Induction of nuclear translocation of mutant cytoplasmic p53 by geranylgeranoic acid in a human hepatoma cell line

Chieko Iwao and Yoshihiro Shidoji*

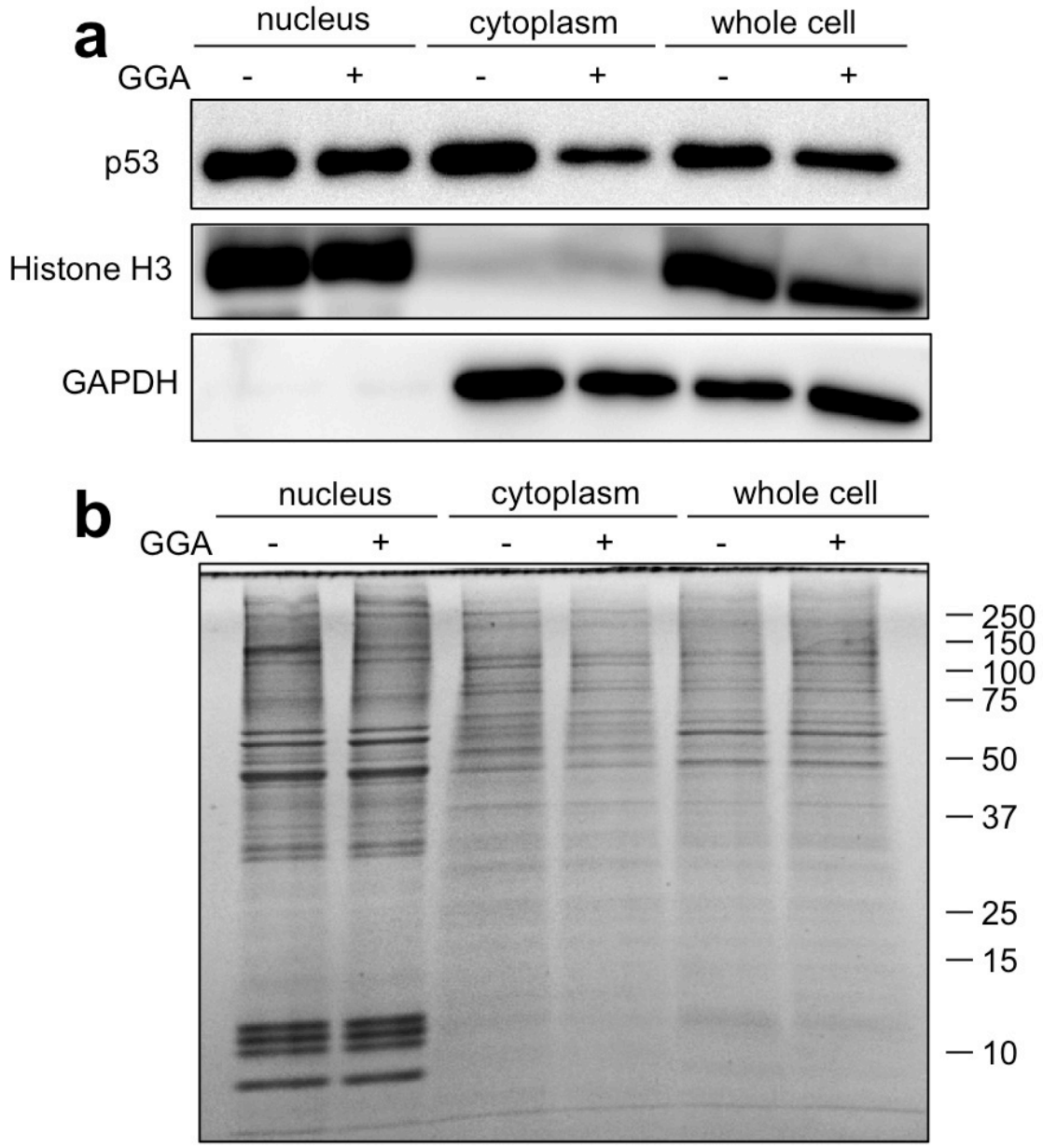
Molecular and Cellular Biology, Graduate School of Human Health Science, University of Nagasaki, Academy Hills 1-1-1, Nagayo, Nagasaki 851-2195, Japan

