

S-nitrosylation of B23/nucleophosmin by GAPDH protects cells from the SIAH1–GAPDH death cascade

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B23/nucleophosmin is a multifunctional protein that participates in cell survival signaling by shuttling between the nucleolus/nucleoplasm and nucleus/cytoplasm. In this paper, we report a novel neuroprotective function of B23 through regulation of the SIAH1–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) death cascade. B23 physiologically bound to both SIAH1 and GAPDH, disrupting the SIAH1–GAPDH complex in the nucleus in response to nitrosative stress. S-nitrosylation of B23 at cysteine 275 by trans-nitrosylation from GAPDH

dramatically reduced the interaction between SIAH1 and GAPDH. S-nitrosylation of B23 enhanced B23–SIAH1 binding and mediated the neuroprotective actions of B23 by abrogating the E3 ligase activity of SIAH1. In mice, overexpression of B23 notably inhibited *N*-methyl-D-aspartate-mediated neurotoxicity, whereas expression of the C275S mutant, which is defective in binding to SIAH1, did not prevent neurotoxicity. Thus, B23 regulates neuronal survival by preventing SIAH1–GAPDH death signaling under stress-induced conditions in the brain.

Introduction

B23/nucleophosmin is mainly localizes in nucleoli, but it shuttles between the nucleolus/nucleoplasm and nucleus/cytoplasm and exhibits diverse functions including ribosome biogenesis, cell proliferation, and cell survival (Borer et al., 1989; Wu and Yung, 2002; Itahana et al., 2003; Rubbi and Milner, 2003). Moreover, unsumoylated B23 (Liu et al., 2007) and a mutant form of B23 that is not able to bind to ATP (Choi et al., 2008) are accumulated in the nucleoplasm, supporting the notion that B23 trafficking may be critical for its cellular functions. Recently, we proposed B23 as a neuronal survival factor that forms a complex with nuclear phosphatidylinositol 3,4,5-triphosphate and caspase-activated DNase, inhibiting DNA fragmentation in the nucleus of neuronal cells (Ahn et al., 2005). In addition, B23 interacts with nuclear Akt and enhances its protein stability, thereby promoting cell survival (Lee et al., 2008b). Besides its role in neuronal survival signaling, B23 is involved in regulating the cellular distribution of its binding partners. For example, B23 binds to the tumor suppressor p19^{ARF} and targets ARF in the nucleoli, thereby inhibiting its function

(Korgaonkar et al., 2005), and also interacts with HDM2, an E3 ligase of p53, and abrogates its nucleolar residency, thus protecting p53 from HDM2-mediated degradation (Kurki et al., 2004).

Diverse apoptotic stimuli activate nitric oxide (NO) synthase and generate NO. S-nitrosylation of protein by NO is a crucial mode of regulation for many cellular proteins, including the nuclear proteins HDAC2 (histone deacetylase 2) and PARP1 (Yu et al., 2006; Nott et al., 2008). S-nitrosylated glyceraldehyde-3-phosphate dehydrogenase (GAPDH) binds to SIAH1 (seven in absentia homologue), which contains a nuclear localization signal and conveys S-nitrosylated GAPDH (SNO-GAPDH) into the nucleus. The nuclear GAPDH–SIAH1 complex stabilizes SIAH1 and enhances its E3 ligase activity, thereby causing neuronal cell death (Hara et al., 2005). In addition, SNO-GAPDH has been considered as a nitrosylase for nuclear proteins such as HDAC2 and DNA-activated protein kinase through trans-nitrosylation (Kornberg et al., 2010).

In the present study, we demonstrate that B23 is a novel binding partner of the nuclear GAPDH–SIAH1 complex.

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Abbreviations used in this paper: ANOVA, analysis of variance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSNO, S-nitroso-glutathione; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; shRNA, short hairpin RNA; WT, wild type.

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S-nitrosylation of B23 occurs by trans-nitrosylation from GAPDH and elicits robust binding of B23 to SIAH1, thus disrupting the interaction between SIAH1 and GAPDH. In intact mice and cultured neurons, nitrosylation of B23 by the NO donor S-nitroso-glutathione (GSNO) or the glutamate derivative *N*-methyl-D-aspartate (NMDA) prevented neurotoxicity, whereas expression of the B23 C275S mutant, which is not nitrosylated and cannot bind to SIAH1, or knockdown of B23 failed to inhibit neuronal cell death. These data suggest that B23 impairs the GAPDH–SIAH1 death cascade in the brain that is induced upon cellular stresses, such as NO, by replacing GAPDH as a binding partner for SIAH1 and suppressing the ligase activity of SIAH1, thus contributing to neuronal survival.

Results

B23 associates with the SIAH1–GAPDH complex

Proteomic analyses to search for binding partners of B23 identified both SIAH1 and GAPDH as potential binding partners. Using immunoprecipitation analysis, we verified specific interactions between GAPDH and B23 and between SIAH1 and B23 (Fig. 1, A and B). To ascertain the specificity of the binding, we performed the *in vitro* binding assay with intact forms of purified GAPDH and B23 or purified SIAH1 and B23 (Fig. S1 A). In intact cells, endogenous B23 also bound to endogenous GAPDH and endogenous SIAH1 (Fig. 1 C). *In vitro* binding assays with a series of B23 deletion mutants, expressed as GST fusions, demonstrated that the central region of B23 that contains the acidic clusters is required for interaction with both SIAH1 and GAPDH and that the N-terminal region is also involved in the interaction with GAPDH but not required for SIAH1 interaction (Fig. 1 D).

Because SIAH1 forms a complex with GAPDH under nitrosative stress condition and conveys GAPDH into the nucleus where this complex mediates cell death, we hypothesized that B23 probably interferes with SIAH1–GAPDH binding in the nucleus when the cell is exposed on nitrosative stress to prevent cell death. To test this hypothesis, we determined whether the interaction between B23–SIAH1 and B23–GAPDH occurs in the nucleus. Immunofluorescence staining of GFP-B23–FLAG-SIAH1 showed that SIAH1 localizes to both the cytoplasm and nucleus with some accumulation in the nucleolus and revealed strong colocalization with B23 in the nucleus, especially in the present of GSNO treatment as a NO stressor, consistent with the previous finding that SIAH1 shuttles between nucleus and cytoplasm under certain conditions because it possesses nuclear localization signal (Fig. 1 E and Fig. S1 B). Immunostaining of GFP-B23–MYC-GAPDH cotransfected cells revealed that GAPDH was localized predominantly in the cytoplasm, whereas B23 was mainly localized in the nucleus. However, in the nitrosative stress condition with GSNO treatment, GAPDH translocated into the nucleus and showed colocalization with B23 (Fig. 1 F and Fig. S1 C). Hence, our data suggest that the interaction between GAPDH and B23 probably occurs in the nucleus after formation of the SIAH1–GAPDH complex and its translocation into the nucleus.

S-nitrosylation of B23 is mediated by GAPDH

S-nitrosylation is known to be a major signaling mechanism for NO (Hess et al., 2005). It occurs at cysteine residues of many proteins in the presence of NO and has been shown to affect protein–protein interactions (Schonhoff et al., 2002; Matsumoto et al., 2003). It has also been reported that S-nitrosylation of GAPDH is required for its binding to, and stabilization of, SIAH1 (Hara et al., 2005). In PC12 cells treated with GSNO, the association between SIAH1 and B23 was markedly enhanced to a level similar to the interaction of SIAH1 and GAPDH (Fig. 2 A, first and second blots). This led us to test whether the action of NO involves B23. Surprisingly, a biotin switching assay showed obvious S-nitrosylation of endogenous B23 in cultured PC12 cells after GSNO treatment, revealing that S-nitrosylation of B23 is increased with increased expression of B23 (Fig. 2 B, first blot). Notably, increased S-nitrosylation of B23 elicited a detectable diminution of endogenous SNO-GAPDH in a B23 dose-dependent manner, raising the possibility that S-nitrosylation of B23 might reflect trans-nitrosylation from GAPDH (Fig. 2 B, second blot).

