

Inhibitory effects of high extracellular L-glutamate concentrations on skeletal myogenesis

Himiko Ban^{1,2}, Koji Nobe^{1,2}, Soushi Kobayashi^{1,2}

¹Department of Pharmacology, Showa Medical University Graduate School of Pharmacy, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo, 142-8555, Japan

² Pharmacological Research Center, Showa Medical University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan

Supplemental data

Supplemental methods

C2C12 cells were seeded in 96-well plates and differentiated for 0–3 days after reaching 90% confluency. Cell nuclei were stained with DAPI, then nuclear and differential interference contrast (DIC) images were acquired with a BZ-X810 fluorescence microscopy. The number of DAPI-stained nuclei within $0.5\text{ mm} \times 0.5\text{ mm}$ was counted by ImageJ/Fiji, and the number of cells per well was calculated. It was averaged for 3 wells on each day (Table S1, Number of cells/well). To calculate the volume per cell, the C2C12 surface area was first calculated from the DIC image using ImageJ/Fiji. On day 3 of differentiation, C2C12 cells were immunofluorescently labeled for MHC (F59). Fluorescent images of the cells were obtained at $0.5\text{ }\mu\text{m}$ intervals along the z-axis, covering a range of $50\text{ }\mu\text{m}$ in both upward and downward directions. From the slice images where the cells appeared clearly, the cell thickness was estimated to be $7.57 \pm 0.13\text{ }\mu\text{m}$. Since the typical thickness of mammalian cells is $5\text{--}10\text{ }\mu\text{m}$, we considered this result reasonable. The approximate cell volume was calculated by multiplying the cell surface area by the thickness (Table 1, Single cell volume). The cell lysate ($200\text{ }\mu\text{L}$) of C2C12 cells contained $14\text{--}20\text{ }\mu\text{M}$ of Glu (Fig. 1A). This Glu was entirely derived from intracellular Glu. The total cell volume per well was calculated by multiplying the number of cells per well in a 96-well plate by the volume of a single cell. The concentration of Glu per cell was determined by dividing the total amount of Glu in the cell lysate by the total cell volume (Table S1, Glu concentration per cell, Fig. S1).

Differentiation	Number of cells/well Mean $\pm$ SEM	Single cell volume (mL) Mean $\pm$ SEM	Glu concentration per cell (mM) Mean $\pm$ SEM
day0	60125 $\pm$ 4279	$3.3 \times 10^{-9} \pm 5.4 \times 10^{-11}$	20.7 $\pm$ 0.87
day1	38186 $\pm$ 3562	$3.0 \times 10^{-9} \pm 2.5 \times 10^{-10}$	26.0 $\pm$ 0.52
day2	33632 $\pm$ 937	$4.6 \times 10^{-9} \pm 7.5 \times 10^{-10}$	19.4 $\pm$ 0.50
day3	23587 $\pm$ 1478	$8.8 \times 10^{-9} \pm 6.2 \times 10^{-10}$	15.3 $\pm$ 1.02

Table S1 Variations in cell count, volume, and Glu concentration per cell during differentiation

Figure S1

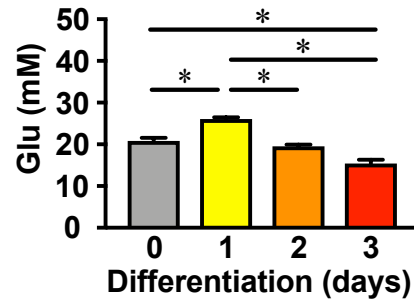


Figure S1 Glu concentration per C2C12 cells during differentiation

The concentration of Glu per cell was calculated using the concentration in the cell lysate and the total number of cells in each well, and the volume per cell. * $P < 0.05$, ANOVA with Tukey's multiple comparisons test, $n=3$.

Figure S2

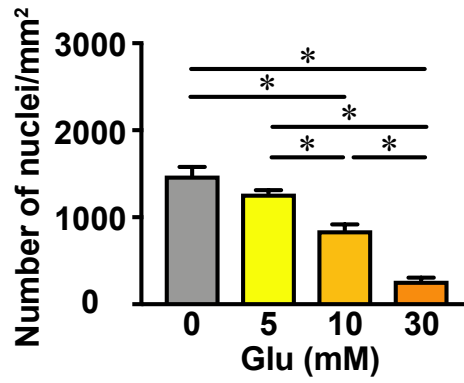


Figure S2 Cytotoxicity of Glu in C2C12 cells

C2C12 cells were differentiated in the presence of Glu (0, 5, 10, or 30 mM) for 3 days. The cells were stained with DAPI, and the number of nuclei was counted. * $P < 0.05$, ANOVA with Tukey's multiple comparisons test, $n = 3$.

Figure S3

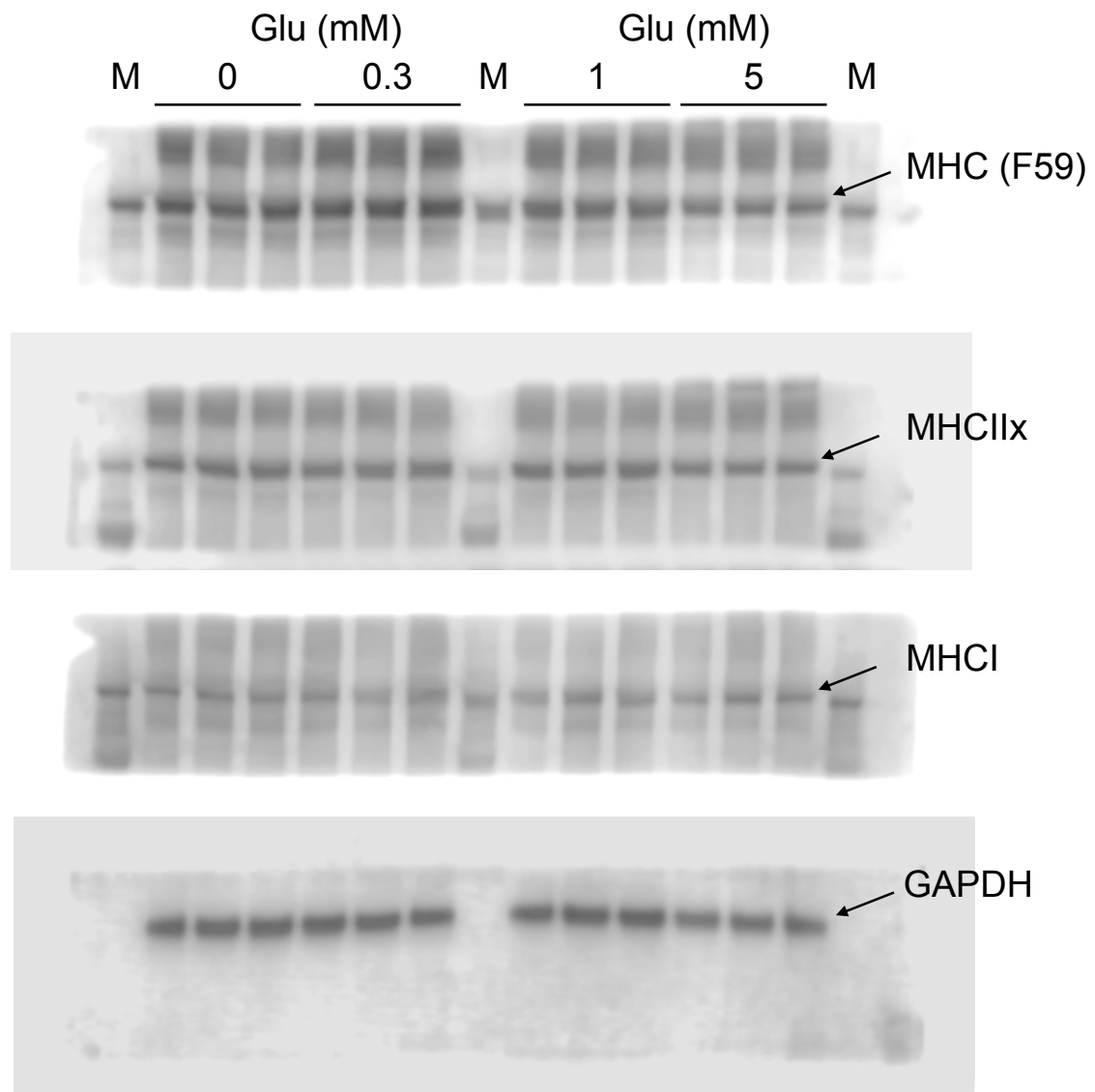


Figure S3 The uncropped western blot data used in Figure 2A

The bands for each protein are indicated by arrows. M indicates the lane with the protein molecular weight marker.