***Corresponding author**:

Y. Shidoji, telephone/fax: +81-95-813-5207, e-mail: shidoji@sun.ac.jp

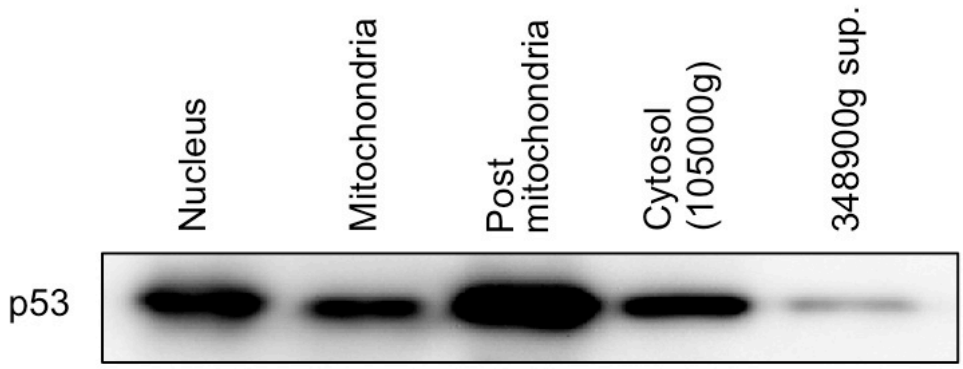
Running title: GGA-induced nuclear translocation of mutant p53

Support: This work was supported in part by a grant-in-aid from the Japan Society for the Promotion of Science (grant number 19590230) and a research-grant B from the University of Nagasaki.