As previous studies have shown that SNO-GAPDH trans-nitrosylates nuclear proteins, such as SIRT-1, HDAC2, and DNA-activated protein kinase (Kornberg et al., 2010), and B23 is a nuclear signaling protein that plays a critical role in neuronal survival (Ahn et al., 2005), we tested whether SNO-GAPDH can transfer its NO group to B23 via a protein–protein trans-nitrosylation reaction. Indeed, a direct *in vitro* S-nitrosylation exchange assay using purified GST-B23 and purified GST-GAPDH. To induce S-nitrosylation of GAPDH, purified GST-GAPDH was exposed to GSNO and desalted to remove excess NO donor. The incubation of SNO-GAPDH with purified B23 followed by a biotin switching assay demonstrated obvious S-nitrosylation of B23 (Fig. 2 C). To ensure B23 trans-nitrosylation from SNO-GAPDH, we conducted a direct trans-nitrosylation assay with purified GST-B23 and GST-GAPDH that had eliminated its S-nitrosylation. Purified GST-GAPDH was preincubated with GSNO followed by exposure to DTT to inhibit GSNO-mediated nitrosylation. Consistent with the aforementioned experiment (Fig. 2 C), the incubation of SNO-GAPDH without exposure of DTT with purified B23 led to B23 nitrosylation. However, GST-GAPDH that has been suppressed to be nitrosylated by the exposure to DTT did not exert B23 nitrosylation (Fig. S2 A). This was supported by the demonstration that knockdown of GAPDH in PC12 cells with or without the GSNO did not result in S-nitrosylation of B23 (Fig. 2 D). Moreover, a C150S mutant of MYC-GAPDH that cannot be S-nitrosylated abrogated endogenous B23 nitrosylation, further supporting the notion that B23 nitrosylation is mediated by SNO-GAPDH (Fig. 2 E).

The B23 protein contains three cysteine residues (Cys 21, 104, and 275). Mutational analysis identified C275 as the single S-nitrosylation site; mutation of this site to serine abolished the biotin switch signal while GAPDH was successfully nitrosylated, whereas B23 C21S and C104S mutants were robustly nitrosylated. Consistent with our previous findings, S-nitrosylation of GAPDH is drastically diminished upon B23

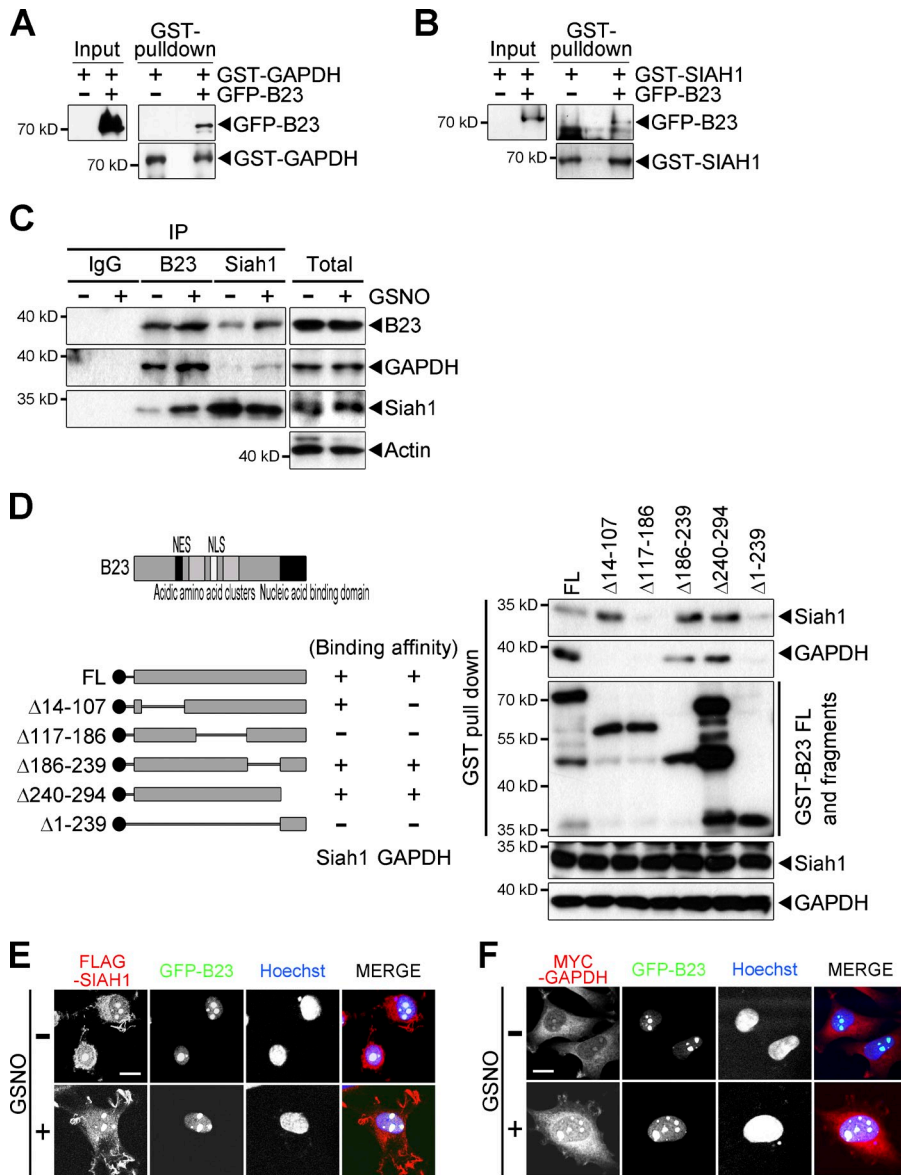


Figure 1. B23 associates with the SIAH1-GAPDH complex. (A) GST pull-down assay. GFP-B23 interacts with purified GST-SIAH1 protein. (B) GST pull-down assay. GFP-B23 interacts with purified GST-GAPDH protein. (C) Endogenous proteins (B23 and SIAH1) were coimmunoprecipitated with GAPDH. (D) SIAH1 and GAPDH bind to B23 domains. A schematic diagram of B23 fragments is shown on the left. SIAH1 interacted with the 117–186-amino acid peptide of B23, whereas GAPDH interacted with the 14–186-amino acid peptide of B23. (E and F) Cellular localization of GFP-B23, FLAG-SIAH1 (E), or MYC-GAPDH (F) in PC12 cells. Cells were transfected with GFP-B23 and FLAG-SIAH1 or MYC-GAPDH. After 24 h, cells were exposed to 200 μ M GSNO for 18 h. FLAG or MYC tags were immunostained with anti-mouse-Alexa Fluor 594 and visualized using a confocal laser microscope (LSM 510; Carl Zeiss). The nucleus was counterstained with Hoechst. FL, full length; IP, immunoprecipitation; NES, nuclear export signal. Bars, 10 μ m.

nitrosylation, supporting the notion that SNO-GAPDH is the physiological source of NO for B23 (Fig. 2 F). Our *in vitro* trans-nitrosylation analysis demonstrated that B23 C275S mutant cannot be trans-nitrosylated from either GST-GAPDH wild type (WT) or GAPDH C150S mutant, whereas B23 WT is nitrosylated from GAPDH WT but not by the GAPDH C150S mutant form, further confirming that B23 can be nitrosylated on C275 residue through trans-nitrosylation from SNO-GAPDH (Fig. 2 G). As GAPDH-SIAH1 is translocated into the nucleus and B23 is obviously redistributed from the nucleolus to the nucleoplasm upon GSNO treatment, B23 WT colocalized with both SIAH1 and GAPDH in the nucleoplasm and nucleolus. However, the B23 C275S mutant, which cannot be S-nitrosylated, seemed to be trapped in the nucleolus regardless of GSNO treatment and disrupted the nuclear distribution of SIAH1 and GAPDH (Fig. 2 H and Fig. S2, B and C). These findings suggest that failure of B23 S-nitrosylation blocks the shuttling of B23 between the nucleoplasm and nucleus, confining B23 to the nucleolus, and alters the intranuclear distribution

of SIAH1 and GAPDH, suppressing their nucleoplasmic residency while increasing nucleolar staining. It is likely that confining B23 in the nucleolus impedes its ability to serve as a nuclear NO acceptor in response to cellular stress, such as NO. Therefore, the GAPDH-SIAH1 complex accumulates in the nucleolus.

B23 interferes with the association of GAPDH and SIAH1

The GAPDH-SIAH1 interaction is augmented by S-nitrosylation of GAPDH. Because B23 can be S-nitrosylated and is able to bind to SIAH1, we wondered whether S-nitrosylation of B23 is important for its interaction with SIAH1. Overexpression of B23 WT with FLAG-SIAH1 demonstrated a low level of interaction under basal conditions that was markedly enhanced in the presence of GSNO. In contrast, an overexpressed B23 C275S mutant did not bind to SIAH1 regardless of GSNO treatment, indicating that S-nitrosylation of B23 is crucial for its binding to SIAH1 (Fig. 3 A).

