

CRISPR/Cas9 Knockout of *N*-acetyltransferase 1 Influences Mesenchymal Transition in Glioblastoma in a Cell Line Dependent Manner

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NAT1 KO Transduction, Selection, and Clonal Isolation

For Cas9 lentiviral transduction, cells were seeded into 24 well plates. Prepackaged lentivirus containing a vector for Cas9 with a blasticidin resistance gene were purchased from GenTarget (San Diego, CA). Cells were allowed to rest for 24 hours, and 50 μ L of lentivirus (1×10^7 IFU/mL with polybrene) was added to each well per cell line, per manufacturer recommendation. Following 18-hour overnight incubation with the lentivirus, media was replaced with complete media, and cells were allowed to rest for 72 hours. Cas9 expressing cells were selected via blasticidin antibiotic selection for 2-3 weeks and were then relabeled U87, U118, or A172 Cas9 respectively.

For gRNA lentiviral transduction, cells were seeded into 10 cm dishes and allowed to rest for 24 hours. Lentiviral prep (1 ml) was added to 9 mL of antibiotic-free media. The media was replaced with fresh complete media the following day. Approximately 48-72 hours following transduction,

hygromycin B (VWR, Radnor, PA, USA, Cat# 45000-806) antibiotic selection was performed for up to 7 days. Cells were then expanded, and stocks were cryogenically stored in liquid nitrogen. For clone generation, cells were seeded at colony formation density. Perspective NAT1 KO colonies were screened for their ability to perform *N*-acetylation of PABA and confirm the KO of NAT1 as previously described (Habil et al. 2022).

Measurement of *N*-acetylation

Para-aminobenzoic acid (PABA) (Sigma Aldrich, St. Louis, MO, USA, CAS 150-13-0) *N*-acetylation was measured as previously described (Wise et al. 2022). A total of 40,000 cells were plated onto a 12 well plate and allowed to rest for 24 hours before treatment with 500 μ M PABA. Immediately after treatment and 48 hours later, 100 μ L cell media was collected for both PABA treated and untreated cell populations. At collection, potential acetylation reactions were stopped with the addition of 10 μ L of 1 M acetic acid. Samples were then centrifuged at 15,000 G for 10 min to precipitate protein. PABA and its *N*-acetylated product *N*-acetyl-PABA were identified by high performance liquid chromatography (HPLC) Agilent 1260 Infinity II (Agilent Technologies, Santa Clara, CA, USA) following injection (40 μ L) onto a 125 $\times$ 4 mm LiChrospher 100 RP-100 5 μ M C18 HPLC column. Standards of 10, 100, 250 and 500 mM PABA and *N*-acetyl-PABA (556-08-1, Sigma Aldrich) were prepared. Cells from each well were then stained with Trypan Blue and counted using a hemacytometer. Measured *N*-acetylation was normalized to cell number. The limit of detection for *N*-acetyl-PABA was 0.005 nmol and three independent experiments were performed for all cell types.

Cell Microscopy and Immunofluorescence

One million U87, U118, and A172 WT, KO1 and KO2 cells were plated in 10 cm plates with CM and allowed to grow undisturbed for either two or seven days. On the seventh day, cells were washed with PBS and imaged using ZOE fluorescent cell imager (Bio Rad, Cat# 1450031).

Immunofluorescence studies were performed following the method previously described (Hood et al. 2004). Prepared cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA, Cat# T8787) in PBS (pH 7.4) for 10 min at room temperature, washed three times in PBS, and blocked in PBS containing 1% BSA for 10 min at room temperature. Antibodies were diluted in blocking buffer to 2 µg/mL including anti-GFAP monoclonal antibody (clone GA5) conjugated to Alexa Fluor 488 (eBioscience/Thermo Fisher Scientific, Cat# 53-9892-82) or anti-Nestin monoclonal antibody (clone 10C2) conjugated to Alexa Fluor 488 (eBioscience/Thermo Fisher Scientific, Cat# 53-9843-80). Following antibody washes, coverslips were removed from the plates, inverted cell-side down onto 10 µL of VECTASHIELD PLUS Antifade Mounting Medium with DAPI (Vector Laboratories, Cat# H-2000-2) on glass microscope slides and gently pressed to exclude air bubbles. Slides were allowed to set for 24 hours at room temperature before imaging. Mounted slides were imaged using a ZOE fluorescent cell imager. Alexa Fluor 488 signal (GFAP or Nestin) was acquired in the green channel and DAPI nuclear counterstain in the blue channel, with a paired image of each identical field captured in both channels. Five non-overlapping fields were captured per condition per marker, yielding 90 marker/DAPI field pairs across all conditions. Fields were selected to reflect the most frequently observed cellular morphology.