Figure S4

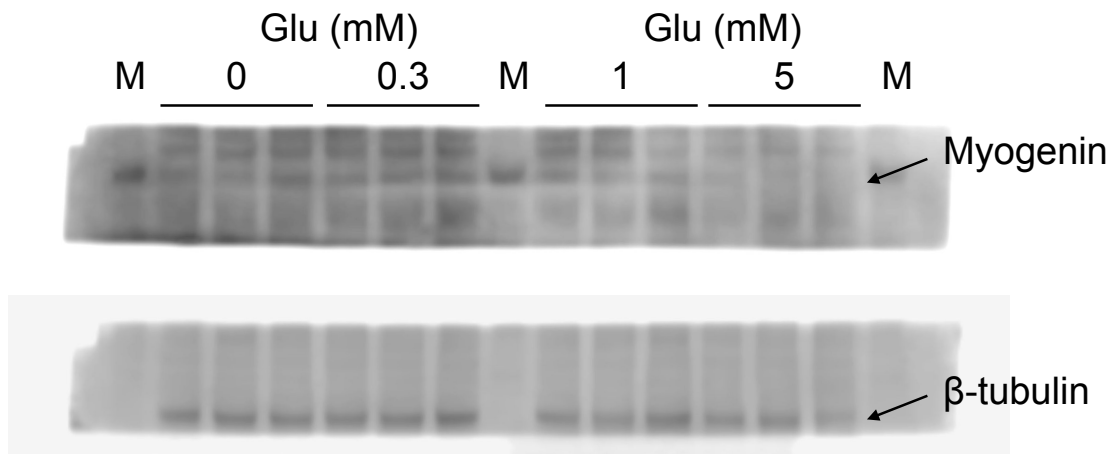


Figure S4 The uncropped western blot data used in Figure 2E

The bands for each protein are indicated by arrows. M: protein molecular weight marker.

Figure S5

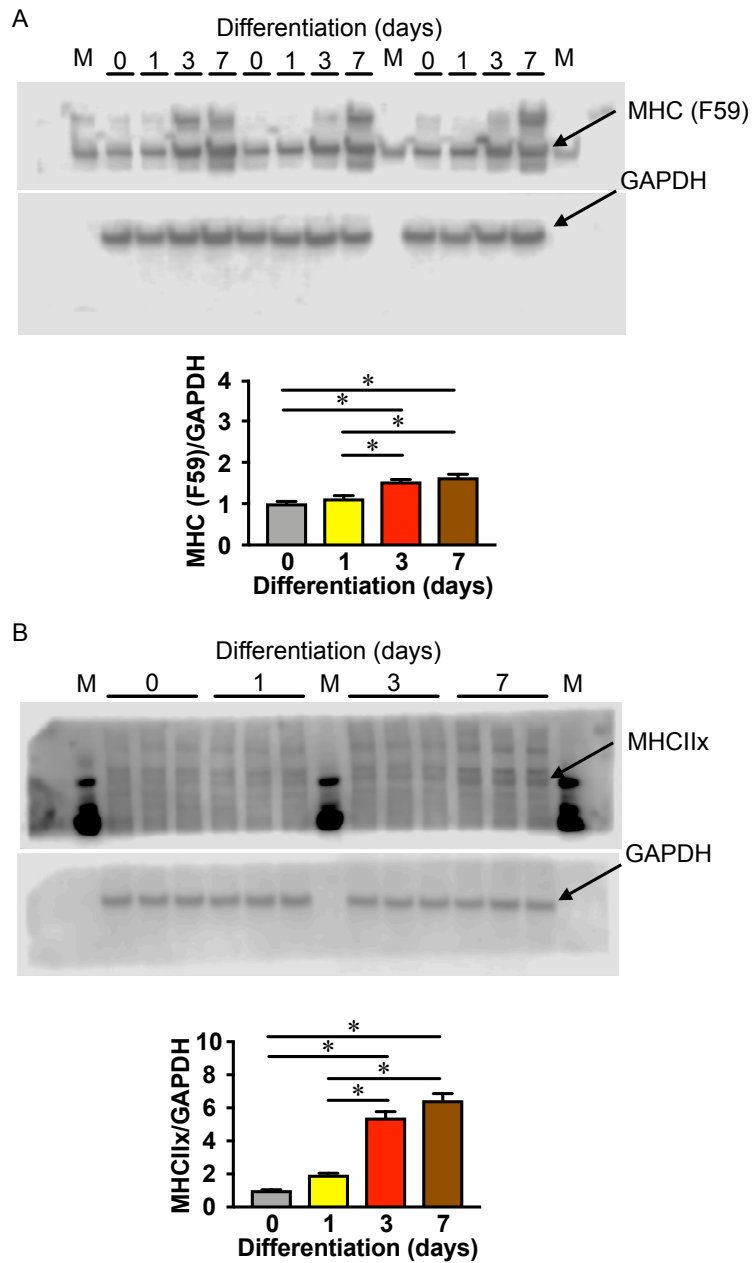


Figure S5 The expression levels of fast-twitch MHC isoforms during C2C12 differentiation. C2C12 cells were differentiated for 0–7 days, and the expression levels of (A) MHC (F59) and (B) MHCIIx were analyzed using western blotting. GAPDH was used as an internal standard. * $P < 0.05$, ANOVA with Tukey's multiple comparisons test, $n = 3$. M: protein molecular weight marker.

Figure S6

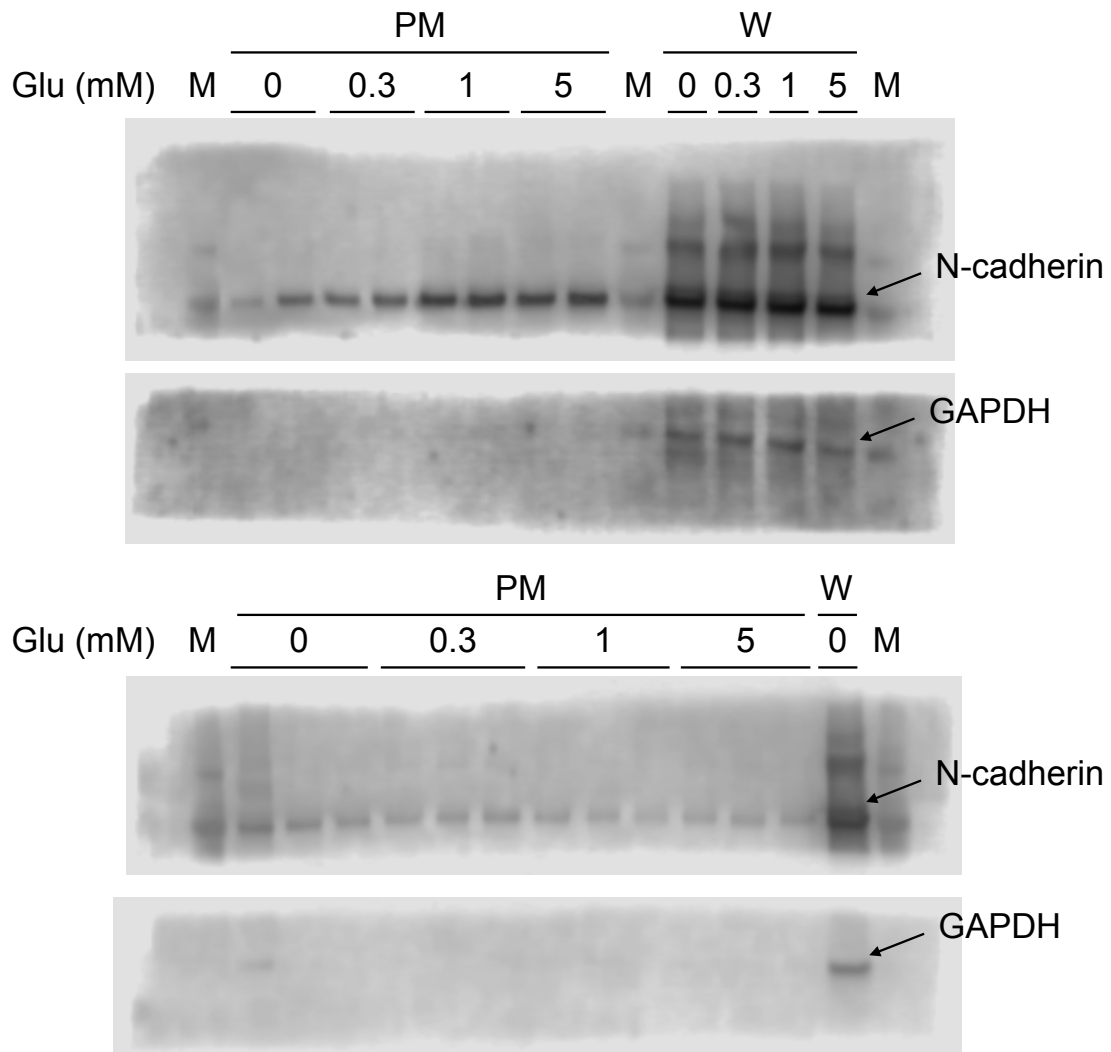


Figure S6 Assessment of the purity of the plasma membrane preparation

After Glu stimulation of C2C12 cells, the plasma membrane fraction was extracted, and the expression of N-cadherin, a plasma membrane marker, was analyzed by western blotting. Similarly, the expression of GAPDH, a cytoplasmic marker, was analyzed. PM: plasma membrane, W: whole cell, M: protein molecular weight marker.

