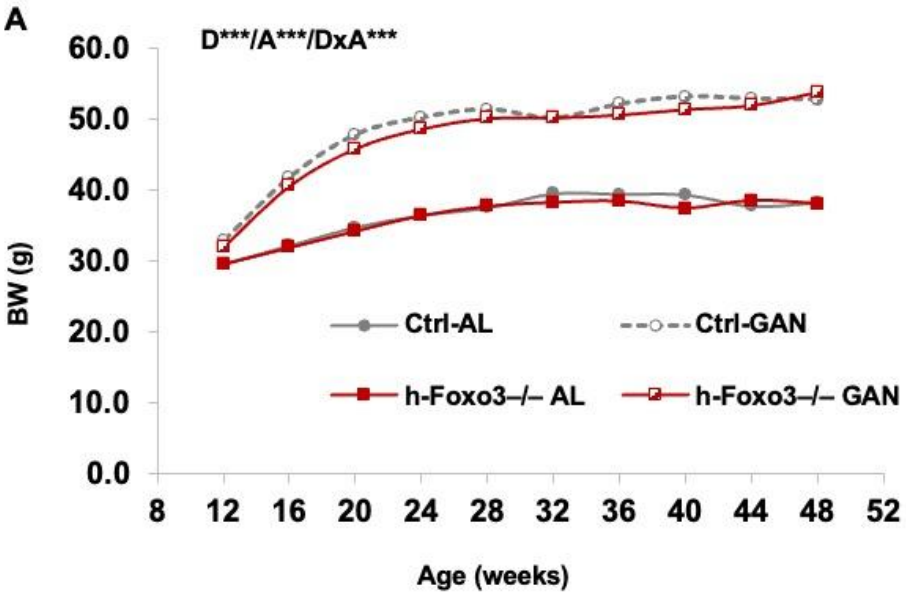
SUPPLEMENTARY DATA

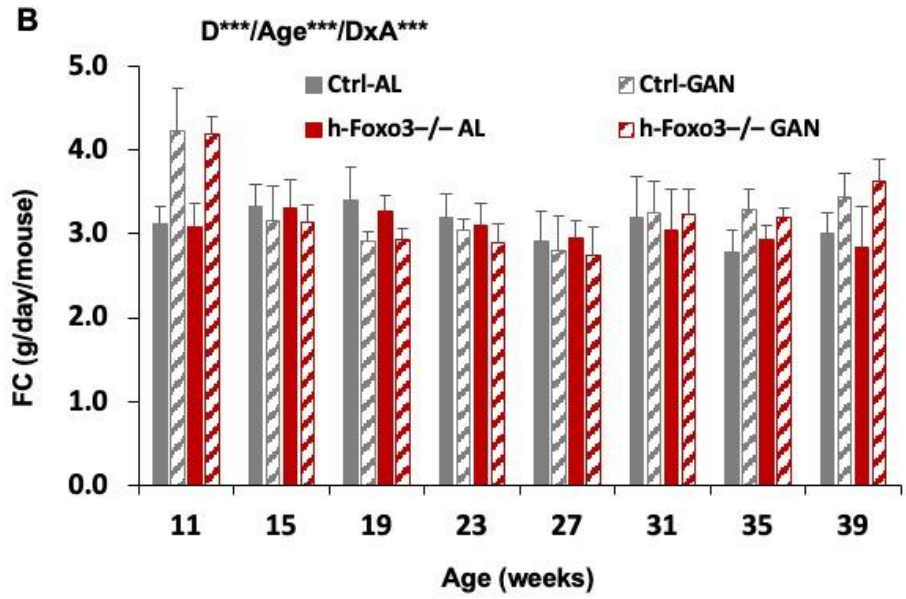


Supplimentary Figure 4E, F

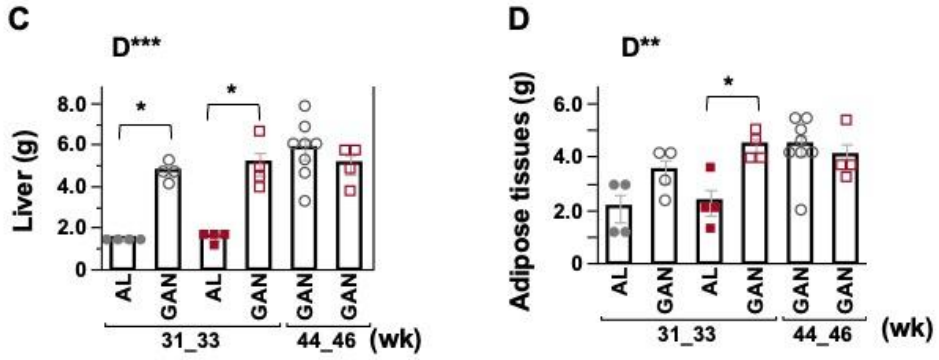
Supplementary Figure 4. Pathway enrichment analyses of genes differentially regulated by overnight fasting in Ctrl liver at 74 wk and genes that were differentially regulated in Ctrl and *h-Foxo3*$^{-/-}$ liver under fasting and fed conditions. Hierarchical clustering of pathways is drawn by ShinyGo 8.2 (17). Enrichment FDR values are inserted before pathway names. Blue circles represent the number of genes. A) Pathways enriched in upregulated genes by fasting in the Ctrl liver. B) Pathways enriched in downregulated genes by fasting in the Ctrl liver. C) Pathways enriched in upregulated genes in fasted h-Foxo3−/− liver. D) Pathways enriched in downregulated genes in fasted h-Foxo3−/− liver. E) Pathways enriched in upregulated genes in fed h-Foxo3−/− liver. F) Pathways enriched in downregulated genes in fed h-Foxo3−/− liver.