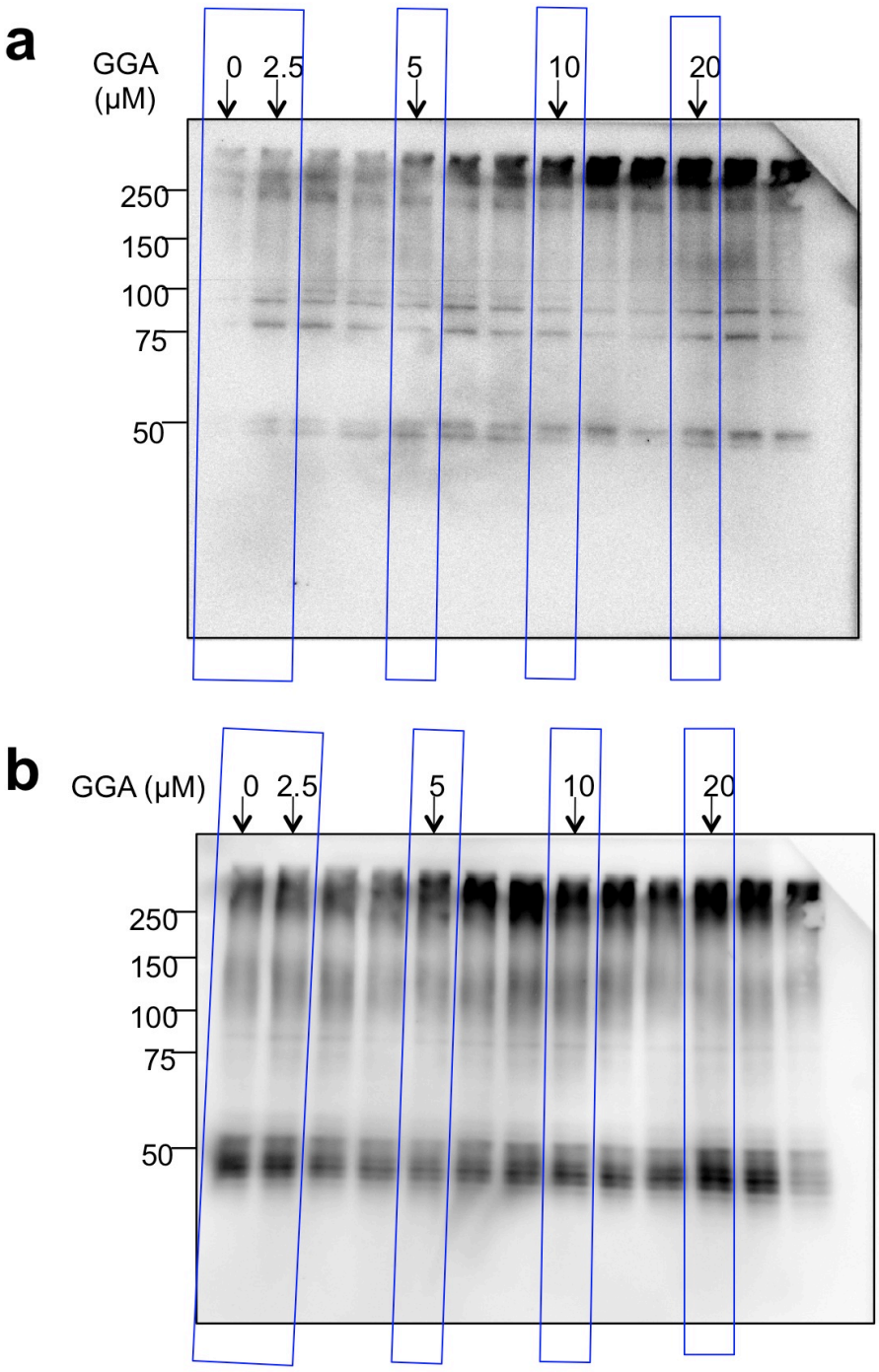
Supplementary Figure S1. GGA effects on nuclear/cytoplasmic distribution of p53 in HuH-7 cells.

Whole-cell lysates of HuH-7 cells were separated into nuclear and cytoplasmic fractions. Protein concentration was determined using Bio-Rad Protein Assay reagent. (**a**) Fraction samples (5 μg) were subjected to immunoblotting with anti-p53 antibody. Histone H3 and GAPDH were used as nuclear and cytoplasmic markers, respectively. (**b**) Coomassie Brilliant Blue stain.



