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Molecules and Cells

Control of TrkA-Induced Cell Death by JNK Activation and Differential Expression of TrkA upon DNA Damage

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TrkA, a receptor for nerve growth factor, plays a crucial role in neuronal cell growth and differentiation. However, overactivation of TrkA signaling leads to cell death in various cell types. TrkA-mediated cell death shows some similarities to DNA damage-induced cell death. In this study, we examined how TrkA-induced cell death is regulated upon DNA damage. Cytoplasmic expression of TrkA protein was differentially modulated during the camptothecin-induced DNA damage response in TrkA-expressing U2OS cells. TrkA-induced cell death was synergistically increased by DNA damage, but it was blocked in the presence of the JNK inhibitor SP600125. Overexpression of a 54-kDa JNK isoform (JNK1α2) aggravated TrkA-induced cell death and was associated with TrkA functional activation. These results suggest that TrkA shares a functional connection with other mediators in the DNA damage response via JNK signaling.

INTRODUCTION

Nerve growth factor (NGF) is a representative neurotrophin that acts through tropomyosin-related kinase A (TrkA) (Reichardt, 2006; Kaplan and Miller, 2000). TrkA plays an essential role in the regulation of cell survival and death, as well as the induction of neuronal differentiation in response to NGF. TrkA promotes cell survival by suppressing c-Myc- or UV-induced apoptosis (Ulrich et al.,1998). On the contrary, TrkA is also involved in NGF-mediated apoptotic cell death in medulloblastoma and neuroblastoma cells (Chou et al., 2000; Lavoie et al., 2005; Ohta et al., 2006). TrkA-induced cell death is largely associated with its tumor suppressor activity (Lucarelli et al., 1997; Nakagawara, 2001). Tumor suppressor p53 has been implicated in TrkA-induced apoptosis (Lavoie et al., 2005), but the mechanistic details of this process still remain to be investigated.

The c-Jun N-terminal kinases (JNKs) are activated by NGF (Waetzig et al., 2008), suggesting that TrkA activation by NGF is associated with JNK signaling. The JNK family consists of JNK1, JNK2, and JNK3 (Bogoyevitch, 2006). JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 appears to be primarily expressed in the brain (Widmann et al., 1999). Mitogen-activated protein kinase (MAP kinase) kinases MKK4 and MKK7 preferentially phosphorylate JNK1 isoforms at tyrosine-185 and threonine-183, respectively, activating the JNK signaling pathway (Fleming et al., 2000). This JNK activation is mostly associated with a number of cellular stress responses and with apoptosis (Lee et al., 2002; Koo et al., 2002; Tournier et al., 2000; Turner et al., 1998).

The TrkA gene can be alternatively spliced, producing an isoform with a deletion of six amino acids (VSFSPV) in exon 9 (Barker et al., 1993; Tacconelli et al., 2004). We have previously reported that this isoform induces apoptotic cell death in SK-N-MC neuroblastoma cells and more strongly in U2OS osteosarcoma cells (Jung and Kim, 2008; Jung et al., 2008). Interestingly, TrkA-induced cell death was prevented by addition of the JNK inhibitor SP600125 (Takamura et al., 2007). Moreover, γ-H2AX production, which is associated with TrkA-induced cell death, was blocked in the presence of a JNK inhibitor (Jung et al., 2008). These findings suggest that JNK is required for TrkA-induced cell death. In addition, we showed that certain hallmarks of apoptosis, including caspase activation and cleavage of BAX, BAK, and PARP, occur during TrkA-induced cell death (Jung and Kim, 2008; Jung et al., 2008). These results suggest that TrkA-mediated cell death signaling resembles DNA damage-mediated pathways via JNK activation. In this report, we investigated how TrkA expression and its biological function are affected by DNA damaging agents such as camptothecin (Hsiang and Liu, 1988), and we found that TrkA signaling is modulated upon DNA damage through JNK activation.