SUPPLEMENTARY DATA



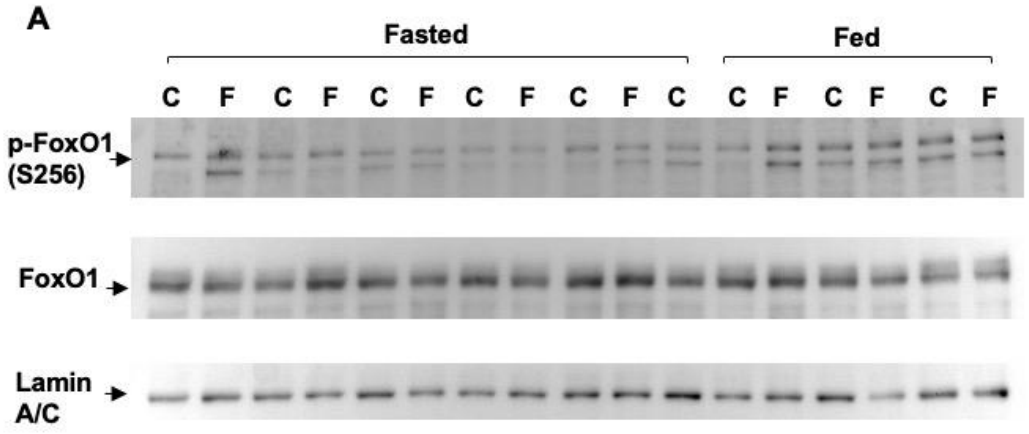


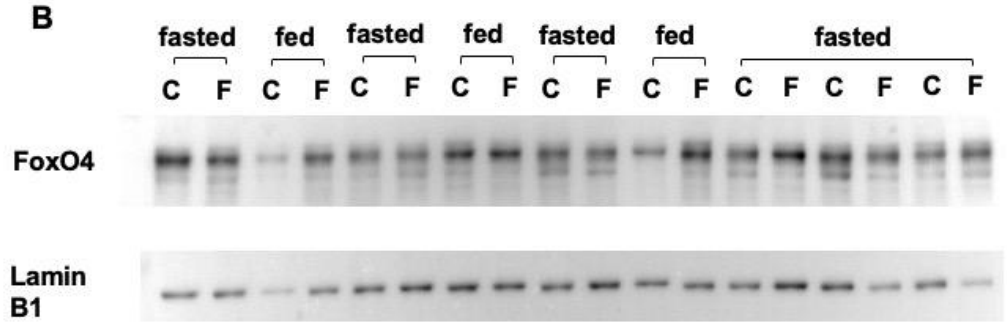
SUPPLEMENTARY DATA

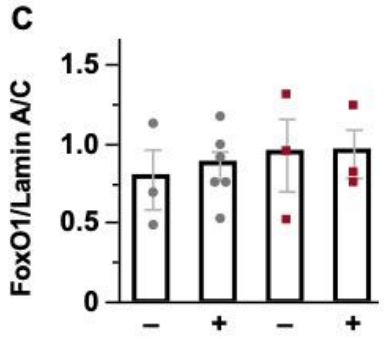


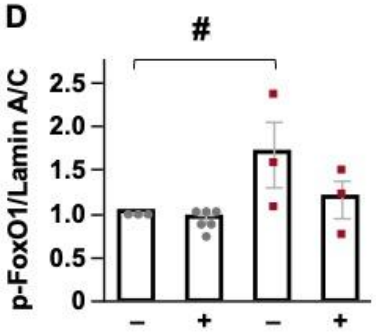
Supplementary Figure 5. Body weight (A), food intake (B), and the liver (C) and adipose tissue (D) weights in mice subjected to the GAN diet. AL and GAN represent the regular CRF-1 and GAN diets, respectively. Gray circles represent Ctrl mice; red squares represent *h-Foxo3*$^{-/-}$ mice. In panels A and B, statistical symbols, D, A, and D×A represent significant effects of diet, age, and diet × age interaction in a mixed model (*** $p < 0.001$). In panels C and D, statistical symbol D represents a significant effect of diet obtained from ART two-way ANOVA with *** $p < 0.001$ and ** $p < 0.01$ in the 31_33 wk. * $p < 0.05$ also represents the significance level obtained for pairwise comparisons using Wilcoxon's method.