Figure S7

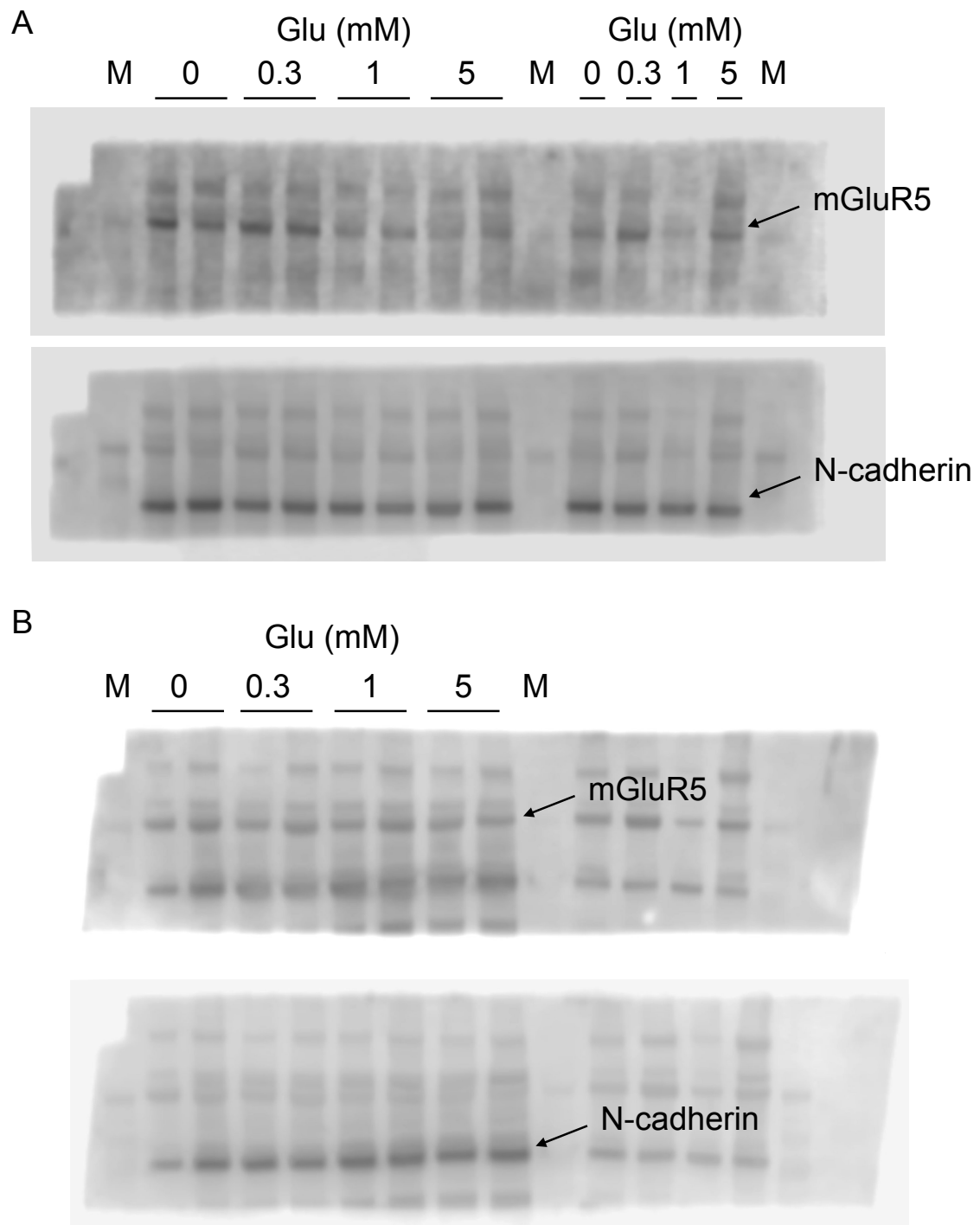


Figure S7 The uncropped western blot data used in Figure 4A

The bands for each protein are indicated by arrows. (A) data used for the typical example and analysis, and (B) additional data for the analysis. M: protein molecular weight marker.

Figure S8

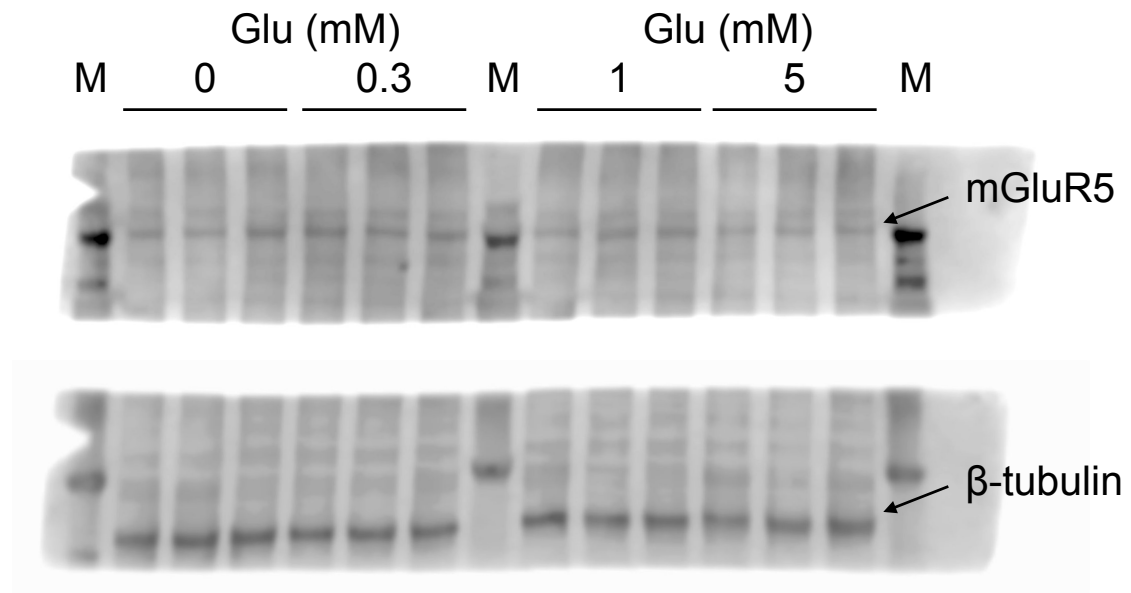


Figure S8 The uncropped western blot data used in Figure 4C

The bands for each protein are indicated by arrows. M: protein molecular weight marker.

