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Valproic Acid Induces Monoamine Oxidase A via Akt/FoxO1 Activation

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Running title: Valproic Acid Induces MAO A

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine/serotonin; AAA, constitutively active; CA, constitutively active; ChIP, chromatin immunoprecipitation; DA, dopamine; EMSA, electrophoretic mobility shift analysis; ERK, extracellular signal-regulated kinase; FoxO1, Forkhead box O1; GABA, γ-aminobutyric acid; GSK3β, glycogen synthase kinase-3β; HDAC, histone deacetylase; *luc*, luciferase; MAO, monoamine oxidase; NE, norepinephrine; PI3K, phosphoinositide 3-kinase; VPA, valproic acid; WT, wild-type

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ABSTRACT

Valproic acid (VPA) has been widely used in clinics for the treatment of multiple neuropsychiatric disorders, such as epilepsy and bipolar disorder. One of the mechanisms by which VPA exerts its effect is through regulating the brain levels of serotonin. However, the molecular basis of this VPA action is not fully understood. Here, we report for the first time that VPA activates monoamine oxidase (MAO) A catalytic activity, mRNA level and promoter activity. MAO A is a key enzyme which degrades a number of monoamine neurotransmitters including serotonin. Our results show that VPA increased the phosphorylation of both Akt and FoxO1, whereas pretreatment of cells with LY294002 (a PI3K inhibitor) reduced the VPA activation of MAO A. Overexpression of FoxO1 dramatically repressed both the basal and VPA-induced MAO A catalytic and promoter activities to 30-60%. siRNA knockdown of *FoxO1* attenuated the stimulating effect of VPA on MAO A. Moreover, introduction of a constitutively active form of *FoxO1* abolished the activation of MAO A by VPA and Akt. These results suggest that FoxO1 is a repressor for *MAO A* transcription and its phosphorylation is involved in VPA activation of MAO A. Sequence analysis, electrophoretic mobility shift and chromatin immunoprecipitation assays further showed the presence of a functional FoxO1-binding site in *MAO A* core promoter. Taken together, these results demonstrate that MAO A is a novel target for VPA via Akt/FoxO1 signaling pathway. This information provides new insights into the pharmacological mechanisms and therapeutic implications of VPA action.

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Introduction

Valproic acid (VPA, 2-propylpentanoic acid, Divalproex) has been used clinically for decades as a mood stabilizer and anticonvulsant, primarily for the treatment of epilepsy and bipolar disorder. It is also used for treating migraine headaches and seizures. It has been suggested that VPA exerts its anticonvulsant effect by regulating multiple brain neurotransmitter systems, including serotonin (5-hydroxytryptamine, 5-HT) and dopamine (DA) via the modulation of brain levels as well as intraneuronal metabolism of these neurotransmitters (Johannessen, 2000; Rosenberg, 2007). For example, chronic administration of VPA induced the differential change of 5-HT level in discrete rat brain regions, such as a decrease of 5-HT in hypothalamus (Baf et al., 1994). Microdialysis studies also revealed an elevation in the metabolites of 5-HT and DA in rats with VPA administration (Horton et al., 1977). However, the molecular basis of VPA action on brain neurotransmission still remains unclear.

Monoamine Oxidase (MAO) oxidatively deaminates a number of biogenic and dietary amines including monoamine neurotransmitters (Shih et al., 1999). MAO exists in two isoenzymes, MAO A and MAO B, with 70% identity in amino acid sequences (Bach et al., 1988). MAO A preferentially oxidizes 5-HT, norepinephrine (NE), epinephrine as well as DA, and is irreversibly inhibited by low concentrations of clorgyline. The catalytic process produces hydrogen peroxide (Shih et al., 1999). MAO A dysfunctions associated with abnormal levels of neurotransmitters, such as 5-HT, have been implicated in numerous neuropsychiatric disorders, such as aggression and antisocial behavior (Bortolato et al., 2008; Shih et al., 1999). MAO A deficiency caused by a spontaneous mutation in *MAO A* gene results in impulsive aggressive behavior and mild mental retardation in affected males in a Dutch family (Brunner et al., 1993). Consistently, *MAO A*-knockout mice also show aggressive behavior (Scott et al., 2008).

In this study, we report for the first time that VPA significantly induced *MAO A* gene expression and demonstrate its molecular mechanisms.

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Materials and Methods

Cell Line and Reagents. The human neuroblastoma BE(2)C cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). BE(2)C cells were grown in a medium containing 1:1 mixture of Eagle's minimum essential medium with Earle's balanced salt solution and Ham's F12 medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 0.05 mM non-essential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. All culture materials were purchased from Mediatech (Manassas, VA, USA). E18 rat primary cortical neurons were kindly provided by Dr. Shuhua Chen (Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, CA, USA). Valproic acid sodium salt was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in ethanol. LY294002 was purchased from Cell Signaling (Danvers, MA, USA) and dissolved in DMSO. The equivalent volume of respective vehicle control (ethanol for VPA and DMSO for LY294002) was added into the cells in the control group for each experiment with drug treatment. Monoclonal anti-β-Actin (A5441) and anti-FLAG (F1804) antibodies were purchased from Sigma-Aldrich. Rabbit monoclonal anti-phospho-Akt (Ser473) (4060), anti-phospho-Akt (Thr308) (2965), anti-Akt (4691) and anti-phospho-FoxO1 (Ser256) (9461) antibodies were purchased from Cell Signaling. Polyclonal anti-FoxO1 (A00427) antibody was purchased from GenScript (Piscataway, NJ, USA). Human *FoxO1* siRNA (sc-35382) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Plasmids. Human *MAO A* 2-, 1.6-, 1.3-, and 0.24-kb promoter-luciferase (*luc*, Firefly) reporter constructs were generated as described previously (Chen et al., 2005; Ou et al., 2006). pGL2-Basic vector was purchased from Promega (Madison, WI, USA). A constitutively active (CA) form of *Akt* expression construct and the parental pIRES empty vector were a gift from Dr. Bangyan Stiles (Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, CA, USA). FLAG-tagged wild-type (WT) *FoxO1* and constitutively active (AAA) *FoxO1* with three mutated phosphorylation sites (T24A, S256A

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and S319A) expression constructs in pcDNA vector were a gift from Dr. Kun-Liang Guan (Department of Pharmacology, University of California, San Diego, CA, USA) (Tang et al., 1999).

MAO A Catalytic Activity Assay. One hundred micrograms of total protein (~1x10^6 cells) were incubated with 1 mM ^{14}C-5-HT in the assay buffer (50 mM sodium phosphate buffer, pH 7.4) at 37 ^{o}C for 20 min, and the reaction was terminated by the addition of 100 µl of ice-cold 6 N HCl. The reaction products were extracted with benzene/ethyl acetate (1:1) and centrifuged at 4 ^{o}C for 7 min. The organic phase containing the reaction products was extracted, and the radioactivity was determined by liquid scintillation spectroscopy (Wu et al., 2009a).

RNA Isolation and Quantitative Real-time RT-PCR. Total DNA-free RNA was purified with TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Two micrograms of total RNA were used for reverse transcription by M-MLV reverse transcriptase (Promega) following the manufacturer's instructions. The RT products were used as the template for quantitative real-time PCR. Quantitation of PCR products was determined by SYBR Green reagent (Maxima SYBR Green qPCR Master Mix 2X; Fermentas, Glen Burnie, MD, USA) using the iCycler optical system (Bio-Rad, Hercules, CA, USA). The primers for *MAO A* were forward 5'-CTGATCGACTTGCTAAGCTAC-3' and reverse 5'-ATGCACTGGATGTAAAGCTTC-3'. The primers for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were forward 5'-GACAACAGCCTCAAGATCATCAG-3' and reverse 5'-ATGGCATGGACTGTGGTCATGAG-3'. PCR conditions included an initial denaturation step of 3 min at 95 ^{o}C, followed by 40 cycles of PCR consisting of 30 s at 94 ^{o}C, 30 s at 60 ^{o}C, and 40 s at 72 ^{o}C. The qPCR data were analyzed by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Western Blotting Analysis. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Twenty to fifty micrograms of total protein from cells lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1% NP-40; 0.5% sodium deoxycholate; and 0.1% SDS) supplemented with 1X protease inhibitors (Roche,

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Indianapolis, IN, USA) and 1X phosphatase inhibitors (Cell Signaling, when required) were separated by 8-12% SDS-PAGE and transferred to the 0.45-μm pore size polyvinylidene difluoride (PVDF) membrane. After the transfer, the membrane was blocked at room temperature for 1 h with 2% bovine serum albumin (BSA) in PBST (10 mM sodium phosphate, pH 7.2; 150 mM NaCl; and 0.05% Tween 20). The membrane was incubated with anti-pAkt (Ser473) (1:2000), anti-pAkt (Thr308) (1:1000), anti-Akt (1:1000), anti-β-Actin (1:4000), anti-pFoxO1 (Ser256) (1:1000), anti-FoxO1 (1:1000), or anti-FLAG (1:1000) antibody in 1% BSA in PBST at 4 ^{o}C overnight. After incubating the membrane with horseradish peroxidase (HRP)-conjugated secondary antibody against appropriate species at room temperature for 1 h, bands were visualized with the ECL Western Blotting Substrates (Pierce). The intensity of bands was quantitated (when required) using ImageJ software (NIH, MD, USA) (Wu et al., 2009b).