MATERIALS AND METHODS

Materials

Camptothecin, doxorubicin, and chloroquine were purchased from Sigma. SP600125 was purchased from Calbiochem. Lipofectamine2000 was purchased from Invitrogen. Tetracycline was purchased from Duchefa. Formaldehyde (20%) was purchased from Tousimis. Aqueous mounting media was purchased from Biomeda. Super signal west pico stable peroxide solution was purchased from Pierce. FLUOS-conjugated annexin-V was purchased from Roche. The cell counting kit-8 (CCK-8) was purchased from Dojindo. Dimethyl sulfoxide (DMSO) was purchased from AMRESCO. pcDNA3-Flag-

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Jnk1α2 was obtained from Addgene. Antibodies used in the study were: TrkA (763, Santa Cruz Biotechnology), phospho-TrkA (E6, Santa Cruz Biotechnology), pan-JNK (FL, Santa Cruz Biotechnology), phospho-ERK (Cell Signaling Technology), phospho-JNK (FL, Santa Cruz Biotechnology), PARP (Transduction Laboratories), β-actin (Sigma), secondary goat anti-rabbit/mouse HRP conjugate (Bio-Rad), and anti-rabbit TRITC conjugate (Sigma).

Cell culture and transient transfection

Expression of exogenously transfected TrkA from U2OS-TrkA cells was induced by the addition of 2 μg/ml tetracycline, as previously described (Jung and Kim, 2008; Jung et al., 2008). Cells were maintained in DMEM with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin in a humidified 5% CO_2 incubator at 37°C. Transient transfection experiments using pcDNA3-flag-Jnk1α2 (Addgene) were performed in a 6-well dish using Lipofectamine 2000 reagent (Invitrogen) as described in the manufacturer's instructions.

Western blot analysis

Whole cell proteins were extracted with SDS sample buffer and boiled for 5-10 min at 95°C. The proteins were separated on a 9% SDS-PAGE and transferred to nitrocellulose membranes. The membrane was blocked for 1 h at room temperature in blocking buffer (PBS containing 3% skim milk and 0.1% Tween-20) and then incubated with primary antibodies in blocking buffer at 4°C overnight. The membrane was washed three times with PBST (0.1% Tween-20/PBS) for 15 min and incubated with horseradish peroxidase-conjugated secondary antibodies in blocking buffer for 1-2 h at room temperature. After washing with PBST three times, specific proteins were visualized using the super signal enhanced chemiluminescence (ECL) detection system.

Cytoplasmic fraction isolation

After washing with PBS to remove dead cells, the attached cells were washed with TBS buffer (20 mM Tris [pH 7.5], 100 mM NaCl), resuspended in hypotonic buffer (25 mM Tris [pH 7.4], 1 mM $MgCl_2$, 5 mM KCl) containing 1% NP-40, and incubated for 5 min on ice. Cell lysates were centrifuged at 7,000 rpm for 1 min at 4°C. These supernatants were designated as the cytoplasmic fractions.

Confocal immunofluorescence microscopy

Cells grown in 6-well dishes on cover slides were fixed with 3% formaldehyde/PBS for 45 min and permeabilized with 0.5% Triton X-100/PBS for 5 min. After blocking with 1% BSA/PBS for 1 h, cells were incubated with primary antibodies for 2 h. Cells were washed with PBS three times and incubated with TRITC-labeled secondary antibody for 1 h. The cells were washed with PBS three times and mounted with aqueous mounting media. Protein expression in cells was visualized using a confocal microscope (Olympus FV-500).

Cell counting kit-8 (CCK-8) assay

Cultured cells were split into a 24-well dish (~5×10^4 cells) and incubated in the presence or absence of tetracycline for the indicated time periods. The CCK-8 assay was performed as described by the manufacturer (Dojindo), and cell viability was determined by measuring absorbance at OD_{485} using a CHAMELEON microplate reader (Hidex).

FLUOS-conjugated annexin-V binding assay

After incubation, all cells (floating and attached) were combined, washed with PBS, and suspended in binding buffer (10 mM HEPES [pH 7.4], 140 mM NaCl, and 5 mM $CaCl_2$). After incubation with FLUOS-conjugated annexin-V in binding buffer, cells were analyzed in the FL1-H (Log) parameter in a flow cytometer (FACS Calibur, BD Biotechnology).

