Supplementary Material and Methods

Clinical patient specimens

Tumor and adjacent non-tumor tissues were surgically collected from human patients and immediately frozen at –78°C until use. *O*-GlcNAc modification within FOXO3 was evaluated in human pancreatic ductal carcinoma (n = 24; male = 15, female = 9; age = 64.95 ± 9.9), hepatocellular carcinoma (n = 10; male = 5, female = 5; age = 59.2 ± 10.3), gastric tubular carcinoma (n = 10; male = 8, female = 2; age = 59.8 ± 12.0), cholangiocarcinoma (n = 5; male = 3, female = 2; age = 63.6 ± 3.6), colon carcinoma (n = 10; male = 7, female = 3; age = 61.5 ± 11.4) and lung carcinoma tissues (n = 10; male = 3, female = 7; age = 63.1 ± 12.4). All tissue samples were obtained from the archives of the Department of Pathology, Yonsei University College of Medicine (Seoul, Korea). This study was approved by the Institutional Review Board (IRB, 4-2015-0474) of Yonsei University of College of Medicine, and PDAC diagnoses were made by pathologists at the Severance Hospital of Yonsei University. All subjects received a written informed consent that the studies were conducted in accordance with recognized ethical guidelines.

Cell culture and plasmid DNA transfection

Pancreatic cancer cell lines (PANC-1, BxPC-3, and HPAC) were purchased from ATCC (Manassas, VA, USA), human pancreatic duct epithelial cell line (HPDE) was kindly provided by Dr. Ming-Sound Tsao (University of Toronto). All cell lines were authenticated by short-tandem repeat (STR) profiling by Korean Cell Line Bank and shown to be negative in mycoplasma test using Mycoprobe mycoplasma detection kit (R&D system). The following ATCC-specified cell culture media were used: Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum for PANC-1, RPMI 1640 with 10% fetal bovine serum for BxPC-3, and DMEM-F12 (1:1) with 10% fetal bovine serum for HPAC. HPDE cells were grown in serum-free media supplemented with bovine pituitary extract and

epidermal growth factor. All cells were cultured in a 37°C incubator with a 5% CO_2 atmosphere. PANC-1 cells were transiently transfected using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) and selected with G418 (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Human FOXO3 DNA was purchased from Origene (Rockville, MD, USA), and human OGT DNA was kindly provided by Dr. Jin Won Cho (University of Yonsei).

Reagents

Antibodies specific for FOXO3 (2497, 1:1000 dilution), p21 (2947, 1:1000), CDK2 (2546, 1:1000), CDK-4 (12790, 1:1000), and Cyclin D1 (2978, 1:1000) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies specific for *O*-GlcNAc (ab2739, 1:500), OGA (ab124807, 1:1000), MDM2 (ab10344, 1:1000), CDK1 (ab18, 1:1000), and Cyclin G2 (ab54901, 1:1000) were purchased from Abcam (Cambridge, UK). Antibodies specific for GAPDH (sc-25778, 1:5000), p53 (sc-126, 1:200), Cyclin A (sc-751, 1:200), Cyclin E (sc-481, 1:200), anti-mouse (sc-2005, 1:5000), and anti-rabbit (sc-2030, 1:5000) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). An OGT-specific antibody (O6264, 1:1000) was purchased from Sigma-Aldrich. Thiamet G ((3aR,5R,6S,7R,7aR)-2-(ethylamino)-3a,6,7,7a-tetrahydro-5-(hydroxymethyl)-5H-Pyrano[3,2-d]thiazole-6,7-diol, OGA inhibitor) was kindly provided by Dr. Jin Won Cho (University of Yonsei).

Immunohistochemistry

Sixteen consecutive pancreatic ductal carcinomas were retrieved from November 2016 to April 2017. Formalin-fixed, paraffin-embedded specimens were obtained from the archives of the Department of Pathology Yonsei University College of Medicine (Seoul, Korea). Four-micrometer tissue microarray sections were subjected to immunohistochemistry for FOXO3 (12829, 1:400, Cell Signaling Technology) using a Ventana BenchMark XT

Autostainer (Ventana Medical Systems, Tucson, AZ, USA). A semi-quantitative H-scoring assessment was performed by multiplying the nuclear staining intensity (0, none; 1, weak or barely detectable; 2, moderate; and 3, strong) by the percentage of positive cells (0–100%).