Stable Cell Line Establishment. WT *FoxO1*, AAA *FoxO1* or the parental pcDNA empty vector was transfected into BE(2)C cells of 10-cm dish separately. All constructs carry the neomycin-resistant gene. After 24 h, geneticin (G418, 750 μg/ml, Sigma-Aldrich), the selective agent, was added into cells. Resistant clones were isolated after the treatment for 10-14 days and cultured under the G418 selection continuously. Overexpression of FoxO1 was verified by Western blot. Established stable cell lines were routinely maintained under the G418 treatment at lower concentration (200 μg/ml) (Chen et al., 2011).

Transient Transfection and Luciferase Reporter Assay. Transfections were performed with Lipofectamine 2000 (Invitrogen) or BioT (Bioland, Cerritos, CA, USA) in 12-well plates. pRL-TK (expressing Renilla luciferase, Promega) was co-transfected as an internal control. The parental empty vector, such as pcDNA, was added to maintain an equivalent amount of DNA in each transfection. After 24- to 48-h incubation, cells were harvested and assayed for luciferase activity using the Dual-Luciferase Reporter 1000 Assay System (Promega).

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siRNA Interference. siRNA was transfected into BE(2)C cells with Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. A non-silencing RNA with sense strand as 5'-UUCUCCGAACGUGUCACGUUU-3' was used as a control (Salahpour et al., 2007).

Site-Directed Mutagenesis of *MAO A* 0.24-kb Promoter. Site-directed mutagenesis was used to mutate the putative FoxO1-binding site as identified in the *MAO A* 0.24-kb promoter. WT *MAO A* 0.24-kb *luc* was used as the template. Mutagenesis was carried out using QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions. The primers used for mutagenesis was forward 5'-CCCCCCGGGTATCAGGGGAAGGATCGGCTCC-3' (mutated nucleotides are *underlined*). Mutated nucleotides were verified by DNA sequencing.

***In Vitro* Translation.** *In vitro* translation was conducted with TNT Coupled Reticulocyte Lysate System (Promega) following the manufacturer's instructions. Both FLAG-tagged WT *FoxO1* construct and the parental pcDNA empty vector carry T7 promoter. *In vitro* translation products were verified by Western Blot.

Electorphoretic Mobility Shift Analysis (EMSA). *MAO A* 0.24-kb promoter-derived oligonucleotide harboring WT FoxO1-bindig site (5'-GGCGGGTATCAAAAGAAGGATCGG-3', FoxO1-binding site is *underlined*) was used as a probe and radioactively labeled by Klenow fill-in reaction. ^{32}P-labeled probe was purified using Nuclear Removal Kit (Qiagen, Valencia, CA, USA). For determining DNA-protein binding, 2 µl of *in vitro* translated FoxO1 protein was diluted with 5X binding buffer [20% glycerol; 5 mM $MgCl_2$; 2.5 mM EDTA; 2.5 mM DTT; 250 mM NaCl; 50 mM Tris-HCl, pH 7.5; and 0.25 mg/ml poly (dI-dC)] in a total volume of 20 µl. One hundred-fold excess of non-radioactively labeled probe (competitor) or 5 µg of anti-FLAG antibody was added, and the mixture was incubated at room temperature for 20 min. ^{32}P-labeled probe (~600,000 cpm) was then added, and the mixture was incubated at room temperature for another 20 min. Samples were analyzed on 5% non-denaturing polyacrylamide gel in 1X Tris

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borate/EDTA buffer at 150 V at room temperature for 3 h. Gel was dried and visualized by autoradiography.

Chromatin Immunoprecipitation (ChIP) Assay and PCR. BE(2)C cells were transfected with FLAG-tagged WT *FoxO1*, 24 h after transfection, cells were treated with 1 mM VPA or vehicle control for another 24 h. Confluent BE(2)C cells in a 10-cm dish were treated with formaldehyde at the final concentration of 1% at room temperature with gentle shaking for 10 min to cross-link nuclear protein with genomic DNA. Cross-link was quenched by incubating cells with glycine at the final concentration of 0.125 M at room temperature with gentle shaking for another 5 min. Cells were quickly washed by cold phosphate-buffered saline (PBS, pH 7.4) twice, harvested by scraping, and centrifuged at 2000 rpm at 4 ^{o}C for 5 min. Cell pellets were lysed in 350 µl of SDS lysis buffer (1% SDS; 10 mM EDTA; 50 mM Tris-HCl, pH 8.0; and 2X protease inhibitors) on ice for 10 min, followed by brief sonication using the Branson 450 Sonifier (Branson Ultrasonics, Danbury, CT, USA) to shear genomic DNA into ~300- to 500-bp fragments. One percent of the supernatant was saved as input. Supernatant was diluted (1:10) in dilution buffer (0.01% SDS; 1.1% Triton X-100; 1.2 mM EDTA; 16.7 mM Tris-HCl, pH 8.0; and 167 mM NaCl), and blocked with 60 µl of sheared salmon sperm DNA/protein G agarose at 4 ^{o}C for 2-4 h. The supernatant was immunoprecipitated (IP) with 5 µg of anti-FLAG antibody at 4 ^{o}C overnight. IgG was used as a negative control for IP. After incubating 40 µl of salmon sperm DNA/Protein G agarose with IP samples at 4 ^{o}C for another 2 h, beads were sequentially washed by low-salt buffer (0.1% SDS; 1% Triton X-100; 2 mM EDTA; 20 mM Tris-HCl, pH 8.0; and 150 mM NaCl), high-salt buffer (0.1% SDS; 1% Triton X-100; 2 mM EDTA; 20 mM Tris-HCl, pH 8.0; and 0.5 M NaCl), LiCl buffer (0.25 M LiCl; 1% NP-40; 1% sodium deoxycholate; 1 mM EDTA; and 10 mM Tris-HCl, pH 8.0), and TE buffer (10 mM Tris-HCl, pH 8.0; and 1 mM EDTA). The DNA-protein complex was eluted by elution buffer (1% SDS and 0.1 M sodium bicarbonate) with gentle rotation at room temperature for 15 min twice, reversely cross-linked by the incubation at 65 ^{o}C overnight, and purified using QIAquick PCR Purification Kit (Qiagen).

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Purified DNA was used as the template for the following qPCR analysis. The primers used to target the *MAO A* promoter region which encompasses the FoxO1-binding site were forward 5'-GTGCCTGACACTCCGCGGGGTT-3' and reverse 5'-TCCTGGGTCGTAGGCACAGGAG-3'.

Statistical Analysis. Data was presented as the mean ± S.D. from three independent experiments and analyzed with unpaired *t* test. A *p* value less than 0.05 was considered as significant.

Results

VPA Activated MAO A Catalytic Activity, mRNA Level and Promoter Activity. We studied the effect of VPA on MAO A and its molecular mechanisms in a human neuronal BE(2)C cell line, which expresses MAO A (Chen et al., 2005) and well responds to VPA in terms of the activation of known VPA downstream target genes (Hahn et al., 2008). To determine the effect of VPA on MAO A catalytic activity, BE(2)C cells were treated with VPA at various concentrations (0.5, 1, 2, 5, and 10 mM) for 24 h. The concentrations of VPA used under current experimental conditions were within the range of its pharmacological concentrations (Loscher, 1999). Moreover, MTT cell proliferation assay indicated that the cell viability was not affected by VPA at concentrations up to 10 mM within a 24-h treatment period (data not shown). As shown in Fig. 1A, VPA significantly activated MAO A catalytic activity in a concentration-dependent manner (up to 305%, EC50: 1.87 ± 0.28 mM). BE(2)C cells were also treated with 1 mM VPA for different times (12, 24, 36, 48 and 72 h), and the results showed a time-dependent induction of MAO A activity (Fig. 1B, up to 340%, maximum effect achieved within 48 h). Notably, the increasing tendency of MAO A activity with time is similar between 24- and 48-h VPA treatment (1 mM) (Supplemental Fig. 1), which suggests that the VPA induction of MAO A within 24 h could be due to a kinetic effect rather than binding effect. Moreover, ethanol, as a corresponding vehicle control for VPA, had no significant effect on MAO A activity within a 72-h treatment period (Fig. 1B). We also determined the stimulating effect of VPA on MAO A catalytic activity in E18 rat primary cortical neurons, and similarly, VPA (0.5 and 1 mM) induced MAO A activity in a

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concentration-dependent manner by up to 230% in this primary neuronal culture system (Supplemental Fig. 2). Next, we investigated whether this induction is at the *MAO A* mRNA level. As determined by quantitative real-time RT-PCR, *MAO A* mRNA increased by 2-fold after 24-h VPA (1 mM) treatment (Fig. 1C). Further, VPA (1 mM, 24-h treatment) activated both *MAO A* 2- and 0.24-kb promoter activities by 122 and 150%, respectively (Fig. 1D). *MAO A* 0.24-kb core promoter contains a 240-bp region immediately upstream from the transcription initiation site and exhibits the maximum *MAO A* promoter activity (Zhu et al., 1992). Taken together, these results demonstrate that VPA activated MAO A catalytic activity, mRNA level and promoter activity.

