A novel radiotracer to image glycogen metabolism in tumors by positron emission tomography

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SUPPLEMENTARY MATERIALS AND METHODS

Senescence β-galactosidase staining
Evaluation of cellular senescence in HEY Rab25 and HCT116 cells seeded into 6-well plates was performed using a Senescence β-Galactosidase Staining Kit (Cell Signaling Technology), according to the manufacturer’s instructions. Cells were treated as described in the main text. Images were acquired on an Olympus IMT-2 inverted microscope (Olympus) using a 200× objective.

α-Glycogen immunofluorescence staining
α-Glycogen immunofluorescence staining was performed according to previously described methodology (1). Cells were washed with 2 x ice-cold PBS on a rotary shaker (200μL; 10 min; 400rpm) prior to fixation in 4% formaldehyde for 15 min at room temperature (RT). Following 3×PBS washing (200μL; 5 min; 400rpm), cells were permeabilized with 0.1% Triton-X100 (Sigma-Aldrich; 100μL; 10 min; RT) and further washed with PBS (3×5 min). Blocking was performed with 1% BSA/0.1% Triton-X100/PBS (PBST-BSA) for 1h at RT.
Mouse α-Glycogen monoclonal antibody was a kind donation from O. Baba (Tokyo Medical and Dental University, Japan). Lyophilized antibody was reconstituted in 500µL distilled water. Antibody was diluted 1/5 in PBST-BSA, with 50µL added per well, prior to overnight incubation at 4°C. The following day, wells were washed in PBS, 2× 0 min, and incubated with Alexa Fluor 594 goat anti-mouse IgG secondary antibody (Life Sciences; 200µL per well; 1/400 dilution; 5 µg/mL) in the dark for 1h. Wells were washed 3×10 min in PBS prior to addition of ProLong gold Antifade reagent with DAPI (Life Sciences) and coverslips. Fluorescence microscopy was performed on an Olympus DP70 microscope (Olympus.) at 400× magnification.

**Tumor spheroid growth and 2-NBDG uptake**

HEY Rab25 cells (1000 cells/well; 200µL) were seeded into 96-well culture plates, pre-coated with 50µL of a 1% UltraPure agarose solution (Invitrogen). Spheroids size was monitored daily by phase-contrast microscopy (Olympus IMT-2) at 400× magnification, with 100µL medium replaced every other day. 2-NBDG uptake was performed on day 5 when spheroids had reached 350-400µm in diameter. Fresh medium containing 2-NBDG (500µM) was added to wells. Following 3h 2-NBDG incubation, spheroids were removed from the well and washed 3× in 500µL ice-cold PBS before placed on microscope slides for analysis. Confocal fluorescence microscopy was performed on a Zeiss Axiovert 200M inverted microscope (Carl Zeiss Ltd), with z-stacking performed at 20µm intervals.

**Turpentine oil-induced inflammation model**

6-7 week old Balb/C mice were anesthetized by isoflurane inhalation. 30µl of turpentine oil (Sigma) was administered intra-muscularly into the right posterior thigh of the mice. The contralateral muscle, which received no treatment, was used as control. The mice were
imaged by either $^{18}$F-NFTG or $^{18}$F-FDG PET, as described in Materials and Methods, at 48h post turpentine oil injection. Static PET scans were carried out on a GENISYS$^4$ (Sofie Biosciences, Culver City, CA, USA) dedicated small animal PET scanner 50–60 min post radiotracer injection. Biodistribution was performed post imaging with $\frac{1}{2}$ of the control and $\frac{1}{2}$ of the turpentine-treated muscle. The remaining muscle was fixed in formalin, embedded in paraffin and sectioned (5µm slices). Sections were counterstained with hematoxylin and eosin (H&E) staining. Representative fields per section (at 200× magnification) were captured using an Olympus BX51 fluorescent microscope.

SUPPLEMENTARY FIGURES

**Supplementary Figure S1.** Glycogen Synthase 1 knockdown by shRNA in HEY Rab25 cells. Four GYS1-specific shRNA sequences in comparison to cells containing scrambled shRNA (Scr) and empty vector (EV) control cells. Actin was used as a loading control.
Supplementary Figure S2. Correspondence between punctate 2-NBDG fluorescence and immunofluorescence staining with the anti-glycogen antibody and Alexa Fluor 594-conjugated secondary antibody, 1, 3 and 6 days post seeding. Scale bar = 20 µm. All images were acquired at 400× magnification and are representative of three independent experiments.
Supplementary Figure S3. Changes in punctate 2-NBDG fluorescence during cell division.

Fluorescent microscopy images of intracellular 2-NBDG fluorescence in HEY Rab25 cells. Cells were incubated in 2-NBDG (500 µM) for 180 min followed by washing and replacement with 2-NBDG-free media. Sequential images (a–d) were taken 20 min apart. The dividing cell is indicated by the white arrow. Scale bar = 20 µm.
Supplementary Figure S4. Effect of HEY Rab25 cell density on cellular senescence.

Cellular senescence was evaluated in 6-well plates 24h post seeding at $1.2 \times 10^6$ cells/well (a), $6 \times 10^5$ cells/well (b), $3 \times 10^5$ cells/well (c), and $1.5 \times 10^5$ cells/well (d) using a Senescence β-Galactosidase Staining Kit from Cell Signaling Technology. Scale bar = 20 µm.
Supplementary Figure S5. Heterogeneous 2-NBDG staining of HEY Rab25 tumor spheroids. Confocal 20 µm Z-scan from HEY Rab25 spheroids following 2-NBDG incubation, characterized by intense staining of the spheroid core and high uptake of the spheroidal rim. Scale bar = 200 µm. Images were acquired at 100× magnification and are representative of three independent experiments.
Supplementary Figure S6. Representative radio-HPLC analysis of mouse plasma extracts.

Representative reference sample of $^{18}$F-NFTG, 5 min plasma extract and 15 min plasma extract are shown ($n = 3$ mice per sample).
Supplementary Figure S7. $^{18}$F-FDG and $^{18}$F-NFTG blood retention. The blood time versus radioactivity curve (TAC) was obtained from the left ventricle over the 60-minute dynamic PET scan. Mean ± SD ($n$ = 3-4 mice per group).
Supplementary Figure S8. 18F-NFTG and 18F-FDG uptake in turpentine-induced inflammatory tissue. A & B, Representative coronal PET images (50–60 minutes post injection) for 18F-NFTG (A) and 18F-FDG (B). C & D, Representative axial PET images (50–60 minutes post injection) for 18F-NFTG (C) and 18F-FDG (D). Turpentine-induced inflammatory tissue is circled in white. E, Normalized uptake of radiotracer derived from treated and untreated muscle ROIs from 50–60 min static PET images. F, 18F-NFTG and 18F-FDG biodistribution (tissue gamma-counting) of one part of the turpentine-treated and control, untreated posterior thigh muscle. Data are mean ± SD (n=3). G, Immunohistochemistry analysis of the other part of the tissue by H&E staining in control and turpentine-treated muscle. Photographic images of H&E-stained sections were acquired at
200× magnification. Scale bar = 100 µm. Abbreviations: NS, Not significant. * $P < 0.05$; *** $P < 0.001$.

**Supplementary Figure S9.** Glycogen content in the stationary phase of growth of 8 human tumour cell lines from ovarian, breast colorectal and lung adenocarcinoma. Mean values (bars, SD) are shown ($n = 3$).

**REFERENCES**