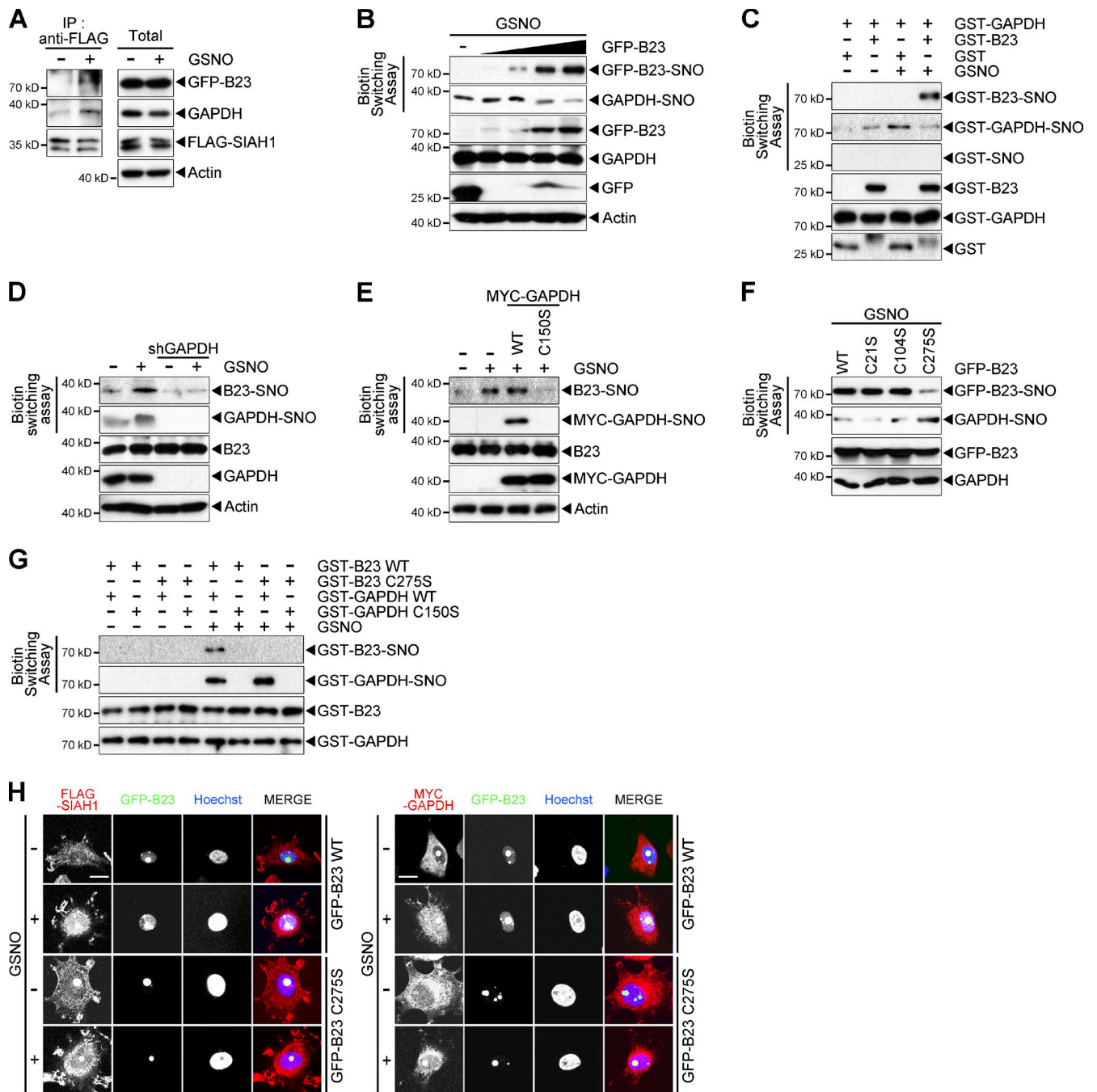


Figure 2. S-nitrosylation of B23 is mediated by GAPDH. (A) The interaction between B23 and GAPDH was affected by GSNO. Cells were treated with GSNO, an NO donor, at a final concentration of 200 μ M for 18 h. Total cell lysates were immunoprecipitated with anti-FLAG and immunoblotted with the indicated antibodies. (B) Biotin switching assay. Increased B23 protein expression affects the S-nitrosylation of GAPDH. Overexpression of B23 decreased levels of SNO-GAPDH and increased levels of SNO-B23. (C) Trans-nitrosylation and biotin switching assay. SNO of B23 originated from SNO-GAPDH. GST-B23 and GST-GAPDH were purified from an *Escherichia coli* expression system. GST-GAPDH was reacted with GSNO and desalted to remove GSNO from the buffer. GST-B23 was incubated with S-nitrosylated or non-S-nitrosylated GAPDH and subjected to the biotin switching assay. (D) Biotin switching assay. PC12 cells were transfected with pGE1/shRNA GAPDH or control. Depletion of GAPDH decreased the S-nitrosylation of B23. (E) Biotin switching assay. PC12 cells were transfected with the GAPDH WT or C150S mutant. Mutagenesis of GAPDH to decrease its S-nitrosylation affected the S-nitrosylation of B23. (F) Biotin switching assay. C275 of B23 is a major S-nitrosylation site. Mutation of C275 inhibited S-nitrosylation of B23 and restored the SNO-GAPDH level compared with the WT and other B23 mutants (C21S and C104S). (G) Trans-nitrosylation and biotin switching assay. GST-B23 (WT and C275S mutant) and GST-GAPDH (WT and C150S) were purified from an *E. coli* expression system. GST-GAPDH was reacted with GSNO and desalted to remove GSNO from the buffer. GST-B23 was incubated with prepared GST-GAPDH and subjected to the biotin switching assay. (H) Localization of B23, GAPDH, and SIAH1 in PC12 cells. Cells were transfected with GFP-B23 and FLAG-SIAH1 or MYC-GAPDH. After 24 h, cells were exposed to 200 μ M GSNO for 18 h. FLAG or MYC tags were immunostained with anti-mouse-Alexa Fluor 594 and visualized using a confocal laser microscope (LSM 510). The nucleus was counterstained with Hoechst. IP, immunoprecipitation. Bars, 10 μ m.

