# HYDROGEN PEROXIDE PROMOTES Aβ PRODUCTION THROUGH JNK-DEPENDENT ACTIVATION OF γ-SECRETASE

**Chengyong Shen[1], Yongfeng Chen[1], Huaqing Liu[1], Kejing Zhang[1], Ting Zhang[1], Anning Lin[2], Naihe Jing[1,*]**

## SUPPLEMAENTAL DATA

## SUPPLEMENTARY MATERIALS AND METHODS

*Plasmids* - Expression vectors encoding GST-c-Jun-(1-79), HA-JNKK2-JNK1, HA-JNKK2(KM)-JNK1 have been described previously (1,2). To construct GST-PS1 bacterial expression vectors, PS1 cDNA was amplified by PCR from the pcDNA3.1-PS1 plasmid (3). The PCR product was digested with BamHI and EcoRI enzymes, and cloned into pGEX-4T-1 vectors (Amersham Pharmacia).

*Subcellular Fractionation* - SH-SY5Y/APP695myc cells were harvested and homogenized in cell lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.25 mM PMSF and cocktails of protease inhibitors. The homogenate was centrifuged for 10 min at 1,000×g at 4°C to prepare a post-nuclear supernatant fraction. Membranes were pelleted from the PNS by centrifugation for 30 min at 13,000×g. The resulting supernatant was designated as cell cytoplasm. All fractions were subjected to Western blot.

*Purification of Recombinant Proteins* - GST-PS1 and GST-c-Jun-(1-79) proteins were purified using glutathione-agarose according to the manufacturer's procedure (Amersham Pharmacia).

*Protein Kinase Assay* - HEK293T cells were transfected with expression vectors encoding HA-JNKK2-JNK1, HA-JNKK2(KM)-JNK1 (1 μg each) for 24 h as indicated. Cells were lysed and HA-JNKK2-JNK1 proteins were immunoprecipitated by anti-HA antibody (1:1,000, Sigma). The activity of the JNK immunocomplex was measured by kinase assays with GST-c-Jun-(1-79) and GST-PS1 as substrates. The reaction was incubated at 30°C for 1 h in 30 μl of kinase buffer in the presence of 10 μM ATP/10 μCi [$\gamma$-$^{32}$P]ATP (10 Ci/mmol). The reactions were terminated with 4× Laemmli sample buffers. The proteins were resolved by 13% SDS-PAGE, followed by autoradiography.

## SUPPLEMENTARY FIGURE LEGENDS

FIGURE S1. **The characteristics of APP processing products.** A. HEK293T cells were transiently transfected with expression vectors encoding AICD57myc (lane 2), AICD59myc (lane 3), SP-C99myc (lane 4) as positive controls to recognize the bands. pcDNA3.1 vectors were transfected as negative controls (lanes 1 and 6). SH-SY5Y/APP695myc cells were subjected to subcellular fractionation followed by Western blot (lanes 7, 8 and 9). APP695myc and C83myc in cytoplasm was a result of membrane contamination from incomplete subcellular fractionation separation. B. HEK293T cells were transiently transfected with expression vectors encoding SP-C99myc (lane 2) and APP695myc (lanes 3, 4, 5 and 6). After 24 h, cells were pretreated with 10 μM DAPT (lanes 5 and 6) or DMSO (lanes 3 and 4) for 3 h, and treated with 1 mM $H_2O_2$ for another hour. Cells were harvested and analyzed by immunoblotting with APP c-terminal antibody (anti-APP676-695, 1:4,000, Sigma). C. HEK293T cells were transiently transfected with expression vectors encoding APP695 (lanes 2 and 3). After 24 h, cells were treated with 1 mM $H_2O_2$ (lane 3) and analyzed by immunoblotting with anti-APP676-695.

FIGURE S2. **Activated-JNK directly phosphorylates GST-PS1 *in vitro*.** HEK293T cells were transfected with expression vectors encoding HA-JNKK2-JNK1, HA-JNKK2(KM)-JNK1. Cells were

lysed and the JNK activity of the immunocomplex was measured by kinase assays with GST-c-Jun-(1-79) and GST-PS1 as substrates. An aliquot of each sample was analyzed for the expression of HA-JNKK2-JNK1 and HA-JNKK2(KM)-JNK1 by immunoblotting using anti-HA antibody (1:10,000). KA, kinase assay. IB, immunoblotting. IP, immunoprecipitation. CS, Coomassie blue staining.

FIGURE S3. **Effects of cell signal inhibitors on $H_2O_2$-triggered cell death.** SH-SY5Y/APP695myc cells were serum-starved for 12 h and pretreated with U0126 (5 μM) (c and d), Wortmannin (20 nM) (e and f), SP600125 (20 μM) (g and h) or the control DMSO (a and b) for 3 h. With exposure to 1 mM $H_2O_2$ for 1 h, morphological images of cells were taken with Olympus BX50 microscopy to determine the effects of cell signal inhibitors on $H_2O_2$-triggered cell death.

## REFERENCES


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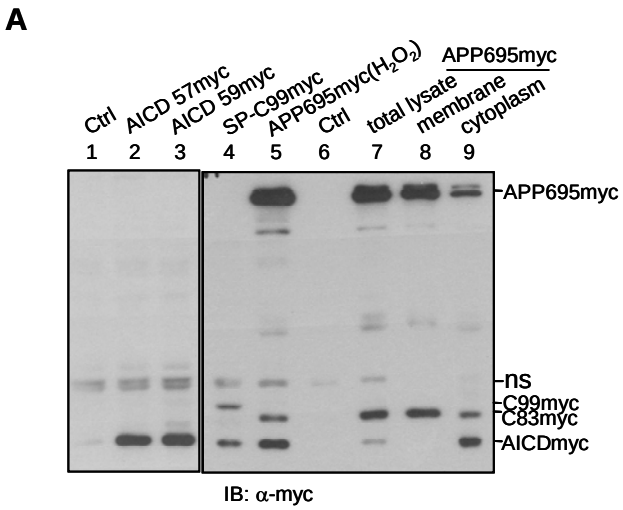
# FIGURE S1


A
Ctrl
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APP695myc(H2O2)
Ctrl
APP695myc
total lysate
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cytoplasm
1 2 3 4 5 6 7 8 9
APP695myc
ns
C99myc
C83myc
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IB: α-myc
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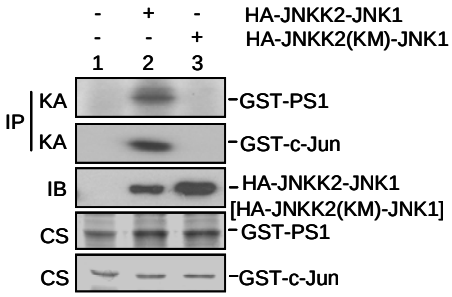
SP-C99myc
APP695myc
DAPT - - - - + +
H2O2 - - - + - +
1 2 3 4 5 6
APP695myc
APP(endogenous)
C99myc
C83myc
AICDmyc
IB: α-APP676-695
C
APP695 - + +
H2O2 - - +
1 2 3
APP695
C83
IB: α-APP676-695

## FIGURE S2


- + - HA-JNKK2-JNK1
- - + HA-JNKK2(KM)-JNK1
1 2 3
IP
KA
KA
GST-PS1
GST-c-Jun
IB
HA-JNKK2-JNK1
[HA-JNKK2(KM)-JNK1]
CS
GST-PS1
CS
GST-c-Jun

## FIGURE S3