Figure S9

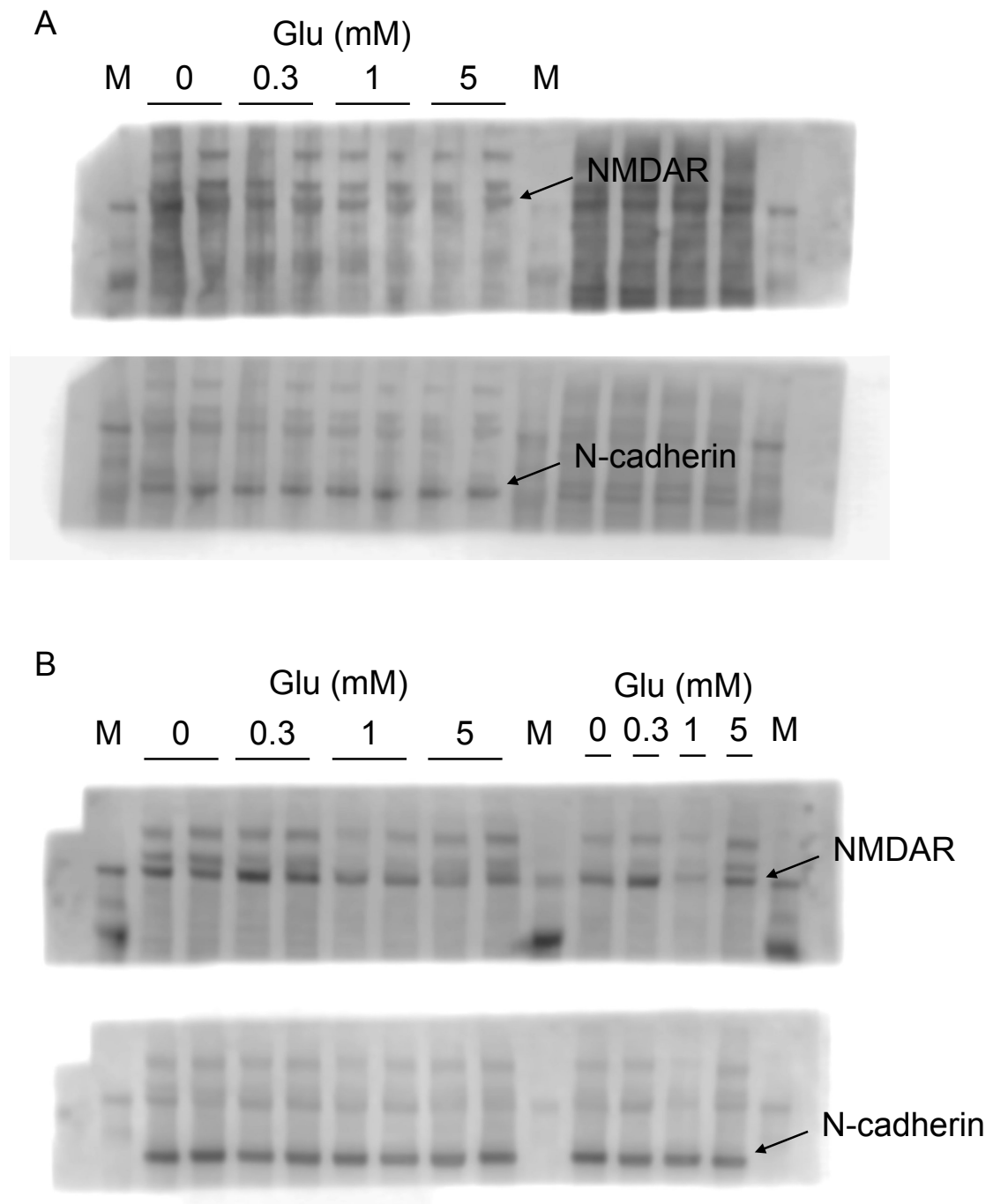


Figure S9 The uncropped western blot data used in Figure 4E

The bands for each protein are indicated by arrows. (A) data used for the typical example and analysis, and (B) additional data for the analysis. M: protein molecular weight marker.

Figure S10

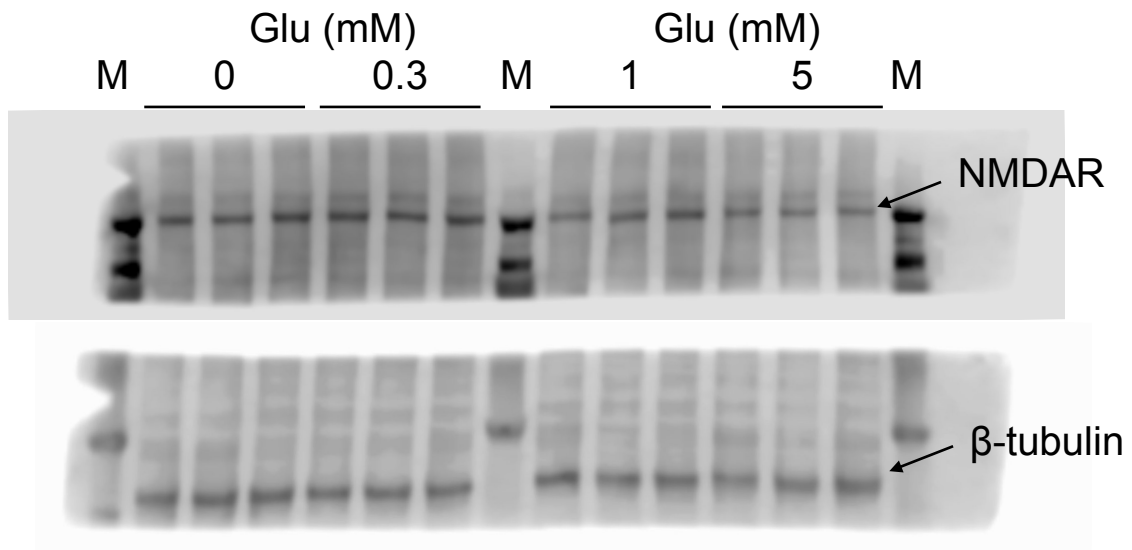


Figure S10 The uncropped western blot data used in Figure 4G

The bands for each protein are indicated by arrows. M: protein molecular weight marker.

Figure S11

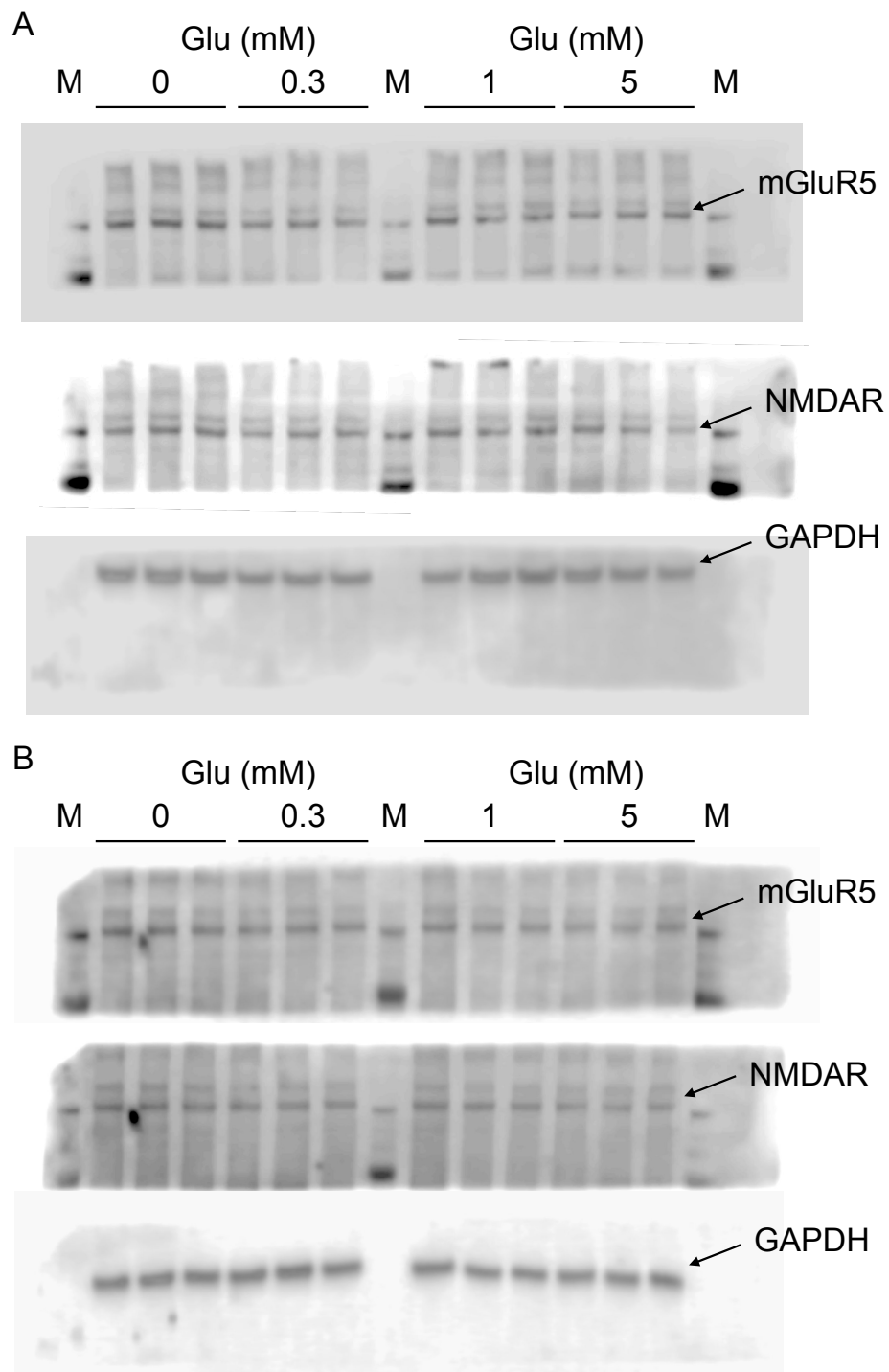


Figure S11 The uncropped western blot data used in Figure 4I

The bands for each protein are indicated by arrows. (A) data used for the typical example and analysis, and (B) additional data for the analysis. M: protein molecular weight marker.