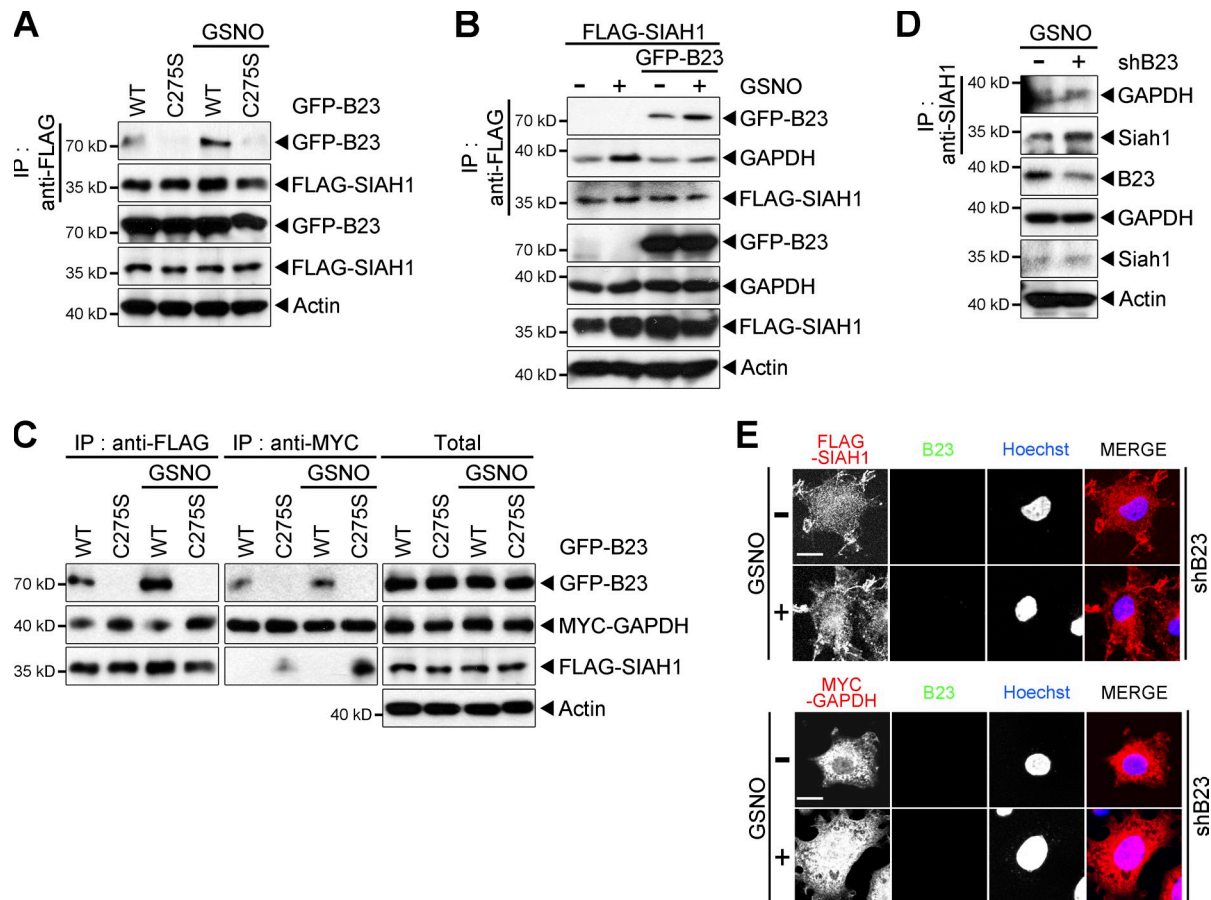


Figure 3. B23 interferes with the association of GAPDH and SIAH1. (A) Mutagenesis of B23 at C275 removes its binding affinity for SIAH1. (B) B23 overexpression disrupts the interaction between SIAH1 and GAPDH. PC12 cells were transfected with FLAG-SIAH1 with or without GFP-B23. Cell lysates were immunoprecipitated with anti-FLAG antibody and analyzed by Western blotting. The binding between B23 and SIAH1 was stronger than that between SIAH1 and GAPDH. (C) B23 WT, but not B23 C275S, binds to SIAH1 and GAPDH to prevent the interaction between SIAH1 and GAPDH. (D) Depletion of B23 augments SIAH1 and GAPDH binding in PC12 cells after GSNO exposure. Cell extracts were immunoprecipitated with anti-SIAH1 antibody and analyzed by Western blotting. (E) Localization of B23, GAPDH, and SIAH1 in PC12 cells after B23 depletion and GSNO exposure. Cells were transfected with shB23 and FLAG-SIAH1 or MYC-GAPDH. After 48 h, cells were exposed to 200 μ M GSNO for 18 h. FLAG or MYC tags were immunostained with anti-mouse-Alexa Fluor 594. B23 was immunostained with anti-rabbit-Alexa Fluor 488 and visualized with a confocal laser microscope (LSM 510). The nucleus was counterstained with Hoechst. IP, immunoprecipitation. Bars, 10 μ m.

Moreover, knockdown of endogenous B23 enhanced the interaction between GAPDH and SIAH1 under basal conditions (Fig. S3 A). As we previously demonstrated that B23 is trans-nitrosylated by SNO-GAPDH, we tested whether B23 competes with GAPDH for binding to SIAH1. The binding of endogenous GAPDH to FLAG-SIAH1 was barely detectable under basal conditions and was enhanced by S-nitrosylation of GAPDH. Overexpression of GFP-B23 dramatically reduced the binding of GAPDH to SIAH1 even in the presence of GSNO. Notably, the interaction between FLAG-SIAH1 and GFP-B23 was detectable under basal conditions and was markedly enhanced by GSNO treatment (Fig. 3 B). Consistent with these findings, coimmunoprecipitation data demonstrated that the presence of B23 WT, but not the C275S mutant, decreased the SIAH1-GAPDH interaction, and this reduction was enhanced by GSNO. However, overexpression of MYC-GAPDH did not interrupt the interaction between B23 and SIAH1 (Fig. 3 C, left, first and second blots). Interestingly, GSNO treatment had no

significant effect on the B23-GAPDH interaction. However, the B23 C275S mutant did not interact with GAPDH, and no interaction of SIAH1-GAPDH occurred in the presence of B23 WT (Fig. 3 C, middle, first and third blots). Together, these data suggest that B23 acts as a nuclear NO acceptor in rapid trans-nitrosylation from SNO-GAPDH and subsequently competes with GAPDH for binding to SIAH1. Moreover, the B23-SIAH1 interaction appears to be favored over the GAPDH-SIAH1 interaction.

To further determine whether endogenous B23 modulates the GAPDH-SIAH1 interaction, we depleted cellular B23 using short hairpin RNA (shRNA). Knockdown of B23 enhanced the GAPDH-SIAH1 interaction and increased endogenous SIAH1 protein levels in GSNO-treated PC12 cells (Fig. 3 D) and NMDA, which activates neuronal NO synthase, treated SH-SY5Y cells (Fig. S3 B), establishing that B23 abrogates the GAPDH-SIAH1 interaction. Immunocytochemical data also revealed enhanced nuclear localization of SIAH1 and GAPDH in the absence of B23 (Fig. 3 E).

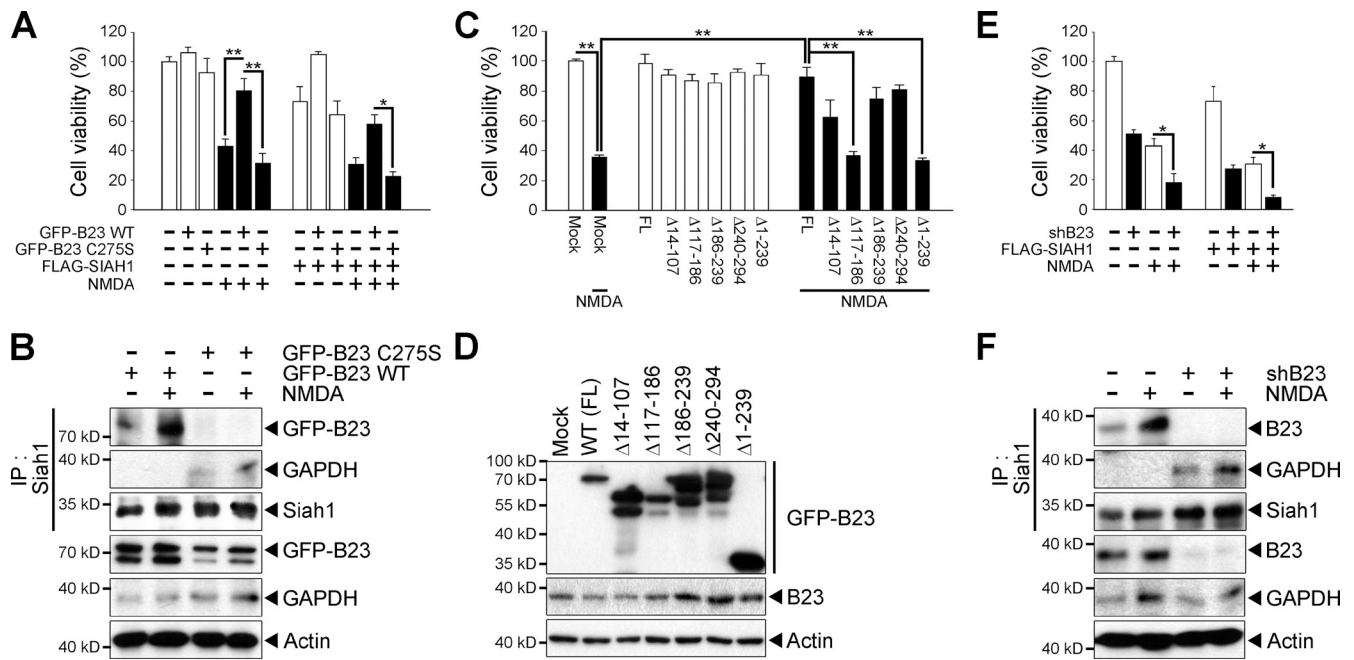


Figure 4. B23 prohibits NMDA neurotoxicity by regulating the function of SIAH1. (A) Overexpression of B23 WT, but not B23 C275S, protects neurons from excitotoxicity after NMDA exposure. Primary neurons were transduced by adenovirus-mediated overexpression of B23 (WT or C275S mutant) and/or SIAH1. B23 WT, but not B23 C275S, protected primary cortical neurons from NMDA-induced cytotoxicity. Cell viability was measured 20 h after transient exposure to 300 μ M NMDA. (B) Overexpression of B23 modulates SIAH1 and GAPDH interaction in primary cortical neurons after NMDA exposure. Primary neurons were transduced by adenovirus-mediated overexpression of B23 (WT or C275S mutant). Cell lysates were immunoprecipitated with anti-SIAH1 antibody and analyzed by Western blotting. (C) Deletion mutants of B23 that failed to bind to SIAH1 impair neuroprotective action of B23 in primary cortical neuron. Cell viability was measured 20 h after transient exposure to 300 μ M NMDA. (D) Expression level of B23 WT and fragment protein in Fig. 4 C. (E) Depletion of B23 enhances excitotoxicity after NMDA exposure. Adenovirus-mediated depletion of B23 and/or overexpression of SIAH1 in the primary cortical neurons decreased neuronal cell viability after NMDA exposure. Cell viability was measured 20 h after transient exposure to 300 μ M NMDA. (F) Adenovirus-mediated depletion of B23 augments SIAH1 and GAPDH interaction in primary cortical neurons after NMDA exposure. Cell lysates were immunoprecipitated with anti-SIAH1 antibody and analyzed by Western blotting. One-way ANOVA resulted in a $P < 0.05$. *, $P < 0.05$; **, $P < 0.005$. Error bars show means \pm SEM. FL, full length; IP, immunoprecipitation.