RESULTS

DNA damage differentially modulates cytoplasmic expression of two TrkA protein isoforms

To understand the cellular consequences of TrkA activation, we generated TrkA-expressing stable SK-N-MC or U2OS cell lines controlled by the Tet-On system (Jung and Kim, 2008; Jung et al., 2008). In both cell lines, TrkA overexpression resulted in cell death accompanied by caspase-7 and ERK activation, and BAX/BAK cleavage. TrkA-induced cell death was likely related to DNA damage responses based on PARP cleavage, p21 induction, and γH2AX production. After tetracycline induction, two TrkA forms of 100 kDa and 120 kDa appeared in U2OS cells stably expressing exogenous TrkA. Expression of the smaller TrkA protein was increased at 48 h after induction, coinciding with the peak of cell death, while the larger TrkA protein was decreased (data not shown). To investigate how TrkA expression is regulated during DNA damage, we treated U2OS-TrkA cells with camptothecin. Expression of the large TrkA form was decreased whereas that of the small form was increased, indicating differential expression upon conditions leading to DNA damage (Fig. 1A, TrkA panel). As expected, we observed PARP cleavage in the presence of camptothecin in a concentration-dependent manner (Fig. 1A, PARP panel). In addition, we found that TrkA was predominantly localized in the cytoplasm, and a large aggresome was observed near the nucleus (Fig. 1B). These data suggest that TrkA expression and conformation can be modified in the cytoplasm in the presence of DNA damage reagent.

We further investigated how the cytoplasmic TrkA forms are altered upon DNA damage. Consistent with Fig. 1A, the large TrkA form (~120 kDa) was markedly down-regulated after camptothecin treatment, but its expression was increased in the presence of chloroquine, a lysosomal degradation inhibitor (Fig. 1C). These results demonstrate that changes in TrkA expression may be associated with DNA damage signaling and lysosome-dependent protein degradation.

TrkA is functionally activated by DNA damage via JNK signaling

To further understand the role of TrkA in cell death, we explored the functional relationship of the two TrkA forms. Expression of the large TrkA protein was reduced upon DNA damage (Fig. 2A, TrkA panel). We further analyzed how ERK is phosphorylated in the presence of camptothecin and found that ERK was phosphorylated when TrkA was overexpresssed. Similarly, ERK phosphorylation was increased in the presence of the DNA damaging drug (Fig. 2A, phospho-ERK panel). As shown in Fig. 2B, TrkA overexpression itself caused severe cell death in control cells with no DNA damage induction, which was also shown previously (Jung and Kim, 2008; Jung et al., 2008). Viability of TrkA-expressing cells was dramatically reduced in the presence of 0.5 μM camptothecin, suggesting that TrkA function is associated with camptothecin-induced DNA damage. We next examined how DNA damage can modulate TrkA-induced apoptotic cell death using an annexin-V binding assay. Either TrkA overexpression or camptothecin treatment alone induced 16.73% and 12.28% cell death, respectively. The percentage of cell death was 71.51% in the presence of 1