Figure S12

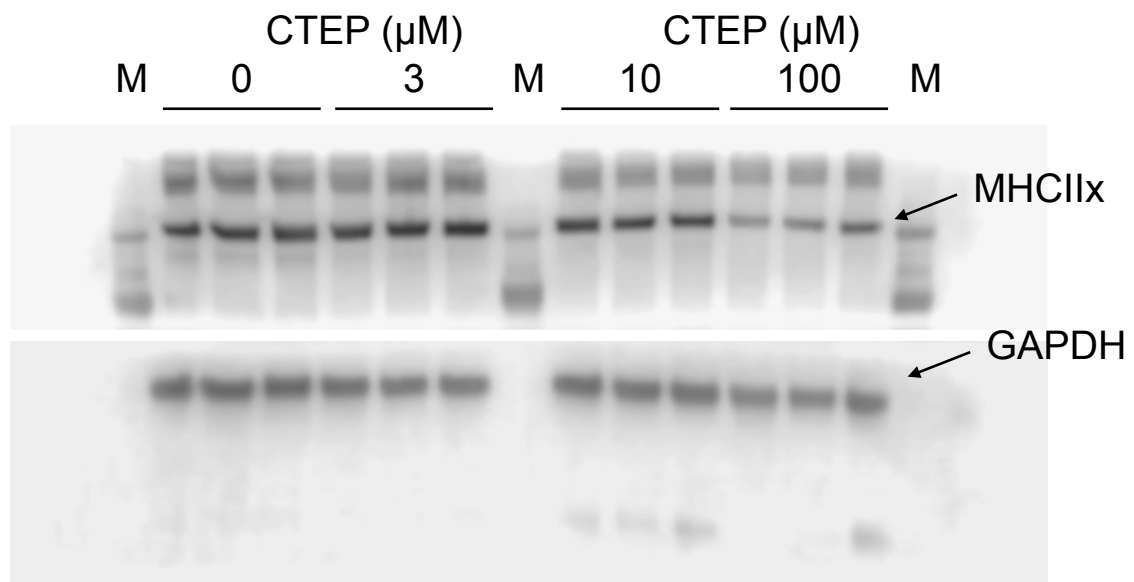


Figure S12 The uncropped western blot data used in Figure 5A

The bands for each protein are indicated by arrows. M: protein molecular weight marker.

Figure S13

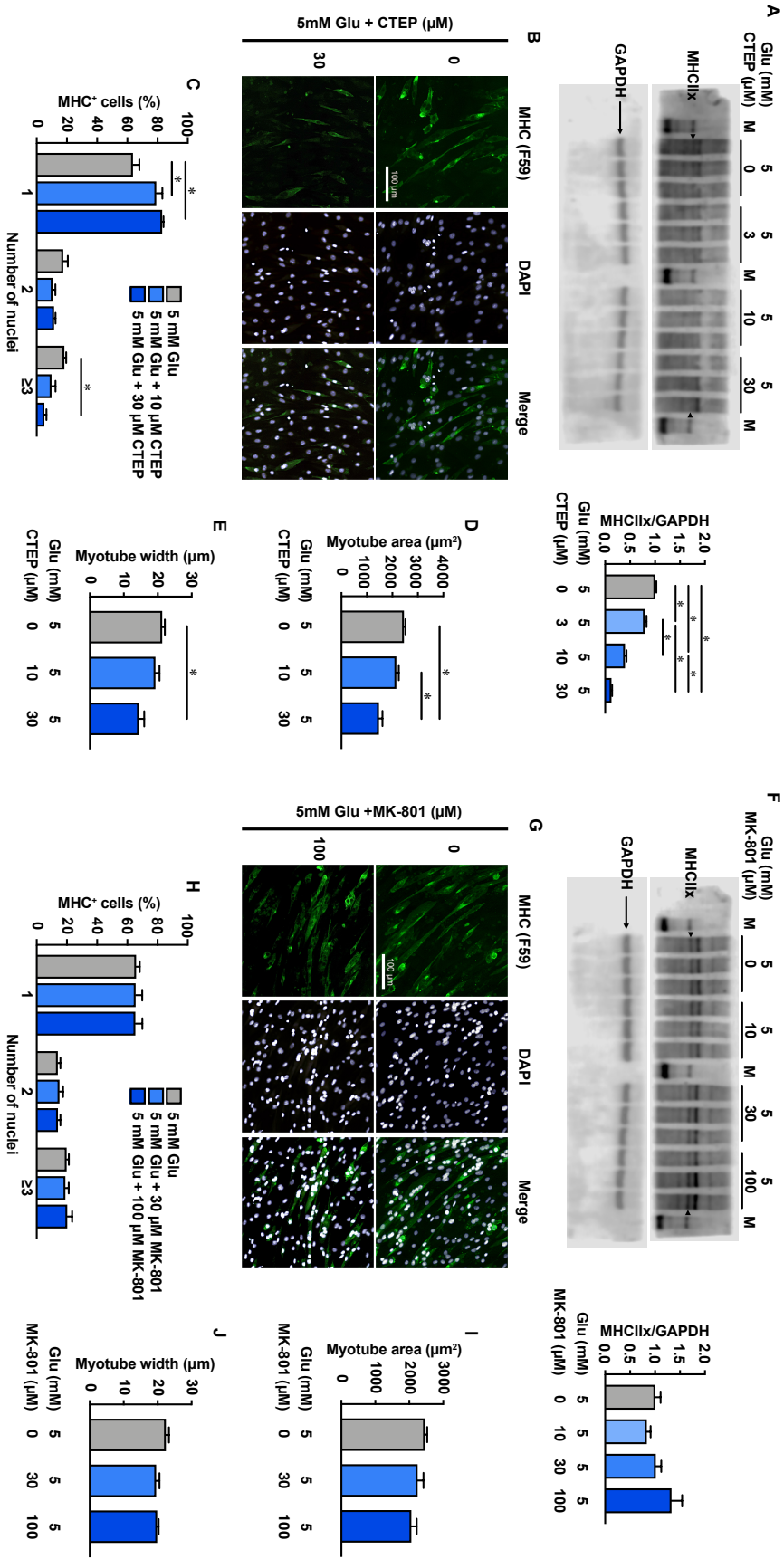


Figure S13 The downregulation of MHCIIx by high concentrations of Glu is enhanced by CTEP, an mGluR5 inhibitor

(A) C2C12 cells were differentiated for 2 days in the presence of 5 mM Glu and 0-30 μ M mGluR5 inhibitor CTEP, and then the expression level of MHCIIx was analyzed using western blotting. GAPDH was used as an internal standard. M indicates the lane with the protein molecular weight marker. The band for MHCIIx is indicated by arrowheads, while the band for GAPDH is indicated by an arrow. (B) Cells were differentiated in the presence of 5 mM Glu and 0-30 μ M CTEP for 3 days, followed by immunofluorescence using the anti-MHC (F59) antibody. Nuclei were stained with DAPI. (C) MHC (F59)-positive (MHC⁺) cells were classified by the number of nuclei as 1, 2, or ≥ 3 . (D) The surface area and (E) width were measured in MHC⁺ cells with ≥ 3 nuclei. (F) Cells were differentiated in the presence of 5 mM Glu and 0-100 μ M NMDAR inhibitor MK-801 for 2 days, and then the expression level of MHCIIx was analyzed. GAPDH was used as an internal standard. M indicates the lane with the protein molecular weight marker. The band for MHCIIx is indicated by arrowheads, while the band for GAPDH is indicated by an arrow. (G, H) Cells were differentiated in the presence of 5 mM Glu and 0-100 μ M MK-801 for 3 days, followed by immunofluorescence and nuclear classification. (I) The surface area and (J) width were measured in MHC⁺ cells with ≥ 3 nuclei. * P < 0.05, ANOVA with Tukey's multiple comparisons test, n=3.