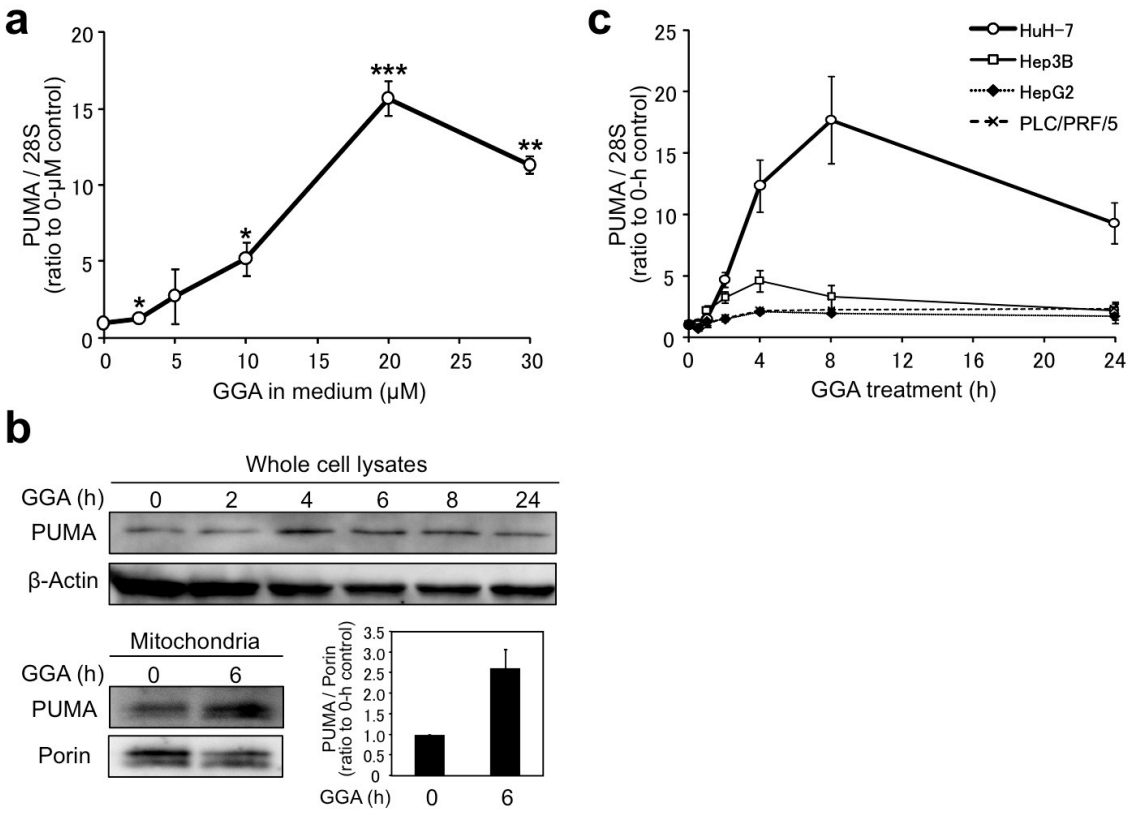
Supplementary Figure S2. Subcellular distribution of p53 in HUH-7 cells.

Whole-cell lysates of HuH-7 cells were separated into nuclear, mitochondrial, post-mitochondrial, cytosolic, and 348900 *g* supernatant fractions. Protein concentration was determined using the Bio-Rad Protein Assay reagent. Fraction samples (5 μg) were subjected to immunoblotting with an anti-p53 antibody.