Immunoprecipitation and western blot analysis

Cell lysates were incubated with specific antibodies and lysis buffer for 1 h. Subsequently, 30 µl of washed agarose beads (Sigma-Aldrich) were added to each lysate and incubated overnight at 4°C. Next, the beads were washed three times with phosphate-buffered saline containing 0.05% Tween-20 (PBS-T), and the antigens were eluted twice using 50 µl of PBS pH 2.0, neutralized with 1 N NaOH, and concentrated. The resulting samples were separated by SDS-PAGE (10%) and transferred onto nitrocellulose membranes using an iBLOT dry blotting system (Invitrogen). The membranes were then blocked with 5% skim milk in Tris-buffered saline + Tween-20 (TBS-T; 20 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH 7.6), incubated for 2 h with a 1:1000 dilution of anti-FOXO3 or *O*-GlcNAc antibody, and subsequently incubated for 1 h with a 1:5000 dilution of a horseradish peroxidase–conjugated goat anti-rabbit or anti-mouse secondary antibody (Santa Cruz). Immunoreactive proteins were detected using ECL Plus western blotting detection reagents (GE Healthcare, Milwaukee, WI, USA) and detected using a Typhoon 9400 scanner (GE Healthcare). The band intensity was densitometrically evaluated using ImageQuant TL software (GE Healthcare).

Agilent 6530 Q-TOF-MS/MS protein identification

A nano LC–MS/MS analysis was performed on a nano HPLC system (Agilent Technologies, Santa Clara, CA, USA). A nanochip column (Agilent, 150 mm × 0.075 mm) was used for peptide separation. For the LC separation, mobile phase A comprised 0.1% formic acid in deionized water, and mobile phase B comprised 0.1% formic acid in acetonitrile (ACN). The chromatography gradient was designed to yield a linear increase

from 3% B to 45% B over 40 min, 45% B to 60% B over 5 min, 95% B over 10 min, and 3% B for 15 min. The flow rate was maintained at 400 nl/min. Product ion spectra were collected in the information-dependent acquisition mode and analyzed using Agilent 6530 Accurate-Mass Q-TOF with continuous one-full-scan TOF MS cycles from 350 to 1200 m/z (5 spectra/s) plus three product ion scans from 100 to 1700 m/z (2 spectra/s). Starting with the most intense ion, the precursor m/z values were selected at a selection quadrupole resolution of 4 Da. The rolling collision energy feature was used to determine the collision energy based on the precursor value and charge state. The dynamic exclusion time for the precursor ion m/z values was 30 s.

Protein fractionation on an Agilent 3100 OFFGEL fractionator

Proteins were purified on an Agilent 3100 OFFGEL fractionator (Agilent). Samples and rehydration buffer (IPG, pH 3–pH 10; Agilent) were added to each well, followed by a mineral oil overlay to reduce protein losses. The samples were fractionated using increasing voltages from 800 V to 8000 V. The results of the OFFGEL procedure were confirmed by Western blotting with a theoretical pH.

Protein identification using the Q Exactive Orbitrap LC–MS/MS

Liquid chromatography separation was performed on an Ultimate 3000 RS nano LC system (Thermo Scientific, Waltham, MA, USA). A C18 Easy nano column (particle sizes: 150 mm, 75 μm, 3 μm; pore size: 100 Å; Thermo) was used for peptide separation. For LC separation, mobile phase A comprised 0.1% formic acid in deionized water, and mobile phase B comprised 0.1% formic acid in ACN. Fifty micrograms of desalted peptides were loaded onto the column with a starting mobile phase of 4% ACN and 0.1% formic acid. The chromatography gradient was designed as follows: 10 min at 2% ACN, linear increases from 4% B to 40% B over 55 min, and 40% B to 60% B over 5 min, 95% B for 7 min, and 4% B for 11 min with a flow rate of 500 μl/min. Mass spectra for peptide identification or

quantification were acquired using an Orbitrap Q Exactive mass spectrometer (Thermo), and a top-10 method was used to automatically switch between MS and MS/MS scans. MS spectra were acquired at a resolution of 70,000 with an automatic gain control (AGC) target value of 1×10^6 ions or maximum integration time of 100 ms. Full MS scan ranges were acquired from 350 to 1800 m/z, with a minimum threshold of 20,000 ion counts. Peptide fragmentation was performed via high-energy collision dissociation with a set energy of 27 NCE. The ion selection intensity threshold was set at 1.0 e^5 with charge exclusions of $z = 1$ and $z > 7$. MS/MS spectra were acquired at a resolution of 17,500, with a target value of 2×10^5 ions or maximum integration time of 120 ms and a set isolation window of 2.0 m/z.