Akt Mediated the Induction of MAO A by VPA. To determine whether VPA initiates the PI3K/Akt signal in the present study, BE(2)C cells were treated with VPA (1 mM) for various times (3, 6, 12 and 24 h). The results of the Western blot show that the phosphorylation level of Akt at both Ser473 and Thr308 sites markedly increased with time up to 198% (Figs. 2A and 2B)

To investigate whether PI3K/Akt mediates the VPA activation of MAO A, BE(2)C cells were treated with 1 mM VPA in the absence or presence of LY294002 (20 μM), a potent PI3K inhibitor that acts immediately upstream of Akt, for 12 h, and MAO A catalytic activity was subsequently determined. As shown in Fig. 2C, MAO A catalytic activity increased by 61% when cells were treated with VPA alone (column 2 vs. column 1), whereas this increase was substantially reduced by 60% in the presence of LY294002 (column 3 vs. column 2). The efficacy of inhibition of phospho-Akt level by LY294002 was demonstrated by the significantly reduced phospho-Akt band by Western blot (Fig. 2D). This suggests that PI3K/Akt signal mediates the VPA induction of MAO A. To further demonstrate the role of Akt in activating MAO A activity, we transiently introduced a constitutively active (CA) form of *Akt* expression construct into BE(2)C cells. Similarly, overexpression of Akt increased MAO A activity by 64% (Fig. 2E). The transfection efficiency of CA *Akt* construct was confirmed by the presence of both

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transfected phospho- and total Akt bands by Western blot (Fig. 2F). Taken together, these results demonstrate that PI3K/Akt signal mediates the VPA induction of MAO A.

FoxO1 Mediated the VPA Induction of MAO A. As shown in Figs 1C and 1D, VPA increased *MAO A* mRNA level and promoter activity, these results suggest that the VPA activation of MAO A may be at the transcriptional level. Forkhead box O1 (FoxO1), a member of the O subclass of the forkhead family of transcription factors, is an important downstream target of Akt and regulated via the phosphorylation by Akt in PI3K/Akt signaling pathway. Phosphorylated FoxO1 is translocated into the cytoplasma from the nucleus and subjected to ubiquitination (Gross et al., 2008). We have previously shown a negative correlation between the expression of FoxO1 and MAO A activity (Fig. 3F, columns 1, 3 and 5), hence, we speculate that FoxO1 might be a potential contributor to the VPA induction of MAO A. To investigate whether VPA induces the phosphorylation of FoxO1, we treated BE(2)C cells with VPA (1 mM) for various times (3, 6, 12 and 24 h). As shown in Figs. 3A and 3B, the phospho-FoxO1 (Ser256) level increased with the time by up to 590% in response to VPA, but the total FoxO1 level was not significantly changed. In addition, we established stable BE(2)C cell lines transfected with FLAG-tagged wild-type (WT) *FoxO1* or constitutively active (AAA) *FoxO1* with three mutated phosphorylation sites (T24A, S256A and S319A) into cells followed by geneticin selection. AAA FoxO1 is sequestered in the nucleus without degradation (Tang et al., 1999). The parental pcDNA empty vector was stably transfected into control cells. As shown in Fig. 3C, overexpression of WT and AAA FoxO1 repressed the basal MAO A catalytic activity to 31% (column 3 vs. column 1) and 20% (column 5 vs. column 1), respectively. The VPA-induced MAO A activity was also reduced to 32% in the cells with WT FoxO1 overexpression (Fig. 3C, column 4 vs. column 2), which is further exacerbated in the cells with AAA FoxO1 stably expressed (column 6 vs. column 4). The successful transfection of WT and AAA *FoxO1* into the genome of stable cells was confirmed by Western blot using anti-FLAG antibody (Fig. 3D). Moreover,

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FoxO1 showed a competitive effect with Akt on the regulation of MAO A, given that overexpression of AAA FoxO1 counteracted the activation of MAO A activity by CA Akt (Fig. 3E, column 3 vs. column 2). This suggests higher potency of FoxO1 than Akt for regulating MAO A activity.

To demonstrate the effect of VPA and FoxO1 on the *MAO A* promoter, *MAO A* 2-kb promoter luciferase (*luc*) reporter construct (*MAO A* 2-kb *luc*) was co-transfected with WT or AAA *FoxO1* expression construct into cells followed by the VPA (1 mM, 24 h) treatment. The result showed that co-transfection of WT and AAA *FoxO1* repressed the basal *MAO A* promoter activity to 66% (Fig. 3F, column 3 vs. column 1) and 42% (Fig. 3F, column 5 vs. column 1), respectively. Similar to the VPA effect on MAO A activity, AAA FoxO1 was more effective than WT FoxO1 in attenuating the activating effect of VPA on the *MAO A* promoter (Fig. 3F, column 6 vs. column 2 and column 4 vs. column 2). These results indicate that FoxO1 mediates the induction of both MAO A catalytic and promoter activities in response to VPA.

Knockdown of Endogenous FoxO1 Diminished the Activation of MAO A by VPA. We further studied the role of FoxO1 in mediating the VPA induction of MAO A by siRNA interference technology. Knockdown of FoxO1 elevated both the basal MAO A catalytic (Fig. 4A, column 3 vs. column 1) and promoter (Fig. 4B, column 3 vs. column 1) activities by ~2-fold, which is similar to the extent of the VPA induction of MAO A activity in the control cells transfected with non-specific (NS) siRNA (Figs. 4A and 4B, column 2 vs. column 1). Successful knockdown of endogenous FoxO1 was confirmed by Western blot (Fig. 4C). The knockdown of FoxO1 diminished the activation of MAO A activities by VPA, as there is no significant VPA induction of either MAO A catalytic (Fig. 4A, column 4 vs. column 3, p=0.27, considered as not significant) or promoter (Fig. 4B, column 4 vs. column 3, p=0.11, considered as not significant) activity in FoxO1-knockdown cells. Most importantly, knockdown of FoxO1 enhanced the activating effect of VPA on MAO A activity by 20-40% with significance (Figs. 4A and 4B,

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column 4 vs. column 2). These data further suggest the involvement of FoxO1 in triggering the expression of MAO A in response to VPA.

Identification and Validation of a Functional FoxO1-Binding Site in *MAO A* 0.24-kb Core Promoter. To investigate if the regulation of MAO A by FoxO1 is due to a direct binding of FoxO1 to the *MAO A* promoter and to locate putative FoxO1-binding sites in the *MAO A* promoter, we did a serial deletions of *MAO A* 2-kb promoter linked with *luc* reporter gene and co-transfected each of them with WT *FoxO1* expression construct into BE(2)C cells. Interestingly, *MAO A* promoter-*luc* constructs consisting of 2-, 1.6-, 1.3-, or 0.24-kb promoter were repressed by FoxO1 to approximately the same extent (Fig. 5A), suggesting that the FoxO1-binding sites are most likely located in the 0.24-kb core promoter region. Sequence analysis of this 0.24-kb region identified a putative FoxO1-binding site (-116/-110) (Fig. 5B, middle panel) which has only one nucleotide different from the canonical site (Fig. 5B, top panel) (Jonsson and Peng, 2005; Pierrou et al., 1994). Mutation of selective nucleotides at this site (Fig. 5B, bottom panel) increased the basal *MAO A* 0.24-kb promoter activity (Fig. 5C, column 3 vs. column 1) but abolished the repressing effect of FoxO1 on the *MAO A* core promoter (Fig. 5C, column 4 vs. column 3, p=0.46, considered as not significant). Moreover, the extent of activation of *MAO A* 0.24-kb promoter by VPA was reduced to ~50% when the FoxO1-binding site was mutated (Fig. 5D). These mutation analyses suggest that this FoxO1-binding site is functional and important for the VPA activation of MAO A.