Figure S14

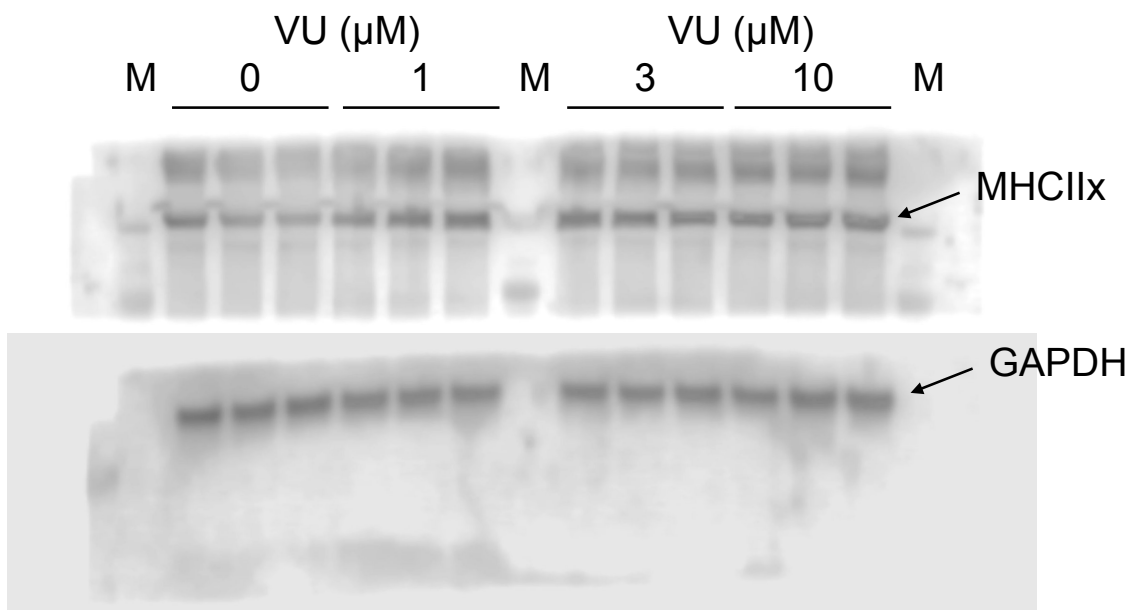


Figure S14 The uncropped western blot data used in Figure 5F

The bands for each protein are indicated by arrows. M: protein molecular weight marker.

Figure S15

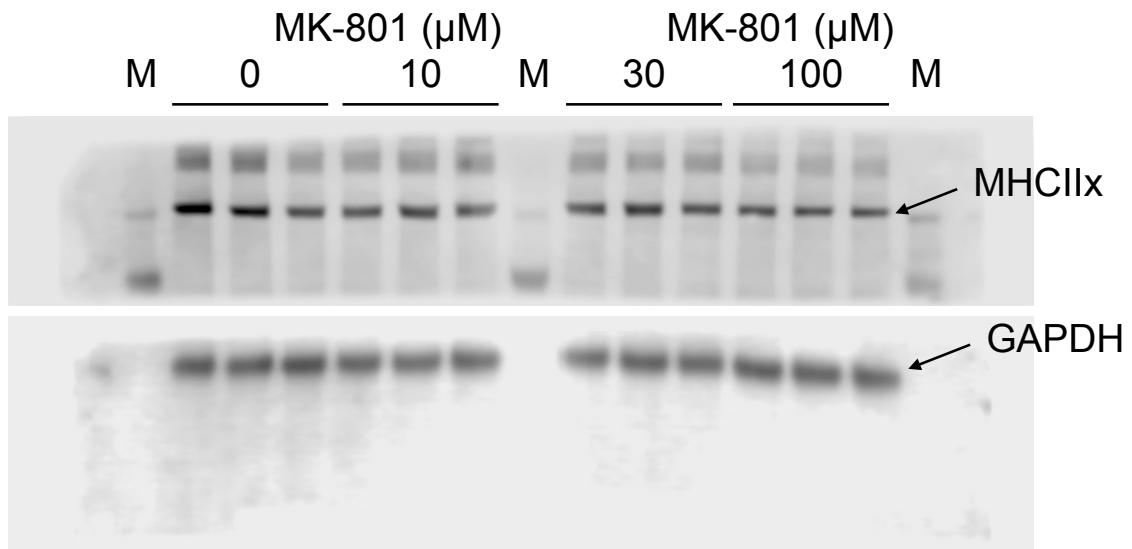


Figure S15 The uncropped western blot data used in Figure 5K

The bands for each protein are indicated by arrows. M: protein molecular weight marker.

Figure S16

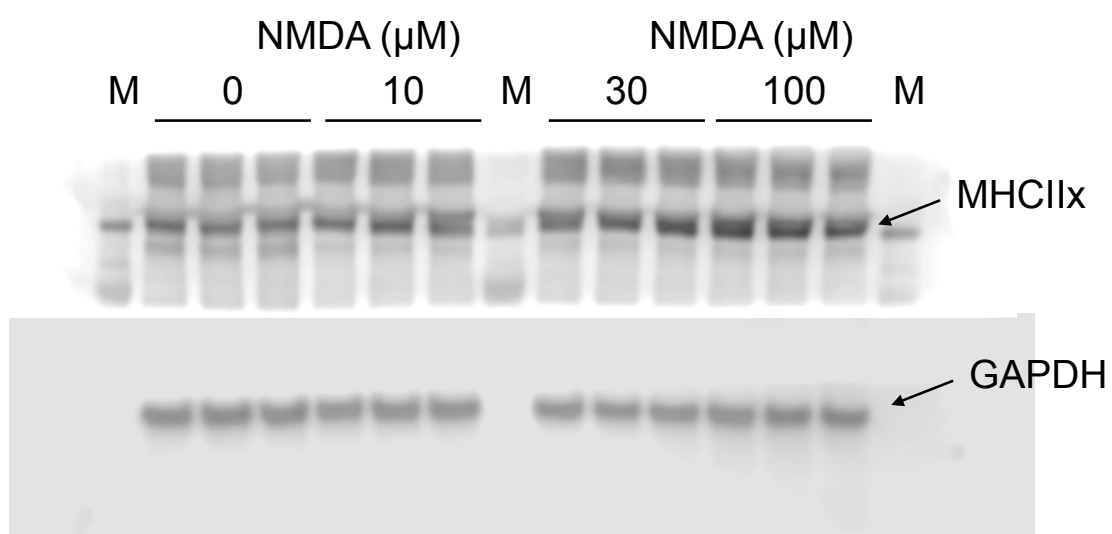


Figure S16 The uncropped western blot data used in Figure 5P

The bands for each protein are indicated by arrows. M: protein molecular weight marker.

Figure S17

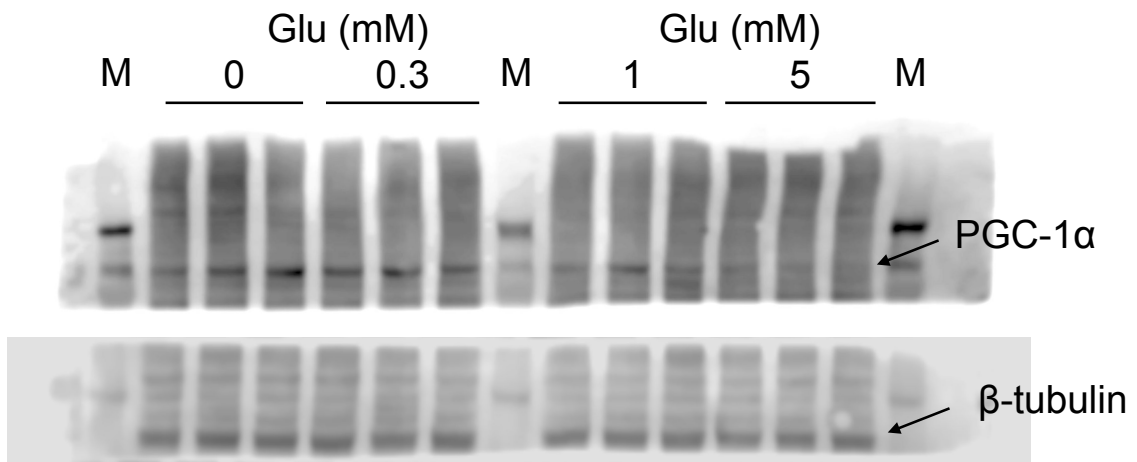


Figure S17 The uncropped western blot data used in Figure 6A

The bands for each protein are indicated by arrows. M: protein molecular weight marker.

Figure S18

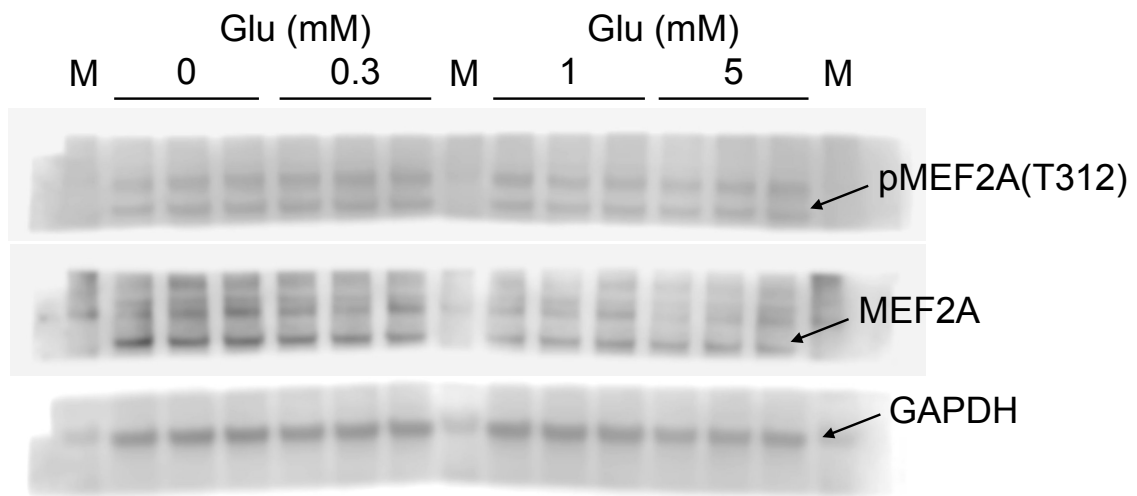


Figure S18 The uncropped western blot data used in Figure 6B

The bands for each protein are indicated by arrows. M: protein molecular weight marker.