B23 prohibits NMDA neurotoxicity by regulating the function of SIAH1

Based on our observations that B23 interacts with the SIAH1–GAPDH complex in the nucleus and, upon nitrosative stress, B23 snatched NO from SNO-GAPDH, dissociating GAPDH from SIAH1 and enhancing its binding to SIAH1, we hypothesized that excess expression of B23 or S-nitrosylation of B23 prevents SIAH1-mediated cell death in the neuron. To test this hypothesis, we infected primary cortical neurons with adenovirus that overexpressed the B23 WT or C275S mutant with FLAG-SIAH1 before NMDA treatment because a previous study showed that NMDA elicits NO generation and GAPDH–SIAH1-mediated neurotoxicity (Hara et al., 2005; Hara and Snyder, 2006). Overexpression of B23 WT notably inhibited NMDA-induced neurotoxicity, whereas the B23 C275S mutant, which is not S-nitrosylated and cannot bind to SIAH1, failed to exert a neuroprotective effect (Fig. 4 A, left). Moreover, coinfection with B23 WT and SIAH1 adenoviruses completely prevented SIAH1-induced cell death in the absence of NMDA stimulation and exerted a neuroprotective effect in the presence of NMDA treatment (Fig. 4 A, right). In contrast, coinfection of the B23 C275S mutant with SIAH1 did not result in a neuroprotective effect (Fig. 4 A, right), suggesting that S-nitrosylation of B23, which is critical for binding to SIAH1 and disruption of the stable complex of SIAH1–GAPDH, is required for the neuroprotective actions of B23.

Consistent with these data, we showed that the interaction of B23 WT–SIAH1 is enhanced by NMDA treatment, whereas no such interaction was observed for B23 C275S–SIAH1 in primary cortical neurons (Fig. 4 B). To support the physiological relevance of the association of B23 and SIAH1, we determined NMDA induced neurotoxicity in primary cortical neurons after the expression of B23 WT and a series of deletion mutant forms of B23 (Fig. 4 C). Deletion mutants (Δ 117–186 and Δ 1–239) of B23, which were prevented to bind to both SIAH1 and GAPDH, notably abrogated the protective effect of B23, implying a crucial role of these protein interactions for the neuroprotective ability of B23. Interestingly, deletion mutant (Δ 14–107) that binds to SIAH1, but not with GAPDH, preserved \sim 60% of the protective effect of B23 (Fig. 4 C). Protein expression was confirmed by Western blotting (Fig. 4 D). Hence, our data indicate that the interruption of SIAH1–B23 binding impairs the neuroprotective effects of B23.

To further explore the physiological effects of B23 on SIAH1–GAPDH death cascades, we infected primary cortical neurons with a shB23-expressing adenovirus. Even in the absence of forced SIAH1 expression, depletion of B23 resulted in robust neuronal death of \sim 50%, and NMDA treatment increased neuron death to \sim 80% (Fig. 4 E, left), consistent with our previous study that B23 is critical for neuronal survival. Moreover, coinfection with shB23 adenovirus and SIAH1

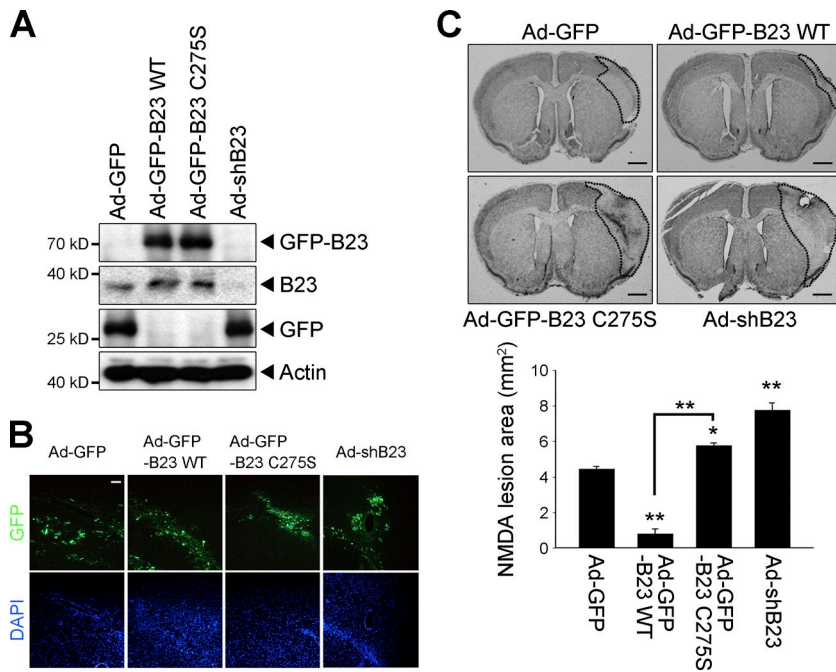


Figure 5. Neuroprotective activity of B23 against the SIAH1 death cascade in a mouse brain model. (A) Validation of protein expression in a transduced mouse brain. Adenoviral infection was used for overexpression of B23 (WT and B23 C275S mutant) and depletion of B23 in the cortex of mouse brains. The same number of viral particles was injected into the right cortex of mouse brains by stereotaxic surgery. (B) Validation of protein expression in a mouse brain model. Brain sections were immunostained with anti-GFP antibody, revealing overexpression of B23 WT and C275S and infection with mock or shB23 vectors. Bar, 50 μ m. (C) Representative images of Nissl-stained mouse brain sections with depletion and overexpression of B23 after adenoviral injection into the right cortex followed by NMDA injection. (top) The dotted lines indicate the area of the brain lesion. Bars, 1 mm. (bottom) Quantitative analysis of damaged areas of brains treated with NMDA. Lesion volumes in brains treated with B23 WT were \sim 82% smaller than those treated with mock controls. However, lesion volumes in mice treated with B23 C275S and shB23 were \sim 130 and 175% larger than the controls, respectively. One-way ANOVA resulted in a $P < 0.05$. *, $P < 0.05$; **, $P < 0.005$. Error bars show means \pm SEM. Ad, adenovirus.

elicited synergistic cell death, and NMDA treatment of shB23- and SIAH1-coexpressing neurons elicited $>90\%$ neuronal death (Fig. 4 E, right). We also confirmed that endogenous B23–SIAH1 binding is increased by NMDA, and depletion of endogenous B23 abolishes the interaction of B23–SIAH1 and enhances the interaction of GAPDH–SIAH1 in cortical neurons (Fig. 4 F).

Neuroprotective activity of B23 against the SIAH1 death cascade in a mouse brain model

To verify the *in vivo* neuroprotective function of B23 against SIAH1–GAPDH death signaling, we injected adenoviruses that express control, B23 WT, B23 C275S, and shB23 into the cerebral cortex of mice and elicited neurotoxicity by direct injection of NMDA. Protein expression from the viral vector system was confirmed by Western blotting and immunohistochemistry (Fig. 5, A and B). NMDA-induced lesions (~ 5 mm²) were observed in the brains of mice infected with control virus, whereas lesions in mice infected with B23 WT virus were $\sim 82\%$ smaller. Notably, mice infected with C275S, which is unable to be S-nitrosylated and does not bind to SIAH1, had $\sim 130\%$ bigger lesions than the control, suggesting that S-nitrosylation of B23 and its interaction with SIAH1 are critical for the prevention of NMDA-induced neurotoxicity. More interestingly, mice injected with adenovirus that delivers shB23 showed a much greater lesion volume ($\sim 175\%$) than in brains injected with control virus and a $\sim 97\%$ greater volume than that in mice injected with B23 WT, implying that B23 is a critical regulator of neuroprotection in the brain (Fig. 5 C). Thus, our data demonstrated that B23 prevents neuronal death *in vivo* by regulating the SIAH1 cascade, whereas mutant B23 C275S or shB23 exhibited reduced neuroprotective effects.

B23 suppresses the E3 ligase activity of SIAH1

Because SIAH1-mediated neuronal death requires E3 ligase activity, it is conceivable that B23-mediated neuroprotection against SIAH1-mediated cell death might be exerted by altering its ligase activity. To test whether B23 interferes with the ligase activity of SIAH1, we examined the effect of B23 on SIAH1 self-ubiquitination because SIAH1 is responsible for its own ubiquitination. Indeed, cotransfection of HA-ubiquitin with FLAG-SIAH1 revealed detectable ubiquitinated SIAH1, and GSNO treatment enhanced this self-ubiquitination, indicating that stabilization of SIAH1 protein levels is important for its ligase activity (Fig. 6 A, first, third, fifth, and seventh lanes). Interestingly, depletion of endogenous B23 greatly increased the amount of ubiquitinated SIAH1 and GSNO treatment resulted in little increase of ubiquitinated SIAH1 in the absence of B23, suggesting that ubiquitination of SIAH1 is saturated by B23 depletion and removal of B23 permits SIAH1 stabilization to the same extent as GSNO treatment (Fig. 6 A, second, fourth, sixth, and eighth lanes). In addition, overexpression of the B23 C275S mutant greatly enhanced the ubiquitination of SIAH1 compared with overexpression of B23 WT (Fig. 6 B). Accordingly, overexpression of B23 WT prohibited homodimerization of SIAH1, which is required for its self-directed degradation activity (Hu and Fearon, 1999; Polekhina et al., 2002; Xu et al., 2006), even in the presence of GSNO treatment (Fig. 6 C, second and fifth blots). In contrast, expression of B23 C275S or silencing of B23 led to obvious dimerization of SIAH1 compared with control or B23 WT expression (Fig. 6, C and D), implying that B23 probably suppresses the E3 ligase activity of SIAH1 by abrogation of SIAH1 homodimerization.