To determine whether FoxO1 directly interacts with this FoxO1-binding site (-116/-110) in *MAO A* 0.24-kb promoter, we conducted electrophoretic mobility shift analysis with *in vitro* translated FoxO1 protein and radioactively labeled FoxO1-binding oligonucleotide derived from the *MAO A* core promoter as a probe. *In vitro* translated FLAG-tagged WT FoxO1 protein was verified by Western blot using both anti-FLAG and anti-FoxO1 antibodies. The parental pcDNA empty vector was used as the template in the protein synthesis for a control (Fig. 6A). No band

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was observed from the control in which the mock protein synthesized from pcDNA vector was used in the DNA-protein binding reaction (Fig. 6B, lane 2). In contrast, our results showed one radioactive band on the gel when FoxO1 (with FLAG tag) protein was incubated with the probe (lane 3), indicating the presence of a FoxO1-DNA complex. This band was abolished in the presence of 100-fold excess of unlabeled probe as a competitor, suggesting it is specific for FoxO1 (lane 4). This band was further supershifted when anti-FLAG antibody was incubated in the DNA-protein binding reaction (lane 5). To confirm a FoxO1 association with the *MAO A* promoter *in vivo*, we performed chromatin immunoprecipitation assay with BE(2)C cells transiently transfected with FLAG-tagged WT *FoxO1* followed by the VPA treatment (1 mM, 24 h). The anti-FLAG immunoprecipitates were subjected to qPCR amplification with primers specific for the *MAO A* promoter region which encompasses the FoxO1-binding site. As demonstrated in Fig. 6C, the ectopically expressed FoxO1 was indeed associated with this region (column 1 vs. column 2). Moreover, in response to VPA, lower occupancy by FoxO1 in the *MAO A* promoter with a decrease of 44% was determined *in vivo* (Fig. 6C, column 3 vs. column 1). Taken together, these results demonstrate the interaction of FoxO1 with the *MAO A* promoter under both *in vitro* and *in vivo* conditions.

Taken together, the molecular mechanisms of VPA activation of MAO A is schematically represented in Fig. 7. FoxO1 acts as a transcriptional repressor of *MAO A* by directly binding to a functional FoxO1-binding site in the *MAO A* core promoter. In response to VPA signal, the phosphorylation levels of both Akt and its downstream FoxO1 increase. Phosphorylated FoxO1 is subjected to nuclear export, which consequently activates *MAO A* transcription.

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Discussion

We demonstrate for the first time that MAO A is a novel target for the anticonvulsant VPA. One of the mechanisms which has been widely accepted for the action of VPA is the increase of the brain levels of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) by inhibiting GABA transaminase and thus preventing GABA metabolism. In addition to GABA, it has also been suggested that VPA may exert its anticonvulsant effect through the regulation of monoamine neurotransmitters (Johannessen, 2000; Rosenberg, 2007). For example, the levels of 5-HT, NE and DA show significant differential changes in discrete regions of the rat brain after chronic administration of VPA (Baf et al., 1994). MAO A is the major enzyme involved in monoamine metabolism (e.g., 5-HT, NE and DA), and its influences on the behaviors of humans and mice have been well established (Bortolato et al., 2008; Shih et al., 1999). Hence, it is possible that the regulation of MAO A serves as one of the mechanisms by which VPA modulates monoaminergic neurotransmission and exerts its therapeutic effect.

VPA is one of the first-line treatment drugs for many neuropsychiatric disorders, such as bipolar disorder, however, the adverse effect when clinically used in pregnancy has been shown since 1980s, which results in the fetal valproate syndrome (FVS). This disease is initiated and progressed along the developmental course in the human embryo and fetus, and later displays autistic-like behaviors and other physical anomalies in children. Similarly, such VPA effect has been validated in most animal species (Ornoy, 2009). For example, maternal administration of VPA induces behavioral impairments in juvenile rats, which mimic some aspects of autistic phenotypes. Impairments of the serotonergic system, which are often observed in human autism, have been shown in the initiation/progression of FVS and further suggested to be involved in the pathogenesis of FSV. Abnormalities of serotonergic neuronal differentiation and

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migration in animal models of FVS have been demonstrated both *in vitro* and *in vivo* (Dufour-Rainfray et al., 2010; Miyazaki et al., 2005). These studies suggest that VPA could have a direct or indirect action on the embryonic development of the serotonergic system.

The potential mechanisms by which VPA regulates the serotonin level may involve different serotonin regulators in the serotonergic system. Previous studies have revealed that VPA is capable of affecting the activities of tryptophan hydroxylase, the enzyme responsible for serotonin synthesis (Shukla, 1985), and certain subtypes of serotonin receptors such as 5-HT2A (Sullivan et al., 2004) and 5-HT1B receptors (Redrobe and Bourin, 1999). MAO A uses serotonin as a substrate, and MAO A dysfunctions associated with a serotonin imbalance has been implicated in multiple mental disorders including depression, social anxiety and autism (Bortolato et al., 2008; Shih et al., 1999). It would be interesting to investigate whether the region-specific variation of brain serotonin level as observed in the pathogenesis of FVS could be partially resulted from the misregulation of MAO A by VPA. Moreover, current findings may also help us develop better therapeutic strategies for treating the pregnant patients with a required clinical use of VPA, such as a combined treatment with MAO inhibitors.

We show that VPA induces MAO A via the activation of Akt/FoxO1 signaling pathway. VPA has shown the capability to induce selective cellular signaling pathways including PI3K/Akt and extracellular signal-regulated kinase (ERK) pathways, which eventually activate downstream transcription factors such as c-Jun, c-Fos and β-catenin via differential regulations of mediators such as glycogen synthase kinase-3β (GSK-3β), to control the transcription of target genes (Kostrouchova et al., 2007). Pretreatment of cells with LY294002, a potent PI3K inhibitor, reduced the VPA induction of MAO A (Fig. 2C), indicating a mediating role of Akt. However, the effective suppression of Akt activity (Fig. 2D) did not abolish this induction completely (Fig. 2C, column 3 vs. column 1, $p<0.05$). This suggests that Akt activation may act as a central signal coupled with other signals/regulators to mediate the VPA induction of MAO A.

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Transcription factor FoxO1 plays an important role in many physiological processes. FoxO1 is regulated by several modifications, but one of the most critical is phosphorylation and nuclear exclusion by Akt (Gross et al., 2008). The present study demonstrates for the first time the responsiveness of FoxO1 to VPA and the repressing effect of FoxO1 on MAO A (Fig. 3). VPA-induced phosphorylation of FoxO1 allows the nuclear export of FoxO1 and its subsequent ubiquitination. The shuttle of FoxO1 between the nucleus and cytoplasma is a key determinant for its activity as well as a master event for the regulation of MAO A by VPA. Introduction of AAA *FoxO1*, which escapes the degradation, completely abolished the VPA-induced activation of MAO A catalytic and promoter activities (Figs. 3C and 3E). FoxO1 repressed MAO A by directly binding to a functional FoxO1 binding-site in the *MAO A* core promoter. Mutation of this site diminished the repressing effect of FoxO1 on the *MAO A* promoter (Fig. 5C), but still retained ~50% of the activation of *MAO A* promoter by VPA (Fig. 5D), which suggests a potential contributing role(s) of other *MAO A* regulators in this induction. In addition to the activation of the basal MAO A activity, siRNA knockdown of endogenous *FoxO1* also attenuated the stimulating effect of VPA on MAO A (Figs. 4A and 4B). This is, to some extent, consistent with the reported FoxO1-Akt feedback mechanism. The knockdown of FoxO1 decreases the sensitivity of Akt phosphorylation to extracellular signals (e.g. VPA in the present study) (Matsumoto et al., 2006), which reduces the Akt-mediated phospho-FoxO1 level, retains more FoxO1 in the nucleus and further counteracts the VPA induction of MAO A.

Recently, VPA has demonstrated its role as a histone deacetylase (HDAC) inhibitor (Gottlicher et al., 2001; Phiel et al., 2001). HDACs play an important role in the regulation of gene transcription by chromatin structure remodeling and dynamic changes in nucleosomal packaging of DNA. Inhibition of HDAC increases histone acetylation and maintains chromatin structure in a more open conformation, which leads to the transcriptional activation via the recruitment of activator proteins (Marks et al., 2003; Richon and O'Brien, 2002). Two well-known HDAC inhibitors, sodium butyrate and trichostatin A, showed stimulating effect on both MAO A

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catalytic and promoter activities by ~2-fold in the present cellular system (Supplemental Fig. 3). This supports the hypothesis that VPA may induce *MAO A* by modulating the acetylation status of *MAO A* promoter. Moreover, VPA has previously shown the capability to increase Sp1 acetylation (Marinova et al., 2009), a key transcriptional activator of MAO A by directly binding to the Sp1 sites in the *MAO A* core promoter (Shih et al., 2011 ; Zhu et al., 1994). This further suggests the possibility that Sp1 and other potential VPA-responsive MAO A regulators may also contribute to the induction of MAO A by VPA via diverse mechanisms such as histone acetylation.

In summary, we report for the first time that VPA activates *MAO A* gene expression and demonstrate the molecular mechanisms via the activation of Akt/FoxO1 signaling pathway. This study provides new insights into the molecular mechanisms of VPA action and helps understand its therapeutic implications in the treatment of multiple neuropsychiatric disorders.

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Authorship Contributions:

Participated in research design: Wu, J.B. and Shih, J.C.

Conducted experiments: Wu, J.B.

Performed data analysis: Wu, J.B. and Shih, J.C.

Wrote or contributed to the writing of the manuscript: Wu, J.B. and Shih, J.C.