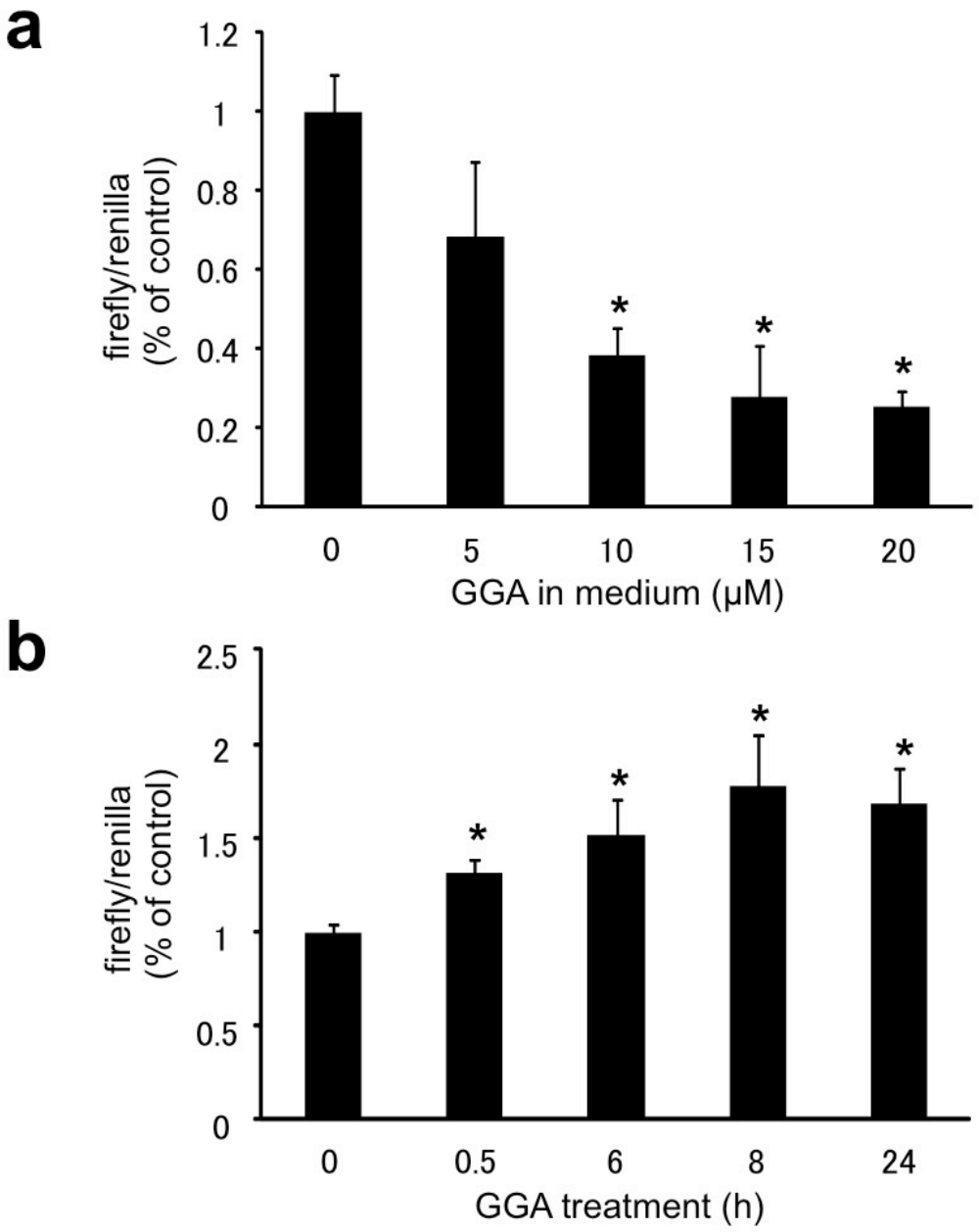
Supplementary Figure S3. Uncropped blots probed with anti-p53 (**a**) and reprobed with anti-β-III-tubulin (**b**).

Panels **c** and **d** of Figure 4 in the main text were cropped by each 4merging blue-lined squares in panels **a** and **b**, respectively. Unmarked lanes show the samples treated with other ditrepenoids than GGA, which are irrelevant to the present study, so that they were removed.



Supplementary Figure S4. GGA upregulates the *PUMA* gene expression selectively in HuH-7 cells.

(**a**) HuH-7cells were treated with 0 — 30 μM of GGA for 8 h, and total mRNA was extracted to analyze PUMA mRNA expression by quantitative RT-PCR. Each point represents the mean ± SE of four independent experiments. (**b**) HuH-7 cells were treated with or without 20 μM of GGA for 2, 4, 6, 8, and 24 h. Whole cell lysates and mitochondrial fractions were prepared and the PUMA level was analysed by western blotting. Total β-actin and porin were used as loading controls. (**c**) HuH-7 cells (p53 Y220C), PLC/PRF/5 cells (p53 R249S), HepG2 cells (p53 wild type), and Hep3B cells (p53 null) were treated with or without 20 μM of GGA for 0.5, 1, 2, 4, 8, and 24 h, and total mRNA was extracted to analyse *PUMA* mRNA expression by quantitative RT-PCR. Each point represents the mean ± SE for HuH-7 (n = 6), Hep3B (n = 3), HepG2 (n = 3) and PLC/PRF/5 (n = 3).



Supplementary Figure S5. Enhancement of transcriptional activation of the *PUMA* promoter by GGA in HuH-7 cells.