Image Analysis and Morphometry

Image analysis was performed in Fiji (ImageJ 2.18.0/1.54p). Spatial calibration was derived from the burned-in scale annotation present in every ZOE export (262 px = 100 µm; 0.3817 µm/px), and the annotated region, comprising 0.09% of the frame and identical in every image, was masked prior to measurement. All images were screened for detector saturation, quantified as the proportion of pixels at the 8-bit ceiling following exclusion of the annotation. For marker quantification, green-channel images underwent rolling-ball background subtraction (50 px radius) followed by a 3 x 3 median filter to suppress single-pixel shot noise inherent to 8-bit

acquisition. The threshold was derived from the residual background distribution across all green-channel images (mean + 4 SD after background subtraction). Threshold sensitivity was confirmed by repeating all measurements at 10 and 16 grey levels, across which condition rankings were unchanged. Percent-positive area was used as the primary readout in preference to mean fluorescence intensity because it is substantially less sensitive to signal clipping. Nuclei were enumerated from the paired DAPI image by Otsu thresholding of the background-subtracted, median-filtered blue channel followed by hole filling, morphological opening, and watershed separation of contacting nuclei, counting objects of 20–500 μm^2 . Marker-positive area was normalized to nucleus count to yield positive area per cell. Nuclear morphometry (area, perimeter, circularity, aspect ratio, and solidity) was recorded for all segmented nuclei ($n = 24,944$). Brightfield cells were segmented by local texture rather than intensity, as cell interiors and background differ minimally in mean grey level. A local standard-deviation filter (9×9 kernel) was computed, Gaussian-smoothed ($\sigma = 2$), and threshold determined by Otsu's method. The resulting mask was closed (7×7), hole-filled, and opened (3×3). Watershed separation was not applied, as it fragments elongated cells transversely. Objects contacting the image border or falling outside 150–8,000 μm^2 were excluded, yielding 3,645 cells across 45 fields. Because this approach identifies cells by texture contrast, thin and poorly contrasted cells are preferentially missed; detection efficiency was quantified for every field as the proportion of frame area classified as cellular and is reported as a quality-control measure alongside the morphometric data. For image-derived measurements, per-cell values were first collapsed to a per-field median and statistical comparisons were performed on field-level values ($n = 5$ fields per condition; $n = 10$ for nuclear morphometry, comprising five fields from each staining session). WT and NAT1 KO conditions were compared within each parental line using two-sided Mann-Whitney U tests, with statistical significance defined as $p < 0.05$. Because cell-area distributions are strongly right-skewed, medians are reported in preference to means where indicated. No correction for multiple comparisons was applied.

Cell Viability and Proliferation

Cell viability and proliferation experiments were performed using PrestoBlue Cell Viability Reagent (ThermoFisher Scientific, Cat# A13262) and manual cell counting via hemacytometer, respectively, to determine the potential influence of NAT1 KO on cell growth. PrestoBlue assays were performed according to manufacturer recommendations. A total of 50,000 cells were plated in each well of a 24 well plate with 1 mL of CM. Plates were then stored in the incubator for a maximum of four days. On days 1 through 4 in 24-hour intervals, PrestoBlue assays and manual cell counting were performed on the cells. PrestoBlue reagent (111 μ L) was added to all wells, and the plate was allowed to incubate at 37°C for 10 minutes before fluorescence measurement. Manual cell counts were performed through the application of 200 μ L of trypsin and 10 minutes of incubation. Cells were spun for 5 minutes at 300 G and resuspended in 200 μ L of CM. Cell containing media was added to Trypan Blue stain in ratios of 50:50 (days 1 and 2), 10:40 (day 3), and 10:90 (day 4), depending on cell concentration. The same ratio was used for each day in all cell conditions. Trypan Blue cell solution was added to the hemacytometer, and cells were counted. PrestoBlue Cell Viability Reagent fluorescence was measured with Tecan M200 infinite pro microplate reader (Tecan Group Ltd. Männedorf, Switzerland, RRID:SCR_019033). PrestoBlue fluorescence was measured using excitation/emission wavelengths of 560/590 nm. Four separate passages of cells were utilized with 4 technical replicates from each passage.

Reverse Transcriptase Quantitative PCR (RT-qPCR)

mRNA fold change expression was measured using the methods previously published protocol (Bardi et al. 2019). One million cells were plated in 10 cm plates. Cell conditions included WT, 5 ng/mL TGF- β 1-treated WT, KO1, and KO2 cells that were grown in complete media for 48 hours. Cell culture media was then removed, and cells were washed in PBS (Sigma-Aldrich, Cat#

D8537). Pooled sample RNA was isolated using Qiagen's miRNAeasy kit (Qiagen, Hilden, Germany, Cat# 217004) according to the manufacturer's instructions for purifying total RNA from cells. RNA quantity and quality were assessed via 260/280 absorbance ratios using a Tecan M200 infinite pro microplate reader. For each cell condition, 1000 ng of total RNA was converted to cDNA using Qiagen's RT2 First Strand Kit (Qiagen, Cat# 330404) and a T100 Thermocycler (Bio-Rad, Hercules, CA, USA, Cat# 1861096). Following conversion to cDNA, samples were loaded into Epithelial to Mesenchymal Transition (EMT) RT2 Profiler PCR Arrays (PAHS-090ZC) which was prepared according to manufacturer instructions using RT2 SYBR Green qPCR Mastermix (Qiagen, Cat# 330503) and DNase, RNase free 0.1 µm filtered water (Sigma Aldrich, CAS 7732-18-5). Quantitative PCR was performed using an Applied Biosystems QuantStudio 6 Flex Real-Time PCR System (ThermoFisher Scientific, Cat# 4485691).