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Footnotes

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Legends for Figures

Fig. 1. VPA activated MAO A catalytic activity, mRNA level and promoter activity in BE(2)C cells. A, MAO A catalytic activity was determined in BE(2)C cells treated with VPA at various concentrations (0.5, 1, 2, 5, and 10 mM) or vehicle for 24 h (mean ± S.D., n=3). MAO A catalytic activity in cells treated with vehicle was set as 100%. B, MAO A catalytic activity was determined in BE(2)C cells treated with 1 mM VPA or vehicle for various times (12, 24, 36, 48, and 72 h) (mean ± S.D., n=3). MAO A catalytic activity determined at the starting point of the treatment was set as 100%. C, Quantitative real-time RT-PCR analysis of the *MAO A* mRNA level in BE(2)C cells under 24-h VPA (1 mM) or vehicle treatment (mean ± S.D., n=3). *GAPDH* was used as an internal control for the normalization. Data were analyzed by $2^{-\Delta\Delta CT}$ method. *MAO A* mRNA level in cells treated with vehicle was arbitrarily set as 1. D, *MAO A* 2- or 0.24-kb promoter-*luc* was transfected into BE(2)C cells, 18-24 h after transfection, cells were treated with 1 mM VPA or vehicle for another 24 h followed by luciferase activity determination (mean ± S.D., n=3). Activity of *MAO A* 2- or 0.24-kb *luc* under the treatment of vehicle was set as 100%. Ethanol was used as a vehicle for VPA. *, $p<0.05$; **, $p<0.01$.

Fig. 2. Akt mediated the VPA induction of MAO A. A, Western blotting analysis of phospho-Akt level at both Ser473 and Thr308 sites in BE(2)C cells treated with 1 mM VPA for different times (3, 6, 12 and 24 h). Total Akt and β-Actin were used as loading controls. B, Quantitation of phospho-Akt level as normalized to total Akt from three independent Western blots in A (mean ± S.D., n=3). The phospho-Akt level at the starting point of the treatment was set as 100%. C, MAO A catalytic activity was determined in BE(2)C cells treated with 1 mM VPA in the absence or presence of LY294002 (20 μM) for 12 h (mean ± S.D., n=3). Ethanol was used as a vehicle for VPA. DMSO was use as a vehicle for LY294002. MAO A catalytic activity under treatment of vehicle alone was set as 100%. D, Western blotting analysis of the phospho-Akt level in C. Total Akt and β-Actin were used as loading controls. E, BE(2)C cells were transfected with a constitutively active form of *Akt* (CA *Akt*) construct, 24 h after transfection, MAO A catalytic

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activity was determined (mean ± S.D., n=3). The parental pIRES empty vector was transfected in control cells. MAO A catalytic activity in control cells was set as 100%. F, Western blotting analysis of the transfection efficiency of CA *Akt* as used in E. β-Actin was used as a loading control. Representative gels are shown. *, $p<0.05$; **, $p<0.01$.

Fig. 3. FoxO1 mediated the VPA induction of MAO A. A, Western blotting analysis of the phospho-FoxO1 level at Ser256 site in BE(2)C cells treated with 1 mM VPA for different times (3, 6, 12 and 24 h). FoxO1 and β-Actin were used as loading controls. B, Quantitation of the phospho-FoxO1 level as normalized to total FoxO1 from three independent experiments in A (mean ± S.D., n=3). The phospho-FoxO1 level at the starting point of the treatment was set as 100%. C, MAO A catalytic activity was determined in BE(2)C cells which stably overexpress wild-type (WT) or constitutively active (AAA) FoxO1 (both with FLAG tag) with VPA (1 mM, 24-h) or vehicle treatment (mean ± S.D., n=3). The parental pcDNA empty vector carrying the neomycin-resistant gene was introduced in control stable cells. Three phosphorylation sites were mutated in AAA FoxO1 (T24A, S256A and S319A). MAO A catalytic activity under the treatment of vehicle alone in control stable cells was set as 100%. D, Western blotting analysis of FLAG-tagged WT or AAA FoxO1 protein levels in stable cells using anti-FLAG antibody. β-Actin was used as a loading control. E, CA *Akt* expression construct was transiently co-transfected with/without AAA *FoxO1* expression construct into cells, 24 h after transfection, MAO A catalytic activity was determined (mean ± S.D., n=3). The parental vectors were transfected in control cells. MAO A catalytic activity with vectors transfected only was set as 100%. F, *MAO A* 2-kb *luc* was co-transfected with WT or AAA *FoxO1* expression construct into BE(2)C cells, 18-24 h after transfection, cells were treated with 1 mM VPA or vehicle for another 24 h followed by luciferase activity determination (mean ± S.D., n=3). The parental pcDNA empty vector was co-transfected in control cells. Activity of *MAO A* 2-kb *luc* with co-transfection of vector and with the treatment of vehicle was set as 100%. Ethanol was used as a vehicle for VPA. *, $p<0.05$; **, $p<0.01$.

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Fig. 4. Knockdown of endogenous FoxO1 attenuated the VPA induction of MAO A catalytic and promoter activities. A, BE(2)C cells were transfected with non-specific (NS) or *FoxO1* siRNA, 24 h after transfection, cells were treated with 1 mM VPA or vehicle for another 24 h followed by MAO A catalytic activity determination (mean ± S.D., n=3). MAO A catalytic activity with NS siRNA transfected and with the treatment of vehicle was set as 100%. B, *MAO A* 2-kb *luc* was co-transfected with either NS or *FoxO1* siRNA into BE(2)C cells, 24 h after transfection, cells were treated with 1 mM VPA or vehicle for another 24 h followed by luciferase activity determination (mean ± S.D., n=3). Activity of *MAO A* 2-kb *luc* with NS siRNA transfected and with the treatment of vehicle was set as 100%. C, The knockdown efficiency of endogenous FoxO1 in A and B was determined by Western blot. β-Actin was used as a loading control. Ethanol was used as a vehicle for VPA. Representative gel is shown. **, $p<0.01$.

Fig. 5. Identification of a functional FoxO1-binding site in the *MAO A* 0.24-kb core promoter. A, Serially deleted constructs of *MAO A* promoter-*luc* were co-transfected with WT *FoxO1* construct into BE(2)C cells, 24 h after transfection, the luciferase activity was determined (mean ± S.D., n=3). Promoterless pGL2 vector was used as a negative control. The parental pcDNA empty vector was co-transfected in the control group for each promoter construct. Percentage of the repression of *MAO A* promoter-*luc* by FoxO1 is indicated. Activity of *MAO A* 2-kb *luc* without co-transfection of *FoxO1* was set as 100%. B, Sequences of the canonical FoxO1-binding site (top panel), a potential FoxO1-binding site (-116/-110) in the *MAO A* 0.24-kb promoter (middle panel), and the introduced point mutations (*italic*) used to inactivate the potential FoxO1-binding site (bottom panel). C, WT or mutated (Mut, harboring selective nucleotide mutations at the FoxO1-binding site as indicated in B) *MAO A* 0.24-kb *luc* was co-transfected with wild-type *FoxO1* construct into BE(2)C cells, 24 h after transfection, the luciferase activity was determined (mean ± S.D., n=3). The parental pcDNA empty vector was co-transfected in the control group for each promoter construct. Activity of WT *MAO A* 0.24-kb *luc* without co-transfection of *FoxO1* was set as 100%. D, WT or Mut *MAO A* 0.24-kb *luc* as used in C was transfected into BE(2)C

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cells, 24 h after transfection, cells were treated with 1 mM VPA or vehicle for another 24 h followed by luciferase activity determination (mean ± S.D., n=3). Fold of the activation of WT *MAO A* 0.24-kb *luc* by VPA was set as 100%. Ethanol was used as a vehicle for VPA. *, $p<0.05$; **, $p<0.01$.

Fig. 6. FoxO1 binds to a functional FoxO1-binding site in the *MAO A* 0.24-kb promoter both *in vitro* and *in vivo*. A, Western blotting analysis of *in vitro* translated FLAG-tagged WT FoxO1 protein using both anti-FLAG and anti-FoxO1 antibodies. The parental pcDNA empty vector was used as the template for protein synthesis in the control. The protein molecular weight marker is indicated. B, EMSA of *in vitro* translated FoxO1 protein with *MAO A* 0.24-kb promoter-derived ^{32}P-labeled oligonucleotide harboring the FoxO1-binding site. *In vitro* translation product using pcDNA vector as the template was used as the mock protein. *Arrows* show free oligo, FoxO1-oligo complex and supershifted FoxO1-oligo complex conjugated with anti-FLAG antibody. C, BE(2)C cells were transfected with FLAG-tagged WT *FoxO1* construct, 24 h after transfection, cells were treated with 1 mM VPA or vehicle for another 24 h followed by ChIP assay using anti-FLAG antibody and qPCR with primers specific for the *MAO A* promoter region which encompasses the FoxO1-binding site. IgG was used as a negative control for IP. One percent of chromatin before IP was saved as input. Data was presented as the percentage of input from three independent experiments (mean ± S.D., n=3). Ethanol was used as a vehicle for VPA. Representative gels are shown. *, $p<0.05$.