Figure S19

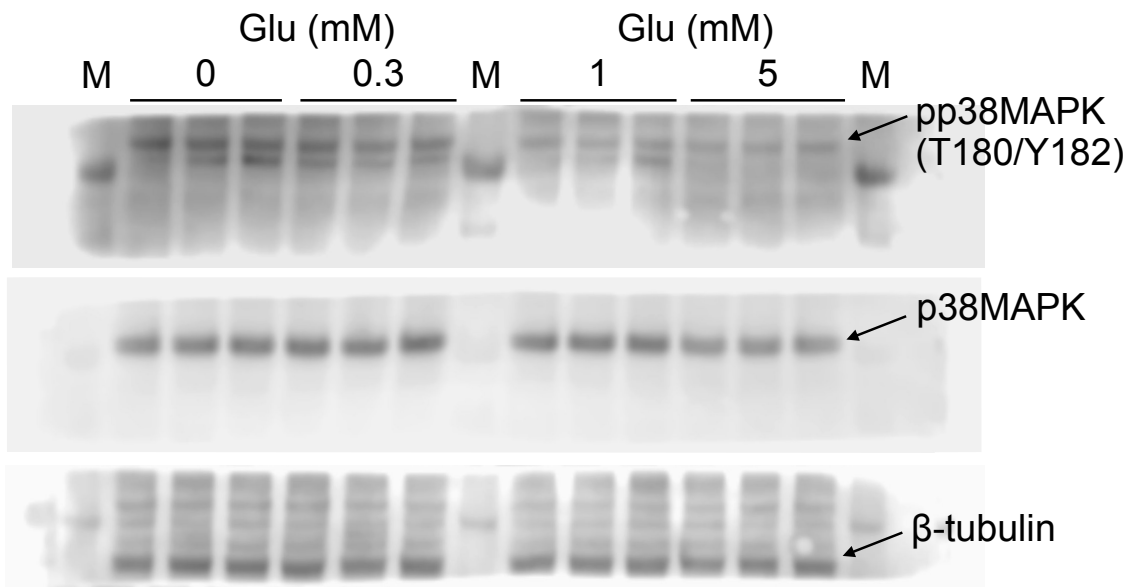


Figure S19 The uncropped western blot data used in Figure 6C

The bands for each protein are indicated by arrows. M: protein molecular weight marker.

Figure S20

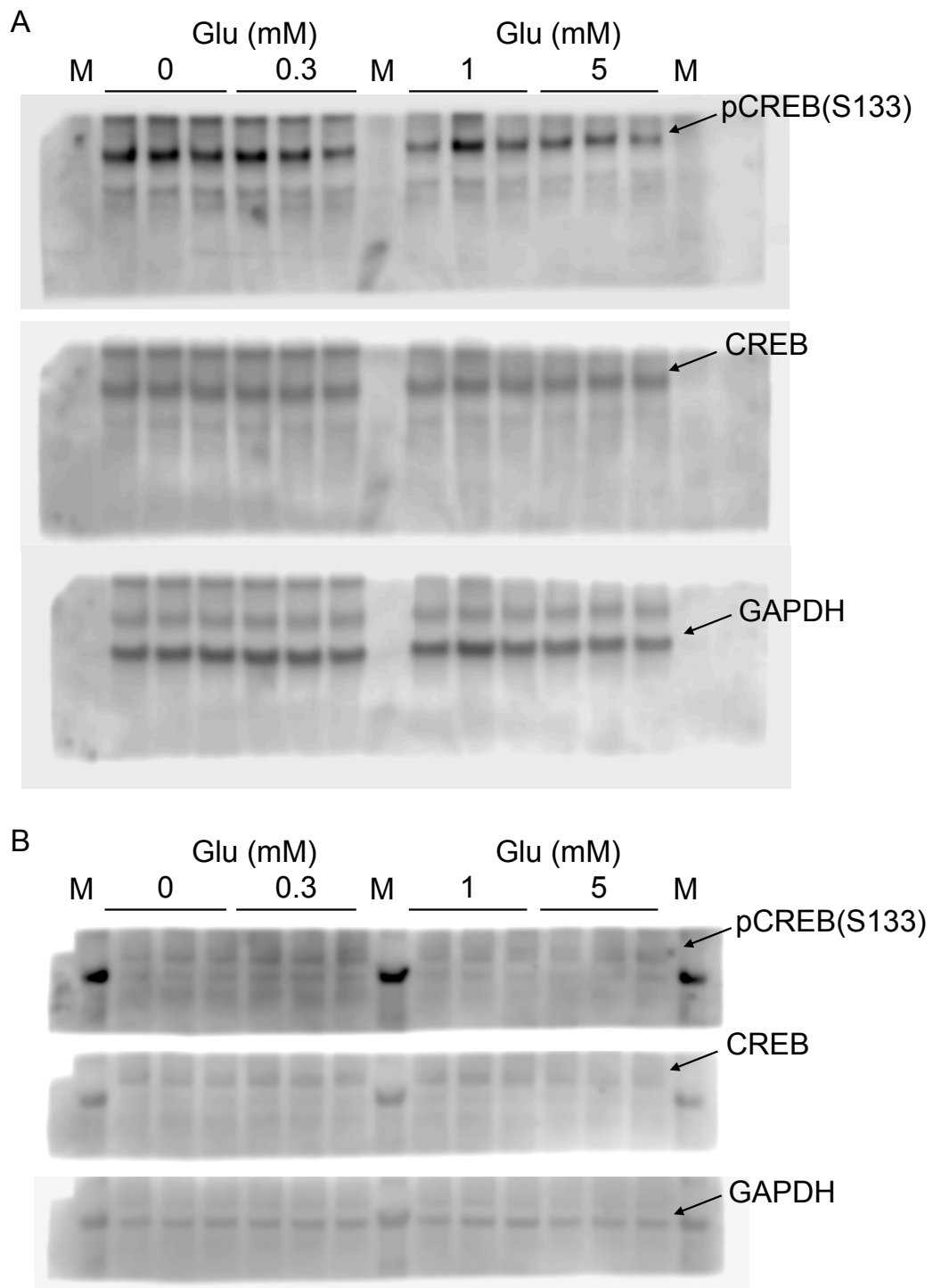


Figure S20 The uncropped western blot data used in Figure 6D

The bands for each protein are indicated by arrows. (A) data used for the typical example and analysis, and (B) additional data for the analysis. M: protein molecular weight marker.