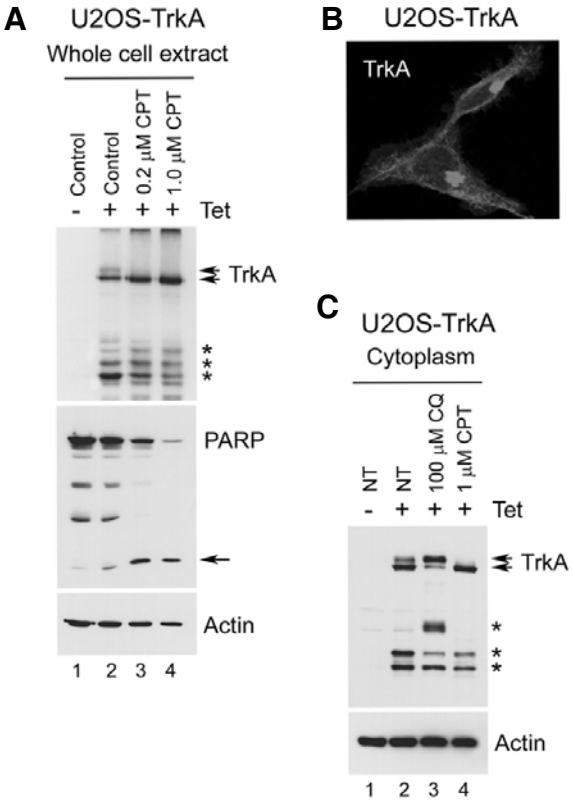

Fig. 1. Differential expression of two TrkA protein isoforms in response to DNA damage. (A) Effect of camptothecin on total TrkA expression. U2OS-TrkA cells were treated with DMSO (control) or the indicated amounts of camptothecin (CPT) in the absence (-) or presence (+) of tetracycline (Tet) for 36 h. Whole cell extracts were analyzed by Western blot analysis using TrkA, PARP, and actin antibodies. *indicates unidentified TrkA breakdown products. Arrow represents cleaved PARP. (B) Intracellular localization of TrkA. TrkA was induced by tetracycline treatment for 16 h in U2OS-TrkA cells, stained with TRITC-labeled antibody and visualized using confocal immunofluorescence microscopy. (C) Effect of chloroquine and camptothecin on cytoplasmic TrkA expression. U2OS-TrkA cells were non-treated (NT) or treated with either chloroquine (CQ, 100 μM) or camptothecin (CPT, 1 μM) in the absence or presence of tetracycline (Tet) for 20 h. The cytoplasmic fraction was isolated from the attached cells as described in "Materials and Methods" and analyzed by Western blot with antibodies against TrkA and actin. *Indicates unidentified TrkA fragments.

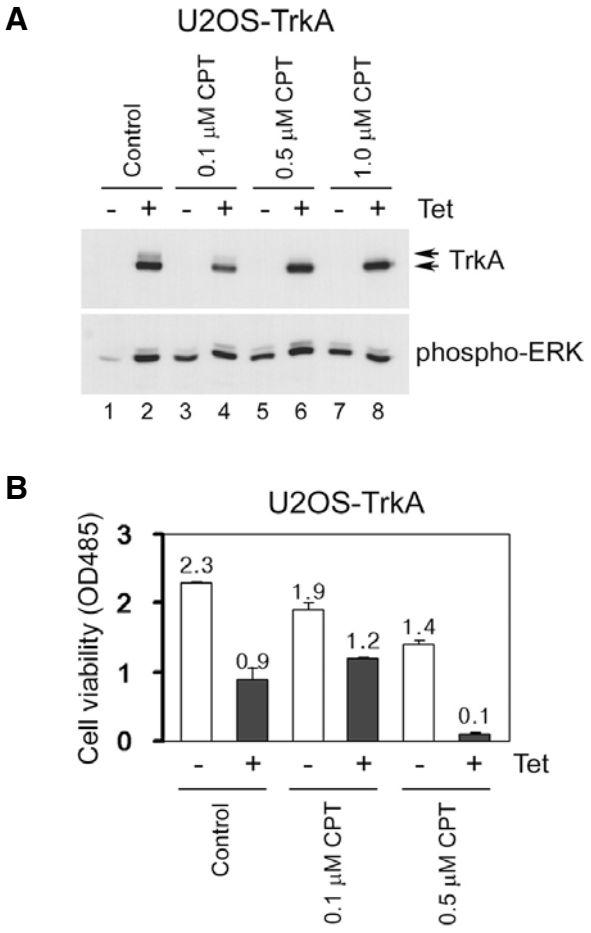

Fig. 2. TrkA expression and cell viability are dependent on camptothecin. (A) Reduction of the large TrkA isoform by camptothecin. U2OS-TrkA cells were treated with DMSO or the indicated amounts of camptothecin in the absence or presence of tetracycline for 24 h. Western blot analysis was performed on the whole cell extracts using TrkA and phospho-ERK antibodies. (B) Cell viability of TrkA-expressing cells in the presence of camptothecin. U2OS-TrkA cells were treated with DMSO or the indicated amounts of camptothecin in the absence or presence of tetracycline for 48 h. Cell viability was determined at OD_{485} by CCK-8 assay using a CHAMELEON microplate reader.

μM camptothecin (Figs. 3B-3D), suggesting a synergistic effect of TrkA and DNA damage. However, the levels of cell death fell to 29.81% with the addition of the JNK inhibitor SP600125 and to 28.08% with the addition of the TrkA inhibitor GW441756 (Figs. 3E and 3F). These results indicate that JNK is essential for TrkA functional activation in response to DNA damage.