Fig. 7. A schematic diagram of molecular mechanisms of VPA induction of MAO A. FoxO1 acts as a transcriptional repressor of *MAO A* by directly binding to a functional FoxO1-binding site in the *MAO A* promoter. In response to VPA signal, the phosphorylation of both Akt and its downstream FoxO1 increase. Phosphorylated FoxO1 is subjected to nuclear export, which consequently activates *MAO A* transcription.

Figure 1

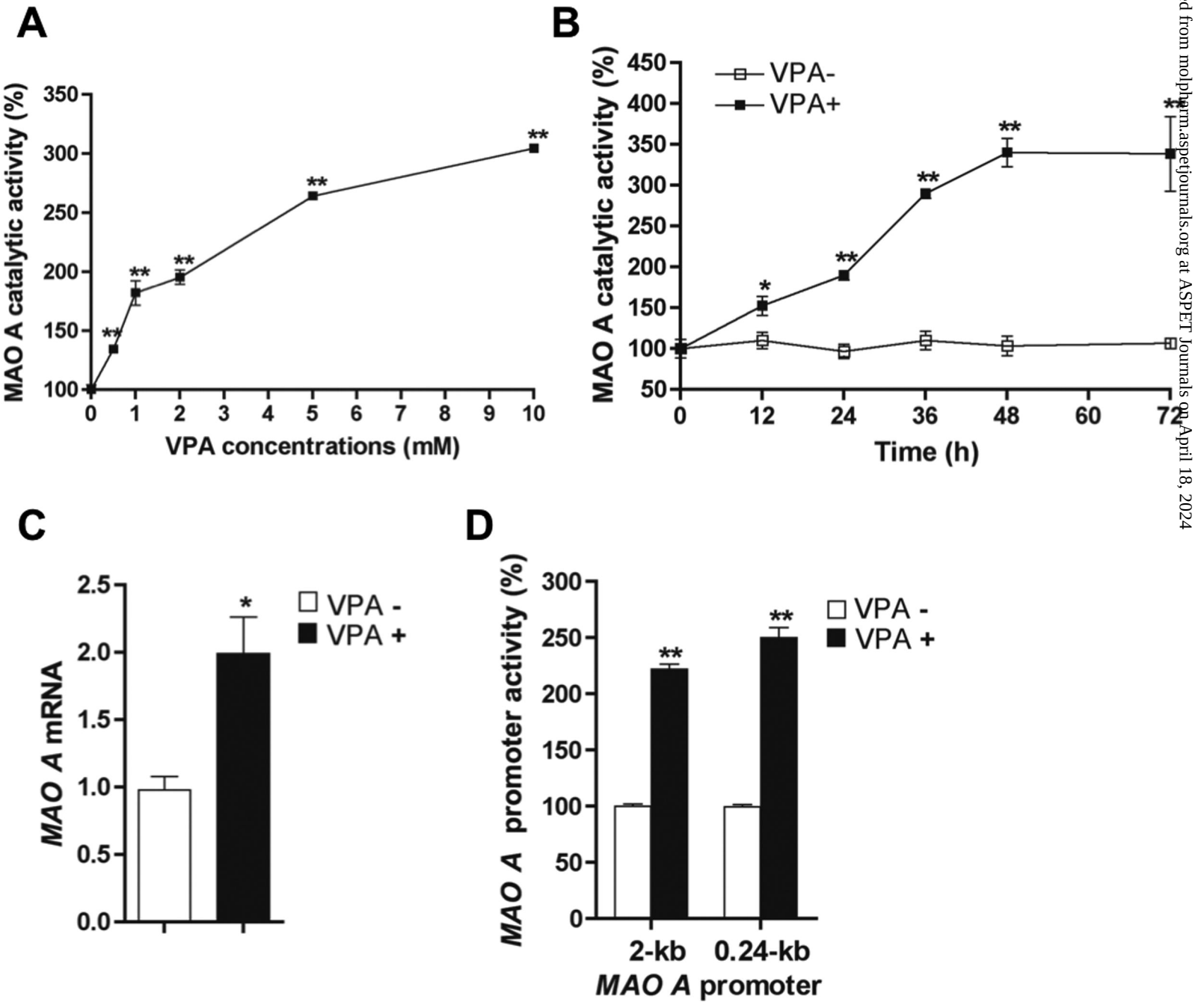

Figure 2

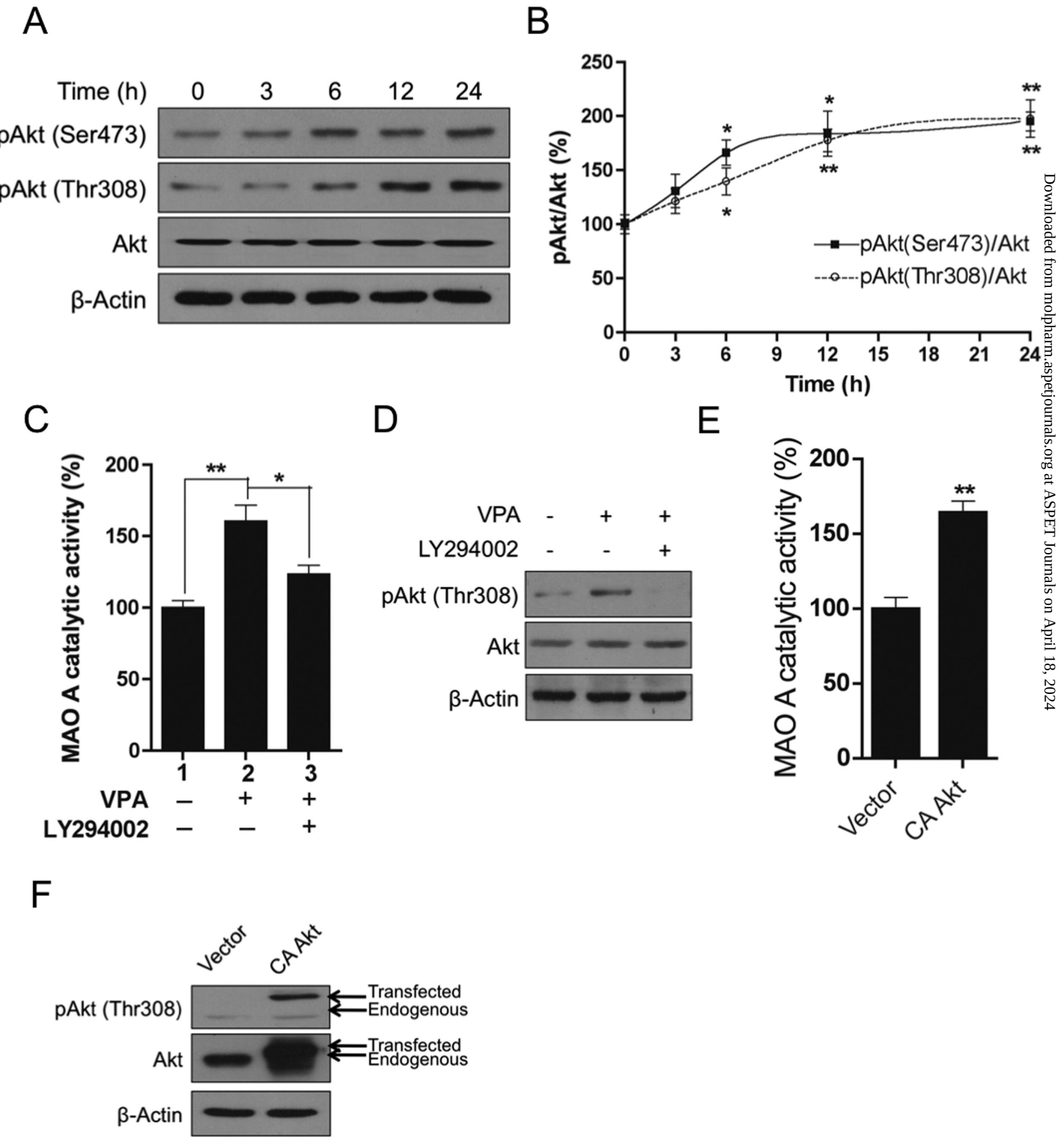

Figure 3

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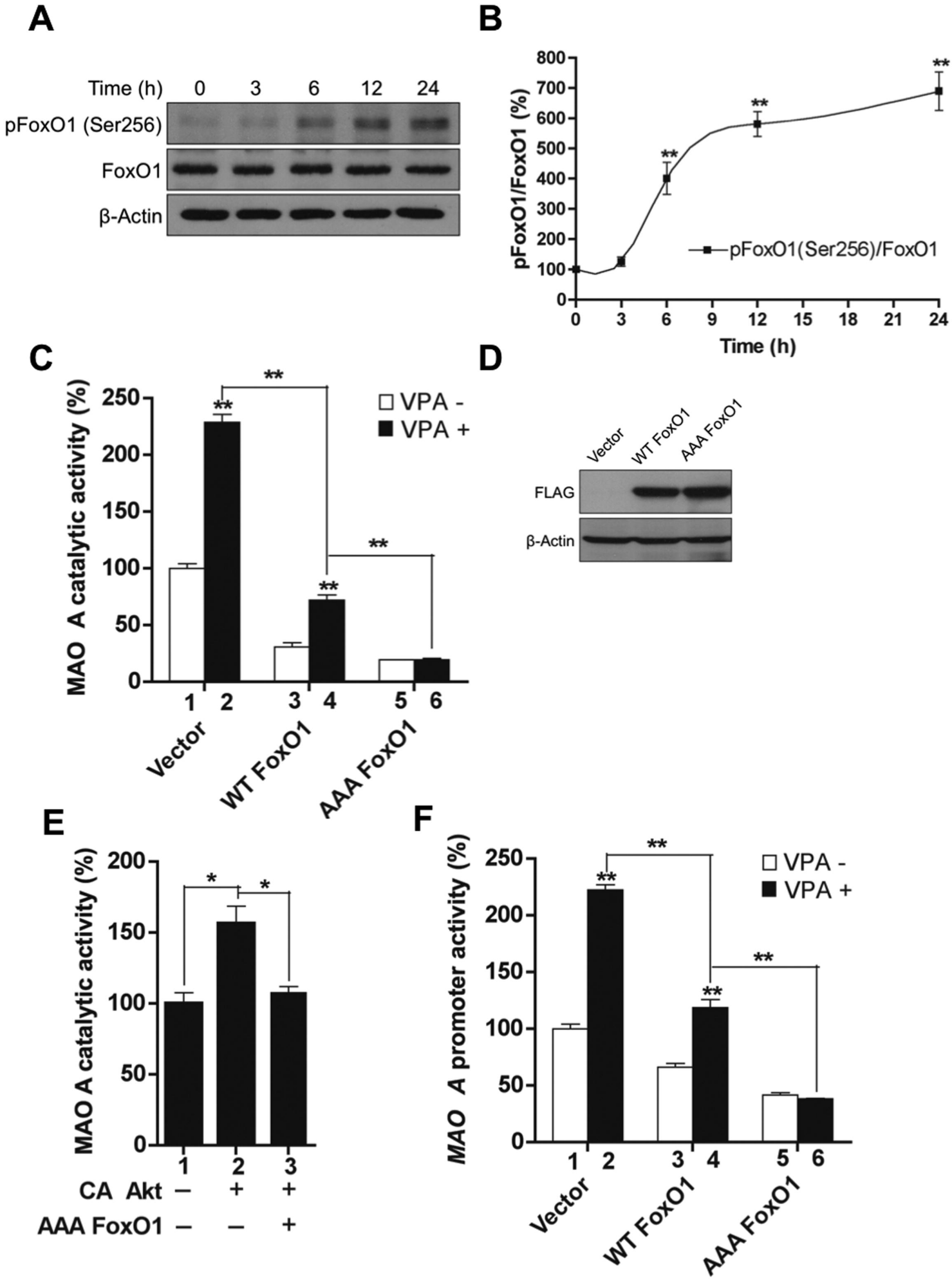


Figure 4

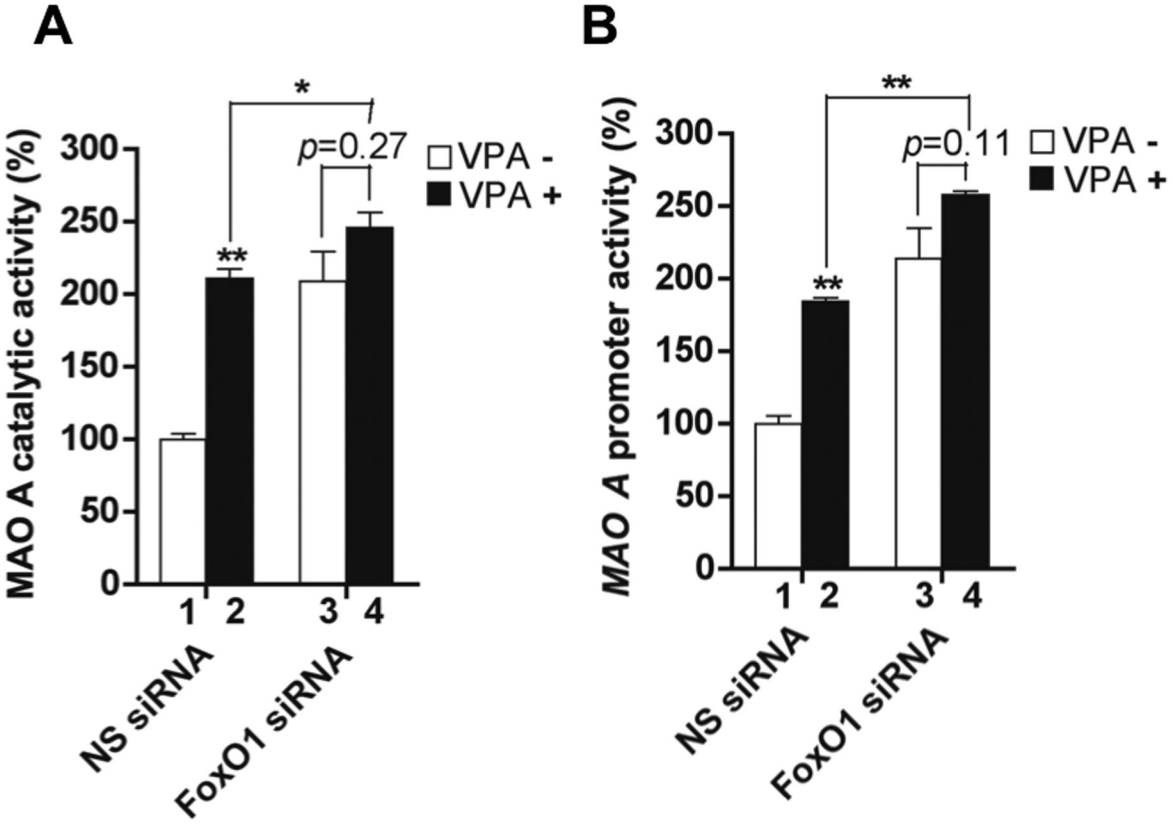
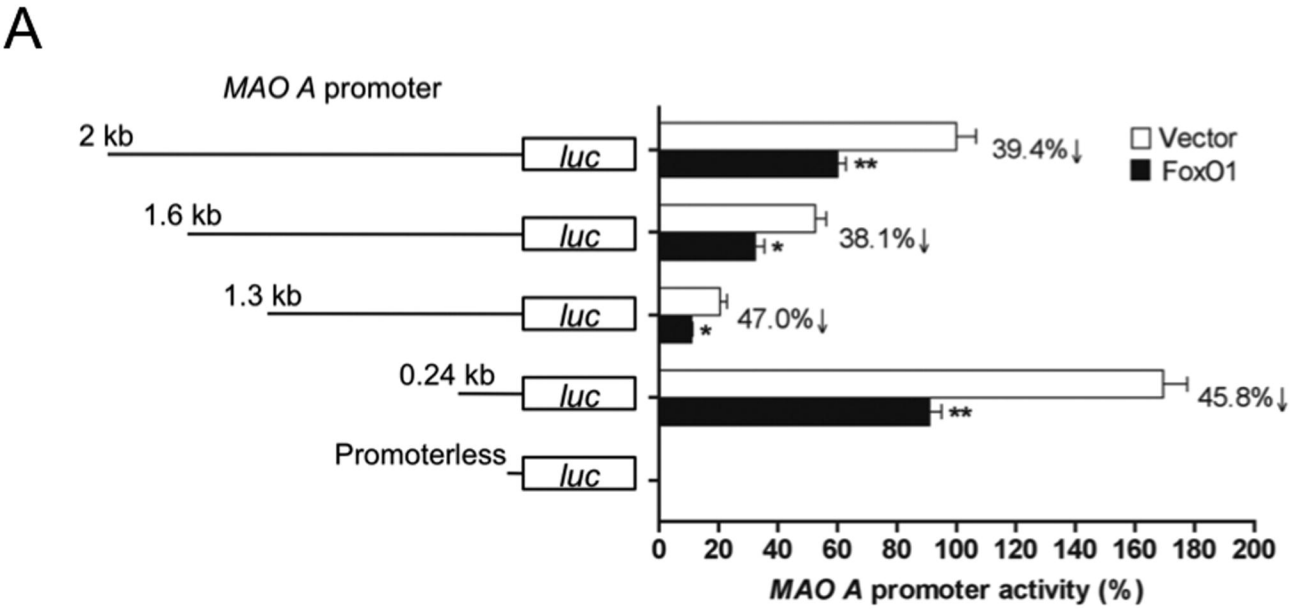
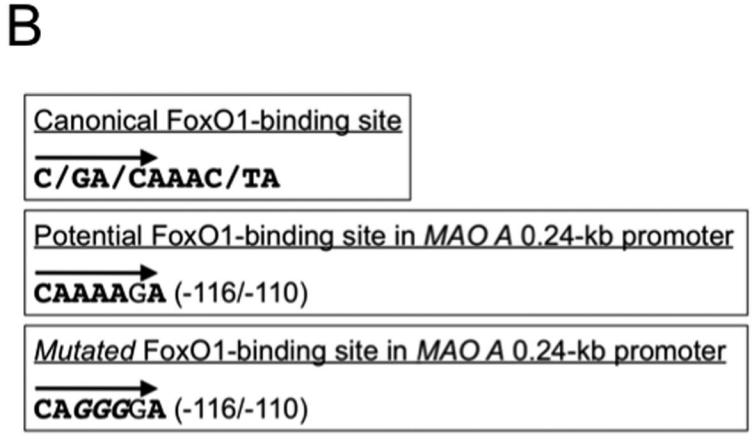
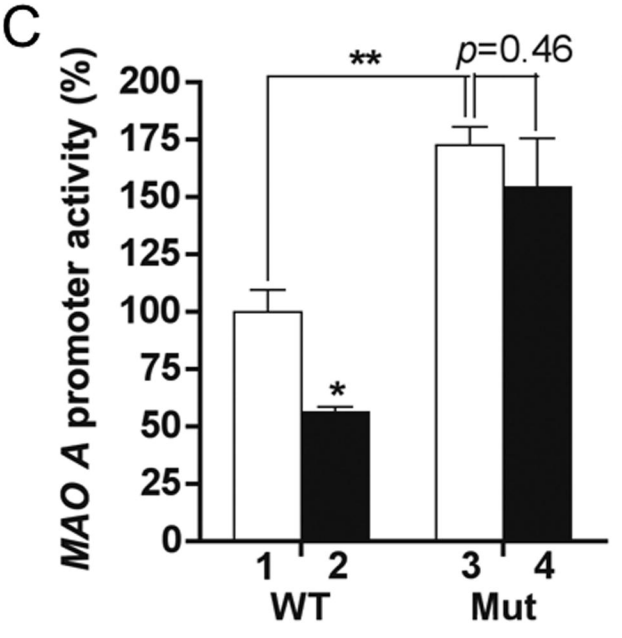