SUPPLEMENTARY DATA

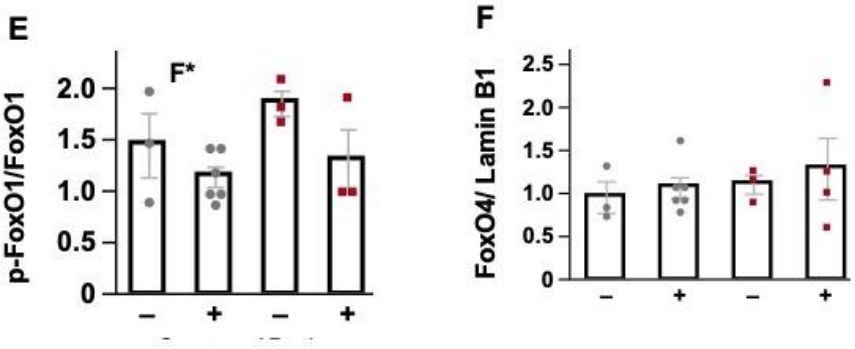






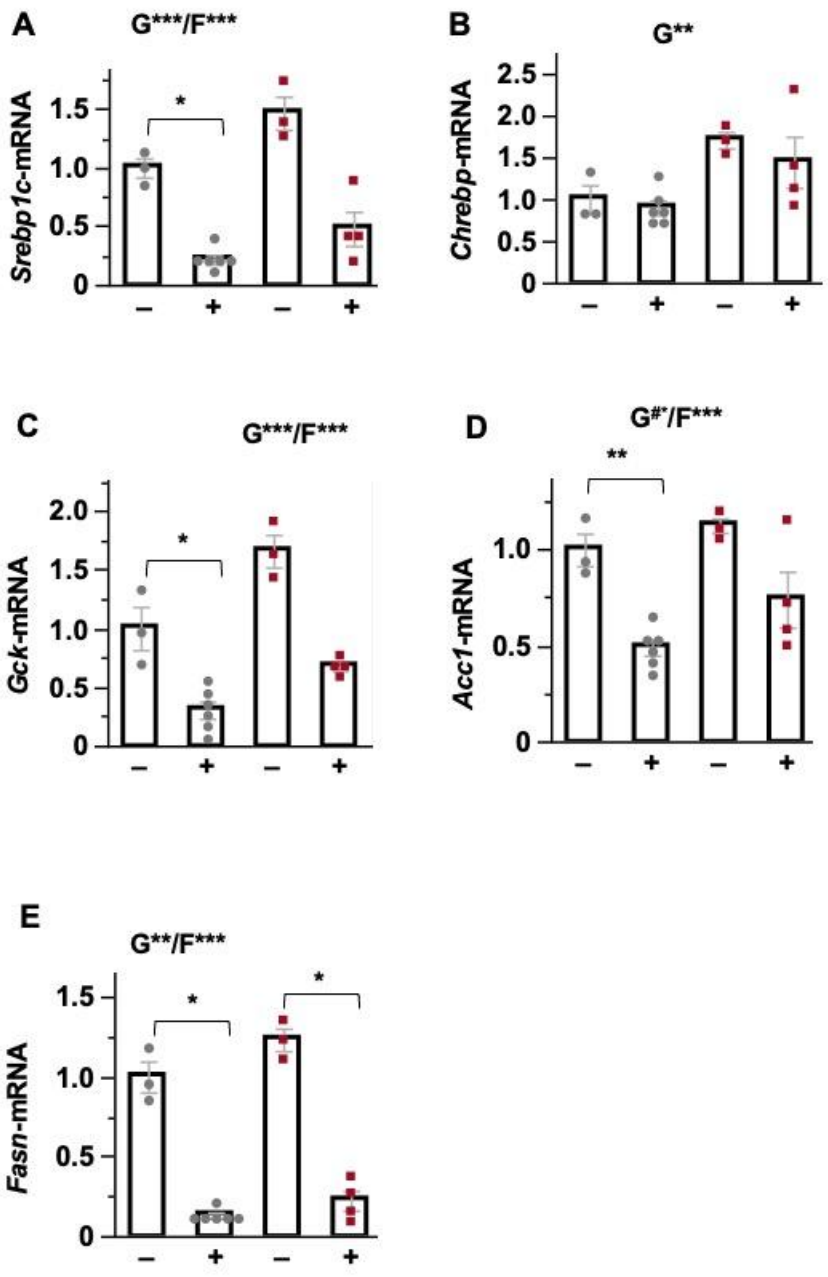


SUPPLEMENTARY DATA



Supplementary Figure 6. Protein levels of FoxO1 and FoxO4 in the nuclear fraction of liver tissues at middle age. A) Western blotting of S256-phosphorylated (p-) FoxO1 (upper panel), FoxO1 (middle panel), and Lamin A/C (lower panel). C and F represent the Ctrl and *h-Foxo3*$^{-/-}$ groups. B) Western blotting of FoxO4 (upper panel) and Lamin B (lower panel). Panels C-E) Protein levels of p-FoxO1 and FoxO1 were normalized to Lamin A/C. FoxO4 levels were normalized to Lamin B1. Bars and lines indicate the means and SE (n = 3 or 4 mice per group). Each point represents a measurement from an individual mouse. #, p = 0.0806 by Wilcoxon's method for pairwise comparisons. F* indicates a significant effect of fasting obtained from ART two-way ANOVA (* $p < 0.05$).

SUPPLEMENTARY DATA



Supplementary Figure 7. mRNA expression levels of lipogenesis genes in the liver at middle age. A) *Srebp1c*-mRNA. B) *Chrebp/Mlxipl*-mRNA. C) *Gck*-mRNA. D) *Acc1/Acaca*-mRNA. E) *Fasn*-mRNA. Expression levels were normalized to *Atp5f1*-mRNA. Statistical symbols: G and F indicate significant effects of genotype and fasting, with *p*-values from ART two-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). * $p < 0.05$ also denotes significance in pairwise comparisons using the Wilcoxon method.