Figure S21

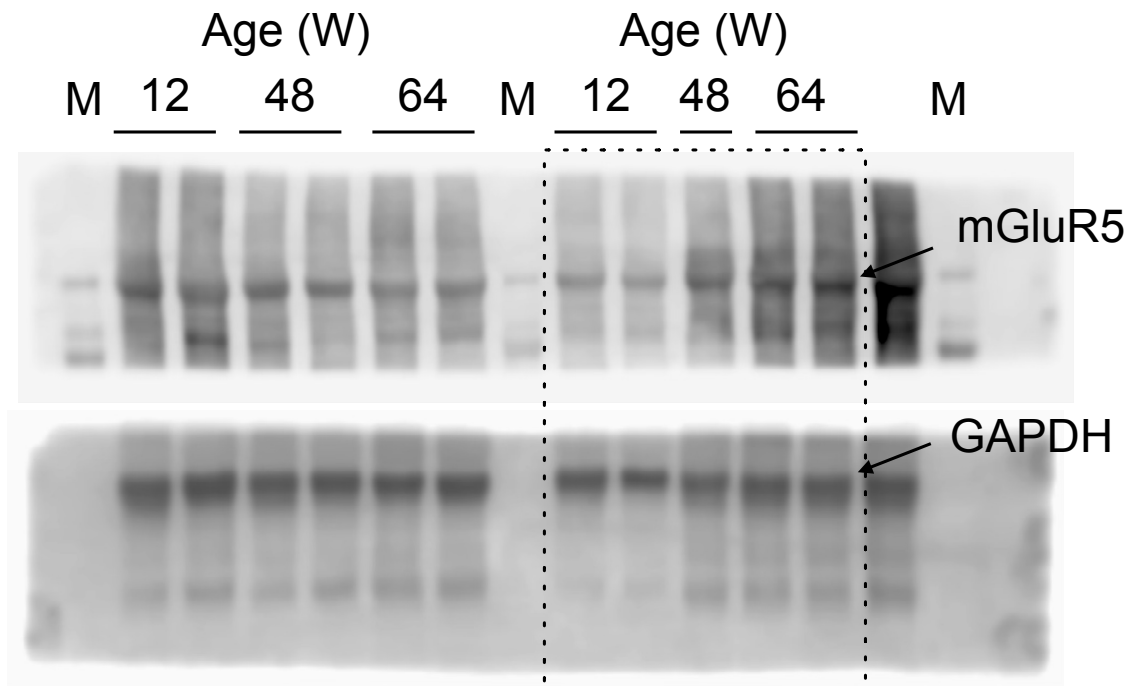


Figure S21 The uncropped western blot data used in Figure 7A

The bands for each protein are indicated by arrows. The bands on the left half were used as typical examples and for analysis, while the bands enclosed by dotted lines were used only for analysis.

M: protein molecular weight marker.

Figure S22

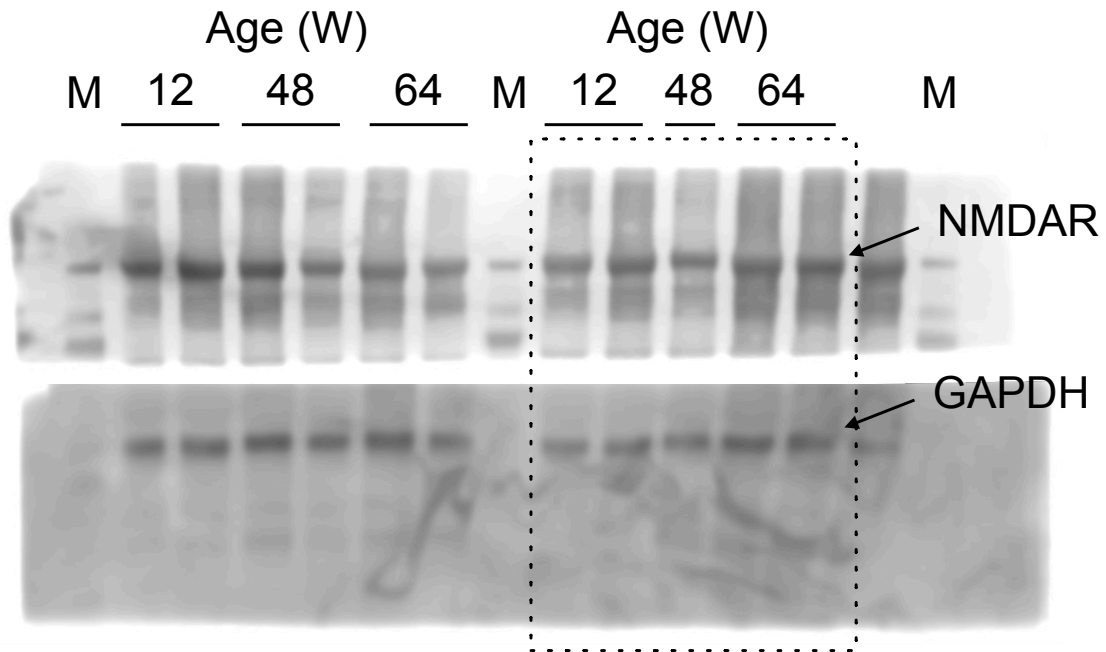


Figure S22 The uncropped western blot data used in Figure 7B

The bands for each protein are indicated by arrows. The bands on the left half were used as typical examples and for analysis, while the bands enclosed by dotted lines were used only for analysis.

M: protein molecular weight marker.

Figure S23

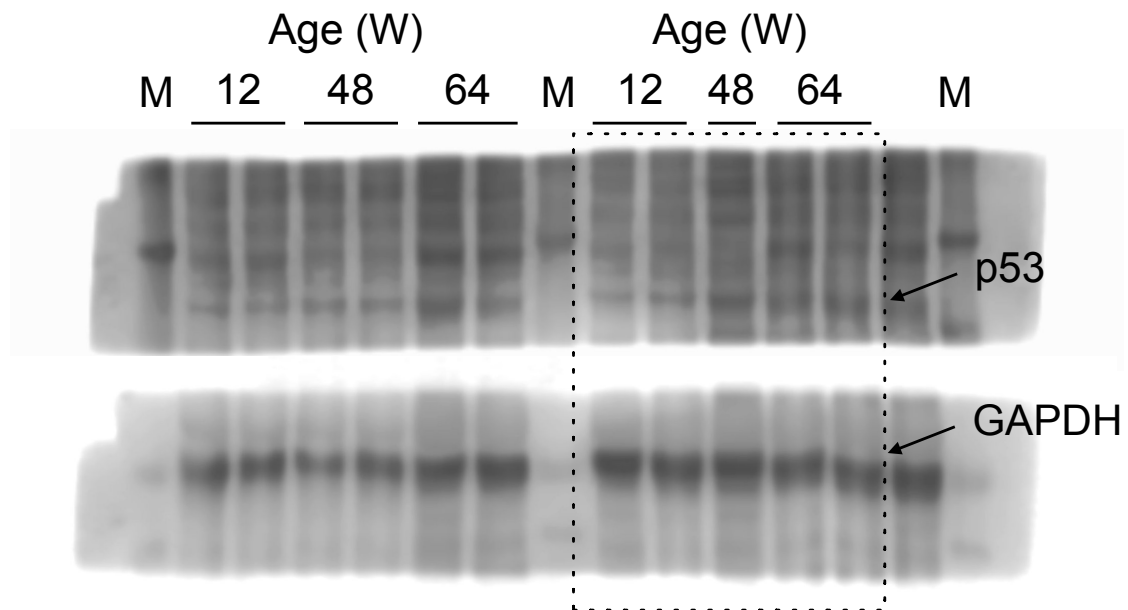


Figure S23 The uncropped western blot data used in Figure 7D

The bands for each protein are indicated by arrows. The bands on the left half were used as typical examples and for analysis, while the bands enclosed by dotted lines were used only for analysis.

M: protein molecular weight marker.

Figure S24

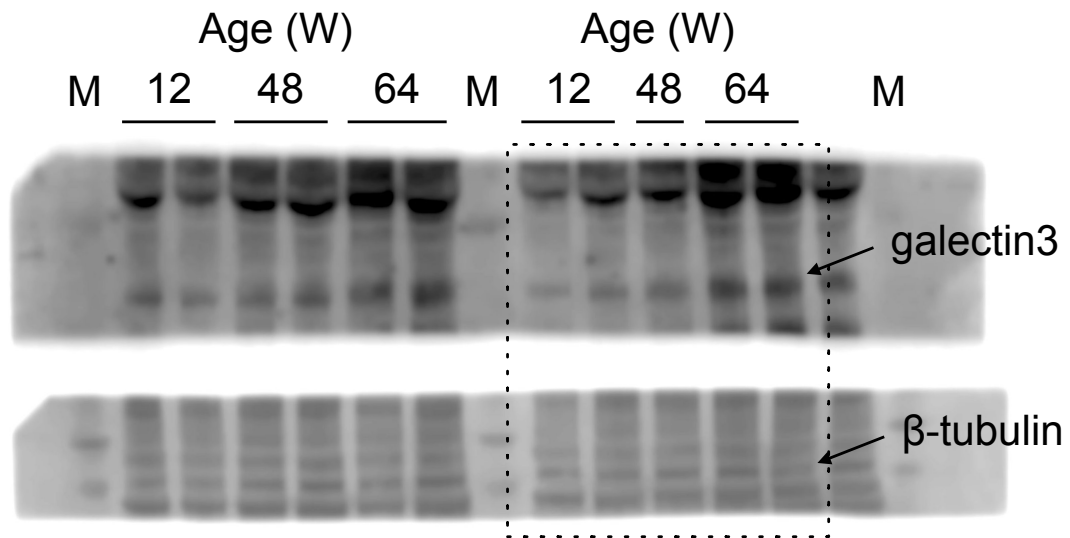


Figure S24 The uncropped western blot data used in Figure 7E

The bands for each protein are indicated by arrows. The bands on the left half were used as typical examples and for analysis, while the bands enclosed by dotted lines were used only for analysis.

M: protein molecular weight marker.