To determine whether B23 suppresses the activity of SIAH1, we monitored the influence of B23 on protein levels of NCoR1 (nuclear receptor corepressor), a well-known ligase

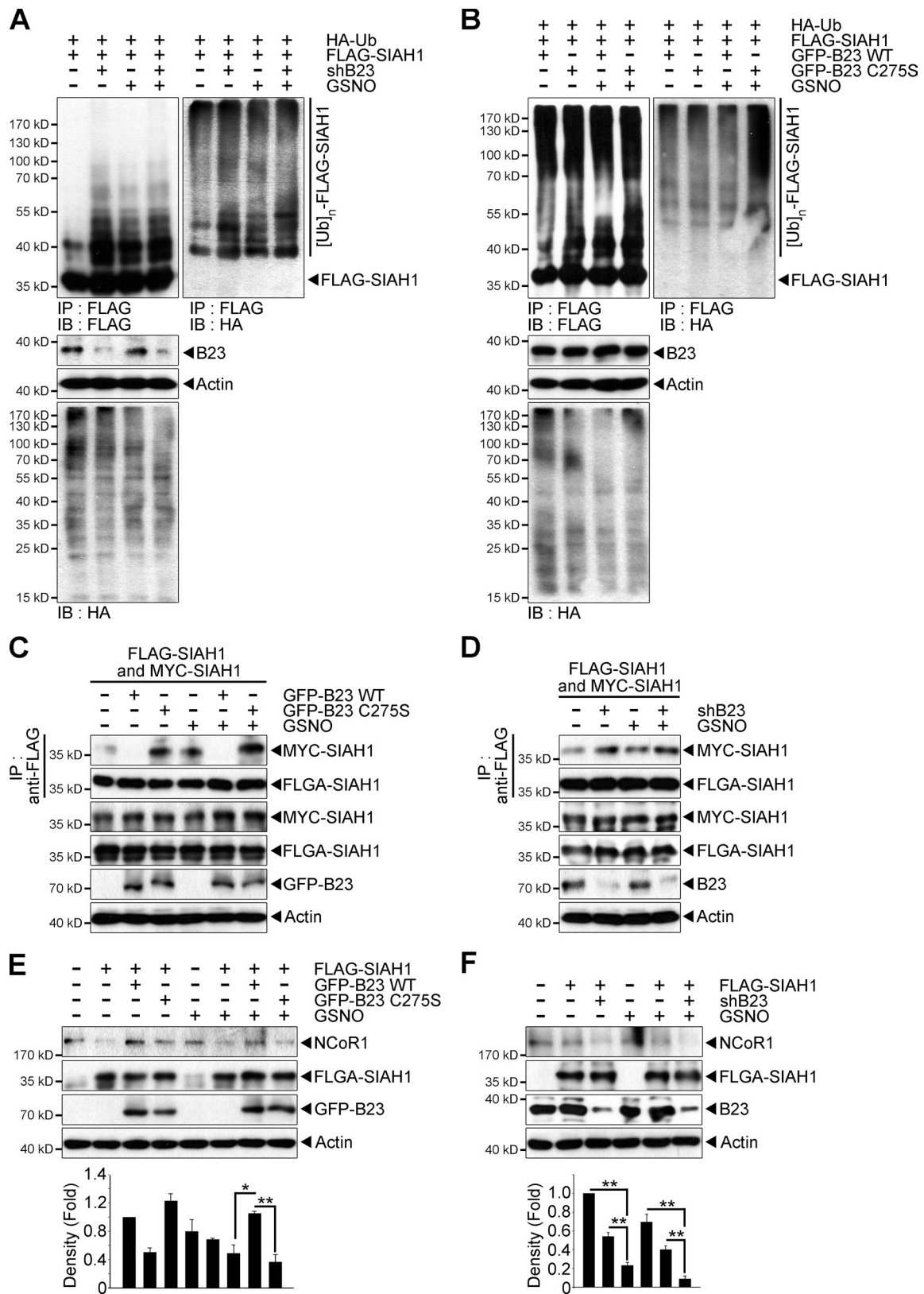


Figure 6. **B23 suppresses E3 ligase activity of SIAH1.** (A and B) SIAH1 self-ubiquitination assay. (C) SIAH1 dimerization is inhibited by B23 WT but not B23 C275S. PC12 cells were transfected with FLAG-SIAH1 or MYC-SIAH1 and GFP-B23 WT or C275S. Cells were exposed to 200 μ M GSNO for 18 h. Cell lysates were immunoprecipitated with anti-FLAG antibody and analyzed by Western blotting. (D) Depletion of B23 enhances SIAH1 dimerization. PC12 cells were transfected with FLAG-SIAH1, MYC-SIAH1, and shB23. Cells were exposed to 200 μ M GSNO for 18 h. Cell lysates were immunoprecipitated with anti-FLAG antibody and analyzed by Western blotting. (E) Degradation of NCoR1 by SIAH1 is inhibited by B23 WT but not by C275S. (F) Depletion of B23 increases degradation of NCoR1 by SIAH1. One-way ANOVA resulted in a $P < 0.05$. *, $P < 0.05$; **, $P < 0.005$. Error bars show means \pm SEM. IP, immunoprecipitation; IB, immunoblot; Ub, ubiquitin.

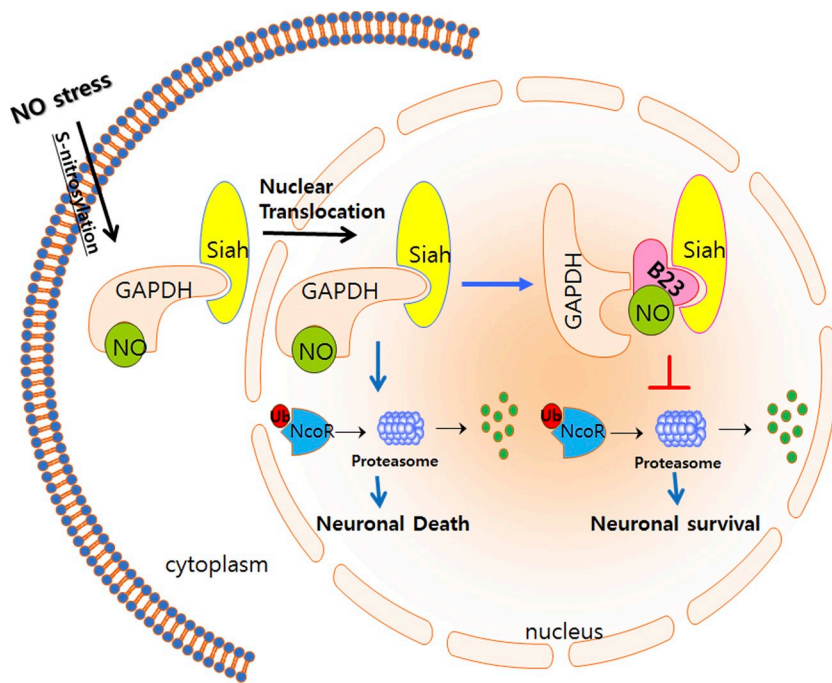


Figure 7. Schematic diagram of cell signal pathway in which B23 suppresses neuronal cell death mediated by SIAH1–GAPDH. Ub, ubiquitin.

target of SIAH1 (Zhang et al., 1998). Overexpression of B23 WT markedly protected against SIAH1-mediated degradation of NCoR1 independent of GSNO (Fig. 6 E). In contrast, neither B23 C275S mutant expression nor removal of endogenous B23 protected against NCoR1 degradation (Fig. 6, E and F), consistent with our observation that both the B23 C275S mutant and silencing of B23 failed to protect against neuron death in intact mice. Thus, our data indicate that B23 suppresses the E3 ligase activity of SIAH1, thereby inhibiting SIAH1-mediated neuronal death.

Discussion

Previously, we demonstrated that B23 is a critical nuclear regulator of neuronal survival, preventing DNA fragmentation and enhancing cell survival through interaction with nuclear Akt. In the present study, we identified a novel mechanism by which B23 mediates neuroprotection by its S-nitrosylation that interferes with nuclear SIAH1–GAPDH death signaling when cell stress activates NO formation (Fig. 7).