(**a**) Dual luciferase reporter assay with the p53-responsive consensus sequence in HuH-7 cells after 24-h GGA (0 — 20 μM) treatment. The luciferase activity was normalized by renilla luciferase. The asterisks (*) indicate statistically significant changes ($p < 0.05$) as determined by the Student's t-test. (**b**) Dual luciferase reporter assay with p53-responsive 5'-upstream regulatory region of the *PUMA* gene in HuH-7 cells after GGA (20 μM) treatment. The luciferase activity was normalized by renilla luciferase. The asterisks (*) indicate statistically significant changes ($p < 0.05$) as determined by the Student's t-test.

Supplementary Table S1. The nucleotide sequences of each primers used for real-time RT-PCR

Genes		Sequence (5' -3')
p21	F	CTGGAGACTCTCAGGGTCGAAA
	R	GATTAGGGCTTCCTCTTGGAGA
PUMA	F	CCCAAGACTGTTGGGTCTG
	R	GCCGTAGTAATCCGTGAAGAG
TIGAR	F	AGACAGCGGTATTCCAGG
	R	AGAGTGGCTGGTAAGGAAC
SCO2	F	AGAAGCCAGGAGAGGGACGG
	R	CCAGGCCCAGAGTGAAGGAG
DRAM	F	CGCCTTCATTATCTCCTACG
	R	CGAAACATCCCACCAATCCA
28S ribosomal RNA	F	TTAGTGACGCGCATGAATGG
	R	TGTGGTTTCGCTGGATAGTAGGT

F: forward primer, R: reverse primer

Supplementary Table S2. The conditions of thermal cycler for real-time RT-PCR of each genes

p21	Temperature	Slope
Denature	95°C, 600 s	20°C / s
PCR (45 cycles)	95°C, 10 s	20°C / s
	65°C, 20 s	20°C / s
	72°C, 10 s	20°C / s
Melting	95°C, 0 s	20°C / s
	57°C, 15 s	20°C / s
	98°C, 0 s	0.1°C / s
Cooling	40°C, 30 s	20°C / s

PUMA	Temperature	Slope
Denature	95°C, 600 s	20°C / s
PCR (40 cycles)	95°C, 10 s	20°C / s
	61°C, 20 s	20°C / s
	72°C, 20 s	20°C / s
Melting	95°C, 0 s	20°C / s
	61°C, 15 s	20°C / s
	98°C, 0 s	0.1°C / s
Cooling	40°C, 10 s	20°C / s

TIGAR	Temperature	Slope
Denature	95°C, 600 s	20°C / s
PCR (45 cycles)	95°C, 10 s	20°C / s
	56°C, 20 s	20°C / s
	72°C, 15 s	20°C / s
Melting	95°C, 0 s	20°C / s
	57°C, 15 s	20°C / s
	98°C, 0 s	0.1°C / s
Cooling	40°C, 10 s	20°C / s

SCO2	Temperature	Slope
Denature	95°C, 600 s	20°C / s
PCR (40 cycles)	95°C, 10 s	20°C / s
	56°C, 20 s	20°C / s
	72°C, 20 s	20°C / s
Melting	95°C, 0 s	20°C / s
	57°C, 15 s	20°C / s
	98°C, 0 s	0.1°C / s
Cooling	40°C, 30 s	20°C / s

DRAM	Temperature	Slope
Denature	95°C, 600 s	20°C / s
PCR (60 cycles)	95°C, 10 s	20°C / s
	55°C, 10 s	20°C / s
	72°C, 20 s	20°C / s
Melting	95°C, 0 s	20°C / s
	65°C, 15 s	20°C / s
	95°C, 0 s	0.1°C / s
Cooling	40°C, 30 s	20°C / s

28S rRNA	Temperature	Slope
Denature	95°C, 600 s	20°C / s
PCR (40 cycles)	95°C, 15 s	20°C / s
	60°C, 30 s	20°C / s
Melting	95°C, 0 s	20°C / s
	65°C, 15 s	20°C / s
	95°C, 0 s	0.1°C / s
Cooling	40°C, 30 s	20°C / s