Site-directed point mutation

Specific primers for serine (S) and threonine (T) to alanine (A) and aspartic acid (D) mutations of FOXO3 were designed and used to insert point mutations in a plasmid vector. A PCR-amplified DNA fragment of pCMV6-FOXO3 was generated using Phusion DNA polymerase (NEB, Ipswich, MA, USA). The primers used in this process are as follows: S284A, 5'-cgaatca gctgacgac GCT cccttcccagctctccaag-3' (Forward), 5'-cttggagagctgggaggg AGC gtcgtcagctgatt cg-3' (Reverse); S284D, 5'-cgaatcagctgacgac GAT cccttcccagctctccaag-3' (Forward), 5'-cttgg agagctgggaggg ATC gtcgtcagctgattcg-3' (Reverse); S286A, 5'-cagctgacgacagtccc GCC cagctc tccaagtggc-3' (Forward), 5'-gccacttggagagctggg CGG gactgtcgtcagctg-3' (Reverse); S411A, 5'-gggactcatgcagcgg GCC tctagcttcccgtatac-3' (Forward), 5'-gtatacgggaagctagaggc CCG ctgc atgagtccc-3' (Reverse); T475A, 5'-cactccaggacctgctc GCT tcggactcacttagcc-3' (Forward), 5'-ggctaagtgagtccga AGC gagcaggtcctggagtg-3' (Reverse); S476A, 5'-ccaggacctgctcact GCG ga ctcacttagccacag-3' (Forward), 5'-ctgtggctaagtgagtc CGC agtgagcaggtcctgg-3' (Reverse); S482A, 5'-ggactcacttagccac GCC gatgtcatgatgacac-3' (Forward), 5'-gtgtcatcatgacatc GGC gtg gctaagtgagtcc-3' (Reverse); S551A, 5'-gtggcagccgtgccttg GCG aattctgtcagcaac-3' (Forward),

5’-gttgctgacagaatt CGC caaggcacggctgccac-3’ (Reverse). After PCR, the non-mutated sequences were cleaved using DpnI (NEB). The mutated vectors were transformed into *Escherichia coli* (DH5α) that were cultured and prepared using a Plasmid Maxi kit (QIAGEN, Venlo, Netherlands).

RNA isolation and quantitative real-time RT-PCR analysis

Total RNA was extracted using Trizol (Invitrogen) and purified using an RNeasy kit (QIAGEN) according to the manufacturers’ instructions. Complementary DNA (cDNA) was obtained via reverse transcription and subsequently combined with primers, a 10 mM dNTP mixture, and SYBR Green PCR mix (Applied Biosystems, Foster City, CA, USA). iQ SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA, USA) was used for qRT-PCR analyses. The reaction products were run on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories). Serial cDNA dilutions were used to generate GAPDH intensity reference standards. The primers used in this process are as follows: *FasL*, 5’-TCCGTGAGTTCACCAACCAAA-3’ (Forward), 5’-GGGGGTTCCCTGTTAAATGGG-3’ (Reverse); *MnSod*, 5’-CAGACCTGCCTTACGACTATGG-3’ (Forward), 5’-CTCGGTGGCG TTGAGATTGTT-3’ (Reverse); *Nib3*, 5’-CTGGGTAGAACTGCACTTCAG-3’ (Forward), 5’-GGAGCTACTTCGTCCAGATTCAT-3’ (Reverse); *p21*, 5’-CCTGGTGATGTCCGACCTG-3’ (Forward), 5’-CCATGAGCGCATCGCAATC-3’ (Reverse); *p27*, 5’-TCTCTTCGGCCCGGT CAAT-3’ (Forward), 5’-AAATTCCACTTGCGCTGACTC-3’ (Reverse); *p53*, 5'-TGCAGCT GTGGGTTGATTCC-3' (Forward), 5'-AAACACGCACCTCAAAGCTGTTC-3' (Reverse); *MDM2*, 5'-GGCTCTGTGTGTAATAAGGGAGA-3' (Forward), 5'-GGACTGCCAGGACTA GACTTTG-3' (Reverse); *OGA*, 5'-GCAGCACCCTCTTTAAATGCCACA-3' (Forward), 5'-C CTGGCACAAACTGCTCCTTGTTT-3' (Reverse); *OGT*, 5'-GCAACGTGGCCGACAGCAC A-3' (Forward), 5'-TGCAGTGTCTCTCAGCTGCCTCA-3'

(Reverse); *PFK1*, 5'-GGAGAAC TTCATGTGGTGTGAGAG-3' (Forward), 5'-GAGAAGTTAGACTTCTCCTTGG-3' (Revers e); *GFAT*, 5'-GGGCGACAAGGCCGTGGAAT-3' (Forward), 5'-CAGCCACTGCGGCGATG TCA-3' (Reverse); *GAPDH*, 5'-ATCAAGAAGGTGGTGAAGCA-3' (Forward), 5'-ACCAG GAAATGAGCTTGACA-3' (Reverse). All reactions were performed in triplicate.