Protein S-nitrosylation in normal physiological condition of cells exerts anticell death effects, whereas S-nitrosylation of specific protein targets elicited by NO induced by exposure to stress is implicated in either procell death effects or pathophysiological events (Foster et al., 2009). Previous studies showed that nuclear translocation of SIAH1–SNO-GAPDH mediates cell death and that binding of SNO-GAPDH to SIAH1 stabilizes SIAH1 in the nucleus and sustains SIAH1-induced neurotoxicity (Hara et al., 2005). Moreover, SNO-GAPDH was suggested to be a NO provider in the nucleus and to trans-nitrosylate its nuclear binding partners. Our current data show that B23 associates with GAPDH–SIAH1 in the nucleus in a ternary complex and is trans-nitrosylated from SNO-GAPDH, thus reducing SNO-GAPDH levels. S-nitrosylation of B23 facilitates its binding

to SIAH1, markedly decreasing the interaction between GAPDH and SIAH1. It is likely that SNO-B23 preoccupies or masks the GAPDH binding site on SIAH1 because, in the presence of B23, GAPDH did not bind to SIAH1 (Fig. 3 C, middle, third blot). This might reflect the rapid cytoprotective effect of B23 by acting as a nuclear acceptor of NO to abrogate cell death signaling upon exposure to nitrosative stress.

In other hands, new cytosolic binding protein of GAPDH, GOSPEL (GAPDH's competitor of SIAH1 protein enhances life) prohibits neurotoxicity by competing with SIAH1 for GAPDH binding (Sen et al., 2009). However, our subcellular fractionation assay showed that B23 did not compete with GOSPEL for GAPDH binding in the cytoplasm, although a small amount of B23 protein was able to reside in the cytoplasm (Fig. S5). A plausible explanation for this observation is a different physiological distribution of these two proteins because although GOSPEL is predominantly localized in the cytoplasm, the majority of B23 is localized in the nucleus, suggesting that B23 probably possesses its own physiological role in neuroprotection. However, it is also conceivable that under nitrosative stress condition, neurons rapidly initiate survival signaling in both the cytoplasm and the nucleus. Although GOSPEL disrupts the GAPDH–SIAH1 complex in the cytoplasm that may not entirely reflect the neuroprotection, B23 abrogates nuclear GAPDH–SIAH1 cascade by decreasing S-nitrosylation of GAPDH and down-regulating SIAH1 death signaling. However, the precise nature of the subcellular response for neurotoxicity in neurons, and the possible cooperation of GOSPEL and B23 await further investigation.

Similar to mitochondria in the cytoplasm that dictate cell survival or death, the nucleolus in the nucleus also determines the cell's fate. In response to a variety of apoptotic stimuli, B23 translocates from the nucleolus to the cytoplasm and/or the nucleoplasm, antagonizing apoptosis (Horky et al., 2001).

We observed that the B23 C275S mutant that cannot be S-nitrosylated or bind to SIAH1 fails to undergo nucleoplasm/nucleolus shuttling and is confined in the nucleus, implying that the dynamic shuttling of B23 is essential to compromise GAPDH-SIAH1 signaling and for its neuroprotective effect. In contrast, nucleolar retention of B23 C275S substantially increased the nucleolar intensity of GAPDH and SIAH1 (Fig. 2 H), indicating that GAPDH-SIAH1-mediated cell death may be controlled in the nucleolus. Numerous nucleolar proteins are implicated in mediating apoptosis, and the nucleolus has been shown to be a preferred target for the apoptotic activity of caspase 3 (Horky et al., 2001). Thus, although it is unclear whether nucleolar localization of GAPDH and SIAH1 augments their cytotoxicity, it is conceivable that failed S-nitrosylation of B23 impedes its ability to displace SIAH1 from GAPDH, thereby allowing the GAPDH-SIAH1 complex to be translocated into the nucleolus and activate the death signal.

Although SIAH1 is well characterized as an E3 ligase that induces protein degradation by interacting with various target proteins, not all SIAH1 binding proteins are targets for degradation (Matsuzawa et al., 1998; Germani et al., 1999; Liu et al., 2001). We did not observe SIAH1-mediated degradation or ubiquitination of B23 (unpublished data), and a half-life assay with cycloheximide showed no difference between B23 WT and B23 C275S, the SIAH1 binding mutant, implying that SIAH1 binding did not alter the stability of the B23 protein (Fig. S4). However, B23 impeded the E3 ligase activity of SIAH1 in the nucleus, disrupting the homodimerization that signals its own proteasomal degradation and causing suppression of self-directed ligase activity (Fig. 6, A–D) as well as perturbation of SIAH1-induced degradation of its nuclear target (Fig. 6, E and F). It has recently been reported that B23 binds to the deubiquitinating enzyme, USP36, enhancing its activity in the nucleolus (Endo et al., 2009). Therefore, it is possible that B23 regulates ubiquitin dynamics by both deubiquitination and ubiquitination of nuclear proteins to provide rapid neuroprotection.

Because of its high mRNA level in the brain (Della et al., 1993), SIAH1 has been implicated in brain disease. For example, SIAH1 monoubiquitinates α -synuclein and promotes apoptotic death (Lee et al., 2008a), contributing to Lewy body formation and the pathogenesis of Parkinson's disease. SIAH1 also mediates the proteasomal degradation of mGluR1 (group 1 metabotropic glutamate receptor), which plays critical roles in neural plasticity (Moriyoshi et al., 2004). Because our study with intact mice clearly demonstrated that overexpression of B23 prevents SIAH1-mediated cell death in the model of NMDA-induced lesions (Fig. 5), identification of a small molecule that mediates the neuroprotective effect of B23 could reveal a novel therapeutic target for several neuronal diseases.

In summary, our study demonstrates that B23 drives cell survival by selectively interfering with GAPDH-SIAH1 cell death signaling. Upon exposure to nitrosative stress, SIAH1-GAPDH is translocated into the nucleus, where B23 dislodges SIAH1 from GAPDH by abolishing the nitrosylation of GAPDH, thereby disrupting the interaction of GAPDH-SIAH1 that is required for neuronal death signaling. Moreover, binding of B23

to SIAH1 attenuates the E3 ligase activity against the nuclear target of SIAH1, NcoR1. This mechanism is of importance in the stress response by which neurons rapidly counteract neurotoxicity and turn a death signal into a survival signal using a preexisting protein that mediates neuronal survival.

Materials and methods

Cell cultures

PC12 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 5% horse serum, and 100 U of penicillin/streptomycin at 37°C under a 5% CO₂ atmosphere. For primary neuron culture, the embryonic day 18 brain cortex was dissected from Sprague-Dawley rats and digested with 0.25% trypsin. The cells were cultured in neurobasal medium supplemented with B27, 1 mM L-glutamine, and penicillin/streptomycin.

Antibodies, shRNA, and chemicals

Anti-B23 antibody was generated in mice using GST-B23 purified from a bacterial expression system. Anti-GAPDH, anti-SIAH1, anti-GFP, anti-GST, anti-MYC, anti-Actin, and anti-NCoR1 were acquired from Santa Cruz Biotechnology, Inc. Anti-FLAG antibody was obtained from Sigma-Aldrich. Alexa Fluor-tagged secondary antibodies were obtained from Molecular Probes. All other chemicals were obtained from Sigma-Aldrich. shRNA oligomers for shB23 (forward, 5'-GATCCGAGGAAGTCTCTTTAAGAAAAGCTTTTCT-TAAAGAGACTTCCTCTTTTT-3'; and reverse, 5'-CTAGAAAAAAGAG-GAAGTCTCTTTAAGAAAAGCTTTTCTTAAAGAGACTTCCTCG-3') were cloned into a pGE1 vector.

Coimmunoprecipitation assay and in vitro binding assay

For coimmunoprecipitation, cells were rinsed with PBS and lysed in buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1.5 mM Na₃VO₄, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM β -glycerolphosphate, 1 mM PMSF, and protease cocktail [EMD Millipore]). Cell lysates (0.5–1 mg protein) were mixed with primary antibody with protein G/A beads and incubated for 3 h at 4°C with gentle agitation. The beads were then washed in lysis buffer, mixed with 2 \times SDS sample buffer, boiled, and analyzed by immunoblotting. For in vitro binding assay, proteins were bacterial expressed and purified with GST-resin. The GST tag from GST fusion protein was removed by thrombin treatment at RT for 24 h. Thrombin was removed using *p*-aminobenzamide-agarose (Sigma-Aldrich) column. Undigested GST fusion protein and GST tag were removed by flow through the GST-resin column. Finally, dialysis was performed to PBS. 500 ng of intact proteins was reacted at 4°C for 1 h with gentle agitation and immunoprecipitated with the indicated antibodies.

Trans-nitrosylation assay

2 μ g bacterial purified GAPDH was incubated with 300 μ M GSNO at 37°C for 1 h. Protein was desalted using desalting column (Thermo Fisher Scientific) to remove the GSNO. For trans-nitrosylation reaction, S-nitrosylated GAPDH or nonnitrosylated GAPDH was incubated with 2 μ g of purified GST-B23 or GST protein at 4°C for 1 h with mild rotation in buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1 mg/ml BSA, and 0.1% Triton X-100), and then, solution was used for biotin switching assay.