Figure 5







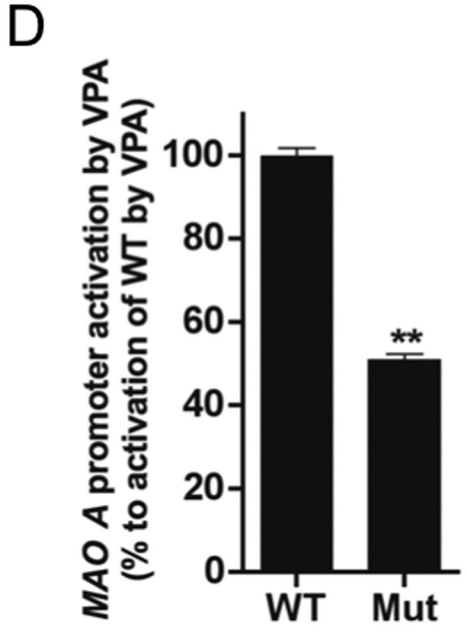
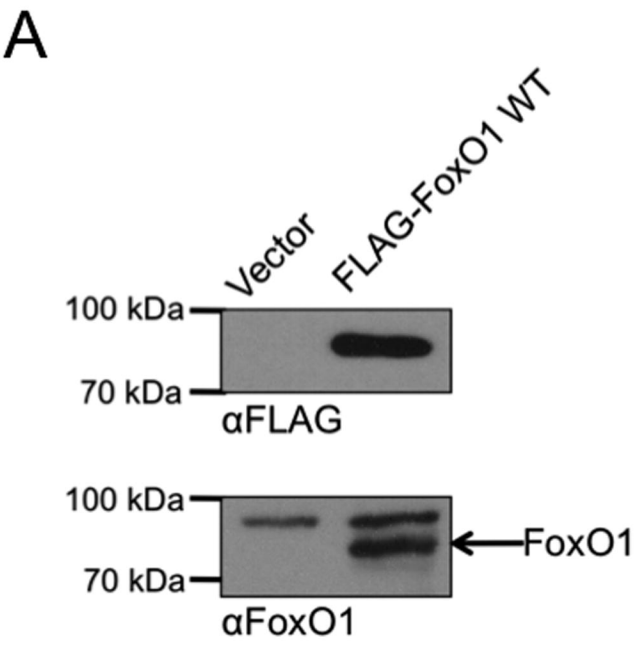
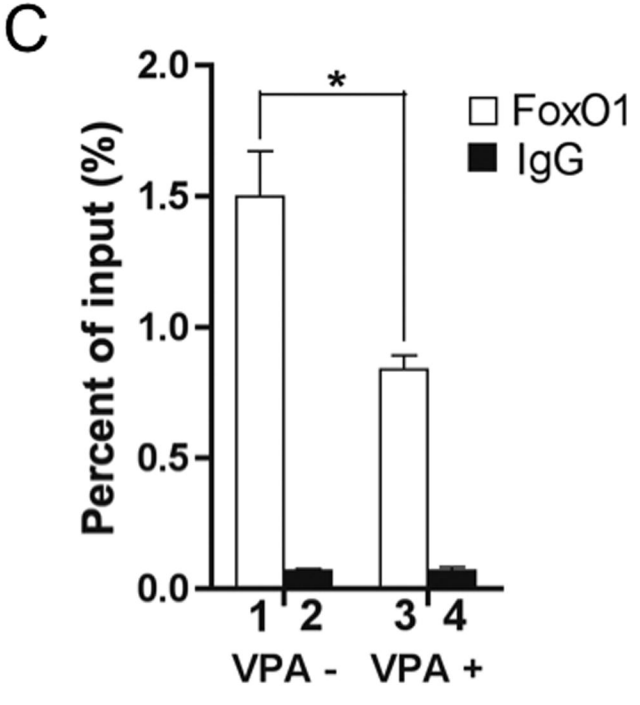
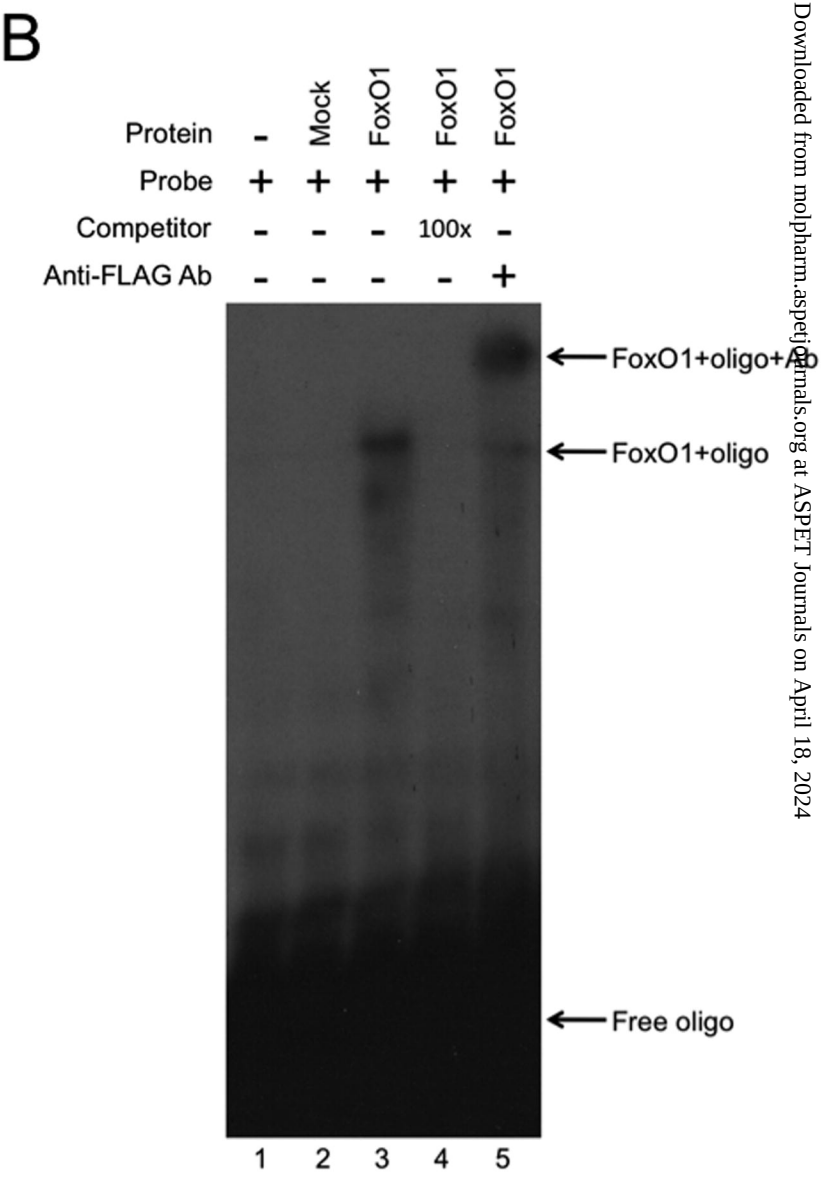


Figure 6







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