Total cell counts, WST-1 assay, and crystal violet staining

Lipofectamine 3000 was used to transfect cells with vectors expressing wild-type and mutated FOXO3. The cells were seeded into 12-well plates, and total cell numbers were counted every 24 h. To evaluate cell proliferation, cells were cultured in 24-well plates in which 10% of the total media was replaced with WST-1. The absorbance of each well at 450 nm was analyzed at several time points using a plate reader. For the crystal violet assay, cells were cultured in six-well plates and stained with crystal violet dye at 1 and 5 days post-transfection. Upon solubilization, the amount of dye taken up by the cell monolayer was quantified using a plate reader at 750 nm.

Label-free MS quantification using LTQ Orbitrap XL LC–MS/MS

Nano LC–MS/MS analysis was performed using an Easy n-LC (Thermo Fisher, San Jose, CA, USA) and an LTQ Orbitrap XL mass spectrometer (Thermo Fisher) equipped with a nano-electrospray source. Samples were separated on a C18 nanobore column (150 mm × 0.1 mm, 3-μm pore size; Agilent). For LC separation, mobile phase A comprised 0.1% formic acid and 3% ACN in deionized water, and mobile phase B comprised 0.1% formic acid in ACN. The chromatography gradient was designed to yield linear increases from 0% B to 40% B over 70 min and from 40% B to 60% B over 5 min, 95% B for 7 min, and 3% B for 8 min. The flow rate was maintained at 1500 nl/min. Mass spectra were acquired using data-dependent acquisition with a full mass scan (350–1200 m/z), followed by 10 MS/MS scans.

For the MS1 full scans, the Orbitrap resolution was 15,000, and the AGC was 2×10^5. For MS/MS in the LTQ, the AGC was 1×10^4.

Peptide identification and quantification

Proteome Discoverer software (version 1.4; Thermo Fisher Scientific) was used for protein identification and quantification. Peptides were identified using UniProt (release date: April 2012; www.uniprot.org). The following database search criteria were used: taxonomy, *Homo sapiens* (86,875 sequences); carboxyamidomethylated (+57) at cysteine residues for fixed modifications; oxidized (+16) at methionine residues for variable modifications; maximum of two allowed missed cleavages; MS tolerance, 10 ppm; collision-induced dissociation, 0.8 Da; and high-energy collision dissociation MS/MS tolerance, 20-mmu. The peptides resulting from tryptic, Asp-N, and Lys-C digests were considered. SIEVE™ (version 2.4, Thermo Scientific) was used to normalize and align the intensities and retention times according to the manufacturer's protocol.

Heat map visualization of gene expression results

We used R statistics software (version 3.2.3; R Project, Vienna, Austria) to generate an expression heat map of the investigated genes and the gplots package to visualize and group gene expression according to similarity. The expression of each main factor was calculated using log-ratios from two group phenotypes (i.e., ratio = log(A/B)). Higher and lower gene expression in the A group relative to the B group were respectively encoded as red and green on the heat map.

Protein networking according to KEGG pathway descriptions and GO annotation using the PANTHER DB

The KEGG, a database designed to evaluate the high-level functions and utilities of biological systems (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/keg/), was used to group related proteins according to functional pathways. Additionally, this study

used the GO database, which contains functional and local gene information, to map all quantified proteins (http://www.pantherdb.org/). These analyses included all proteins quantified by the SIEVE program.

Nuclear fractionation

Nuclear proteins were fractionated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) according to the manufacturer's instructions.

Chromatin Immunoprecipitation assay (ChIP)

Magnetic coated Dynabeads Protein A/G was washed three times with block solution. Used for the ChIP assay were 10 μg of antibodies against FOXO3 (Abcam, ab12162). The results were normalized with input, and the primers in this experiment are as follows: BD1, 5'-CTCCTTCTCCACTCCCACT-3' (Forward), 5'-CTCCCAAGTGCTGGGATTAC-3' (Revers e); BD2-8, 5'-ATATCGCACCATTGCATTCCG-3' (Forward), 5'-GCCTCCCAAAGTGCTG AGAT-3' (Reverse); BD9, 5'-CATTGTGAAGCTCAGTACCAC-3' (Forward), 5'-CCCCTC TGCTTTCAGGCATT-3' (Reverse).

Statistical analysis

Three independent experiments were performed per assay, and the data are presented as means ± standard deviations. Data were analyzed using Student's *t*-test, and *p* values of <0.05 were considered statistically significant.