JNK is required for the modulation of TrkA expression by DNA damage

We observed that expression of two TrkA forms was modulated by DNA damage, and DNA damage-induced cell death in TrkA-expressing cells was suppressed by the addition of a JNK inhibitor. We next examined whether expression of two TrkA forms might be regulated by JNK signaling by addition of SP600125 in the absence or presence of DNA damage. TrkA activation stimulated JNK and ERK phosphorylation (Fig. 4A), and activated TrkA signaling was blocked by SP600125, resulting in the suppression of TrkA, ERK, and JNK phosphorylation (Fig. 4A). Interestingly, expression of the large form of TrkA (~120 kDa) was significantly less at 48 h than at 12 h (compare Figs. 4A and 4B, lane 2 TrkA panels). Since the peak of TrkA-induced cell death occurred at 48 h after tetracycline treatment, these results indicate that TrkA expression is modulated in a cell death-dependent manner. Expression of the large form of TrkA was further decreased by doxorubicin-mediated DNA damage, but it was restored by addition of SP600125, suggesting that JNK activity is required for TrkA modulation upon DNA damage (Fig. 4B).

To further investigate the role of JNK on TrkA-induced cell death, we examined how exogenously transfected JNK1α2 affects cell death in TrkA-expressing cells. We found that JNK1α2 expression increased the ability of TrkA to reduce cell

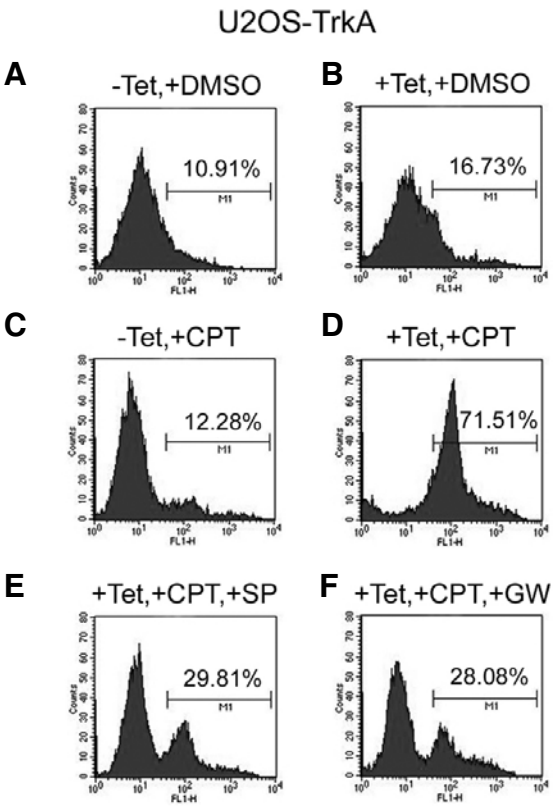

Fig. 3. DNA damage enhances TrkA-induced apoptotic cell death. U2OS-TrkA cells were treated with DMSO, camptothecin (CPT, 1 μM), SP600125 (SP, 2 μg/ml), or GW441756 (GW, 2 μM) in the absence or presence of tetracycline for 48 h, and analyzed using the FLUOS-conjugated annexin-V binding assay via flow cytometry. The arbitrary gate indicates annexin-V positive cells.

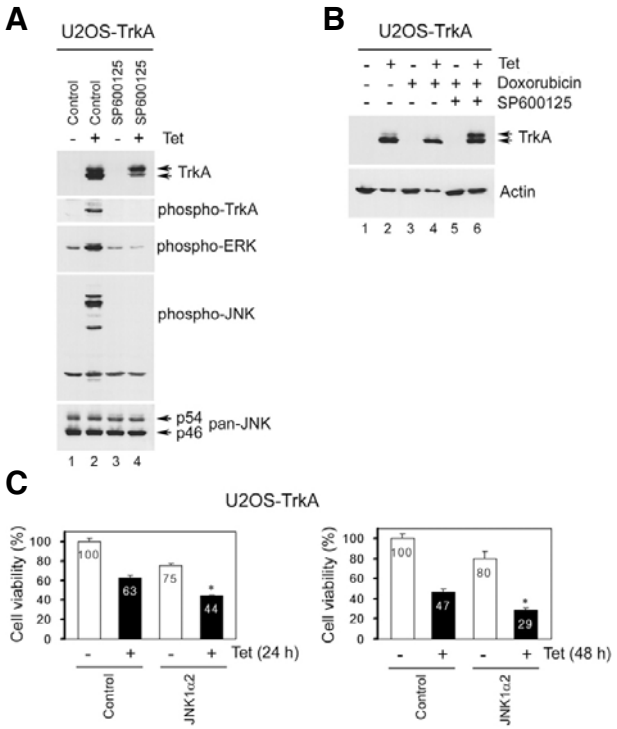

Fig. 4. Modulation of TrkA expression by JNK inhibitor upon DNA damage. (A) Effect of JNK inhibitor on TrkA expression. U2OS-TrkA cells were treated with tetracycline in the absence or presence of SP600125 (2 μg/ml) for 12 h. Protein expression was detected by Western blot using the indicated specific antibodies. (B) Effect of SP600125 on DNA damage-mediated TrkA modulation. U2OS-TrkA cells were uninduced or induced with tetracycline for 12 h and subsequently treated with doxorubicin (0.5 μg/ml) or SP600125 (2 μg/ml) for 36 h in the presence of tetracycline. Proteins were detected by Western blot analysis. (C) Effect of exogenously-expressed JNK1α2 protein on TrkA-induced cell death. pcDNA3 (control) and pcDNA3-Flag-JNK1α2 plasmids (2 μg each) were transfected into U2OS-TrkA and cultured in the absence or presence of tetracycline for 24 or 48 h. Cell viability was determined using the CCK-8 assay reagent. The average percentage of cell viability from triplicate tests is reported; bars, SD. *Represents $p < 0.05$ compared to control.

viability at both 24 h (63% to 44%) and 48 h (47% to 29%) (Fig. 4C), suggesting that functional activation of JNK1α2 is associated with TrkA-induced cell death.

DISCUSSION

TrkA is an intrinsic receptor for NGF that plays an important role in neuronal cell growth and differentiation. Depletion of NGF or overactivation of TrkA leads to massive cell death. To investigate the mechanism of TrkA-induced cell death, we examined the regulation of TrkA expression upon DNA damage. We observed two different TrkA forms (100 and 120 kDa) that were overexpressed in U2OS-TrkA cells and were differentially regulated depending on cellular conditions. The large TrkA protein was down-regulated upon cell death, and was further down-regulated under conditions leading to DNA damage. This suggests that expression of the two TrkA proteins could be modulated by downstream effect(s) of a DNA damage signaling cascade. These two TrkA proteins have also observed in previous reports. Although their molecular sizes are slightly different, two forms of exogenous TrkA (110 kDa, $gp110^{trkA}$ and 140 kDa, $gp140^{trkA}$) are post-translationally modified by glycosylation in PC12 cells (Jullien et al., 2002; Zhou et al., 1995). When TrkA is activated by NGF, $gp140^{trkA}$ expression is decreased (Jullien et al., 2002; Zhou et al., 1995) and its localization changes from the plasma membrane to intracellular compartments including the endosome, lysosome, and Golgi (Saxena et al., 2005a., 2005b; Zhang et al., 2000; 2009). In our study, we observed that exogenously expressed and activated TrkA was cytoplasmically localized, forming a large aggresome near the nucleus. These data suggest that the intracellular localization of TrkA is dependent on its activation, leading to apoptotic cell death as we have previously shown (Jung and Kim, 2008; Jung et al., 2008).

JNK is a member of the mitogen-activated protein (MAP) family and is primarily activated by cytokines (e.g., IL-1, TNF-α), environmental stress (e.g., UV, γ-radiation, reactive oxygen species) (Koo et al., 2002; Lee et al., 2002) or growth hormone receptors (e.g., EGF, PDGF). A previous report has shown that TrkA induces Schwann cell migration in response to neurotrophin 3 through JNK signaling (Yamauchi et al., 2003). Activation of p75NTR, a TNF-family neurotrophin receptor, causes cell death by a typical JNK-p53-BAX pathway (Volosin et al., 2006). However, little is known about the direct functional interactions between TrkA and JNK. In this report, we showed that differential expression of two TrkA proteins is functionally asso-

ciated with JNK activation in response to DNA damage (Figs. 3 and 4). Our results also suggest that JNK1α2 is a critical target in the TrkA-induced cell death signaling pathway. According to our previous data (Jung et al., 2008), TrkA-mediated cell death has similar hallmarks to cell death caused by DNA damage, including BAK/BAX activation, PARP cleavage by caspase activation, and γ-H2AX production. DNA damage-induced cell death is synergistically enhanced by TrkA activation, suggesting that TrkA could be a potent chemotherapeutic target of DNA damage reagents. However, the molecular details of TrkA-induced cell death associated with DNA damage will continue to be investigated.

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