Biotin switching assay

S-nitrosylation biotin switching assay was performed as in Jaffrey et al. (2001). Proteins from the reaction or cell lysates were prepared in 1.8 ml with HEN buffer (100 mM Hepes, 1 mM EDTA, and 0.1 mM neocuproine, pH 8.0). To block free thiol groups, 0.2 ml of 25% SDS was added with 20 μ l of 10% methyl methane thiosulphonate (Sigma-Aldrich) with frequent vortexing. Samples were incubated at 50°C for 30 min to avoid the light. After removing of methyl methane thiosulphonate by acetone precipitation, the proteins were resuspended in HENS buffer (HEN buffer containing 1% SDS) and labeled with nitrosylated thiol amino acids using 4 mM biotin-HPDP in dimethyl sulfoxide and 20 mM sodium ascorbate at 25°C for 1 h in the dark. After the reaction, proteins were purified by acetone precipitation and resuspended in 0.25 ml of 10% HENS buffer. Proteins were neutralized by addition of 0.75 ml neutralization buffer (25 mM Hepes, 100 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100, pH 7.5). Biotinylated proteins were precipitated by streptavidin-agarose beads at 4°C with rotation for overnight. Precipitated proteins on beads were washed in wash buffer (neutralization buffer containing 600 mM NaCl) and eluted in elution

buffer (10% HEN buffer containing 1% β -mercaptoethanol [vol/vol]) with frequent agitation at 25°C. Eluted proteins were analyzed by SDS-PAGE and Western blotting.

GST pull-down assay

Cells were rinsed with PBS and lysed in buffer as described in the Coimmunoprecipitation assay and in vitro binding assay method. Cell lysates (0.5–1 mg of protein) were mixed with glutathione–Sepharose beads and incubated for 3 h at 4°C with gentle agitation. The beads were then washed in lysis buffer, mixed with 2 \times SDS sample buffer, boiled, and analyzed by immunoblotting.

Immunofluorescence

Cells grown on coverslips in 24-well plates were fixed in 4% paraformaldehyde for 15 min, permeabilized in PBS containing 0.25% Triton X-100 for 10 min, and blocked in 2% BSA for 30 min. Cells were immunostained using primary antibodies and the appropriate Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 594 goat anti-mouse secondary antibodies. Nuclei were counterstained with DAPI or Hoechst 33342 dye. Immunostained images were acquired using a laser-scanning confocal microscope (LSM 510 Meta DuoScan; Carl Zeiss) equipped with a 40 \times water immersion C-Apochromat objective (Carl Zeiss) at RT (22°C). The confocal microscope was controlled using the LSM software version 4.2 (Carl Zeiss). The pinhole was open to the 1- μ m thickness of the stack. Excitation of Hoechst 33342 or DAPI was performed using a 405-nm diode laser. Excitation of GFP or Alexa Fluor 488 was performed using a 488-nm argon laser, and excitation of Alexa Fluor 594 was performed using a 543-nm helium-neon laser.

Cell viability assay

Primary cultured cortical neurons were infected with adenovirus and exposed to NMDA. 3 d before NMDA exposure, primary neurons were infected with adenovirus. Neurons were treated with Mg²⁺-free Earle's balanced salt solution containing 0.3 mM NMDA and 0.005 mM glycine for 10 min at 8 d in vitro. Cell viability was analyzed using the luminescent cell viability assay kit (CellTiter-Glo; Promega) at 24 h after exposure to NMDA.

NMDA-induced mice brain lesion

Adult male BALB/c mice (20–25 g) were used for NMDA-induced cytotoxicity measurement. Anesthetized mice were mounted on a stereotaxic frame. The skull surface was exposed, and holes were drilled at the appropriate site. 0.3 μ l (10¹² plaque-forming units/ml) of viral particles expressing GFP, GFP-B23 WT, GFP-B23 C275S, or shRNA/B23 was slowly injected into the cortex (anterior 0.5 mm, lateral 2.5 mm from bregma, and ventral 1.0 mm) using a syringe (Hamilton) connected to a stereotaxic syringe pump. 7 d after viral infection, overexpressed or knocked down proteins in coronal sections were detected by immunohistochemistry with anti-GFP antibody (1:200) and Western blotted in the brain tissue of the injected site (2 \times 2 \times 2 mm). On day 8, 15 nmol NMDA was injected at the same position using a Hamilton syringe connected to a stereotaxic syringe pump. Mice were transcardially perfused and fixed with PBS and 4% PFA at 24 h after NMDA injection. Brains were removed and soaked in 4% PFA overnight and then equilibrated with 30% sucrose. Brain sections containing the area of injection were stained with cresyl violet to estimate the lesion volume. One-way analysis of variance (ANOVA) was used to calculate the difference between the groups.

Statistical analyses

Data are expressed as means \pm SEM. Statistical analysis was performed by SigmaPlot statistical analysis software (Systat Software). All experiments were performed in a blinded manner. Statistical significance was defined as *, $P < 0.05$ and **, $P < 0.005$.

Online supplemental material

Fig. S1 shows in vitro binding of purified proteins and cellular distribution of Fig. 1 (E and F). Fig. S2 demonstrates trans-nitrosylation of B23 from GAPDH and cellular distribution of Fig. 2 H. Fig. S3 shows immunoprecipitation assays under condition of B23 depletion in PC12 and SH-SY5Y cell lines. Fig. S4 shows protein stability of B23 WT and C275S mutant in the treatment of cycloheximide. Fig. S5 exhibits that B23 does not compete with GOSPEL for the binding of GAPDH by immunoprecipitation assay with subcellular fractions. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201205015/DC1>.

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References

- Ahn, J.Y., X. Liu, D. Cheng, J. Peng, P.K. Chan, P.A. Wade, and K. Ye. 2005. Nucleophosmin/B23, a nuclear PI(3,4,5)P(3) receptor, mediates the anti-apoptotic actions of NGF by inhibiting CAD. *Mol. Cell.* 18:435–445. <http://dx.doi.org/10.1016/j.molcel.2005.04.010>
- Borer, R.A., C.F. Lehner, H.M. Eppenberger, and E.A. Nigg. 1989. Major nucleolar proteins shuttle between nucleus and cytoplasm. *Cell.* 56:379–390. [http://dx.doi.org/10.1016/0092-8674\(89\)90241-9](http://dx.doi.org/10.1016/0092-8674(89)90241-9)
- Choi, J.W., S.B. Lee, C.K. Kim, K.H. Lee, S.W. Cho, and J.Y. Ahn. 2008. Lysine 263 residue of NPM/B23 is essential for regulating ATP binding and B23 stability. *FEBS Lett.* 582:1073–1080. <http://dx.doi.org/10.1016/j.febslet.2008.02.059>
- Della, N.G., P.V. Senior, and D.D. Bowtell. 1993. Isolation and characterisation of murine homologues of the *Drosophila* seven in absentia gene (sina). *Development.* 117:1333–1343.
- Endo, A., N. Kitamura, and M. Komada. 2009. Nucleophosmin/B23 regulates ubiquitin dynamics in nucleoli by recruiting deubiquitylating enzyme USP36. *J. Biol. Chem.* 284:27918–27923. <http://dx.doi.org/10.1074/jbc.M109.037218>
- Foster, M.W., D.T. Hess, and J.S. Stamler. 2009. Protein S-nitrosylation in health and disease: a current perspective. *Trends Mol. Med.* 15:391–404. <http://dx.doi.org/10.1016/j.molmed.2009.06.007>
- Germani, A., F. Romero, M. Houlard, J. Camonis, S. Gisselbrecht, S. Fischer, and N. Varin-Blank. 1999. hSiah2 is a new Vav binding protein which inhibits Vav-mediated signaling pathways. *Mol. Cell. Biol.* 19:3798–3807.
- Hara, M.R., and S.H. Snyder. 2006. Nitric oxide-GAPDH-Siah: a novel cell death cascade. *Cell. Mol. Neurobiol.* 26:525–536. <http://dx.doi.org/10.1007/s10571-006-9011-6>
- Hara, M.R., N. Agrawal, S.F. Kim, M.B. Cascio, M. Fujimuro, Y. Ozeki, M. Takahashi, J.H. Cheah, S.K. Tankou, L.D. Hester, et al. 2005. S-nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 binding. *Nat. Cell Biol.* 7:665–674. <http://dx.doi.org/10.1038/ncb1268>
- Hess, D.T., A. Matsumoto, S.O. Kim, H.E. Marshall, and J.S. Stamler. 2005. Protein S-nitrosylation: purview and parameters. *Nat. Rev. Mol. Cell Biol.* 6:150–166. <http://dx.doi.org/10.1038/nrm1569>
- Horky, M., G. Wurzer, V. Kotala, M. Anton, B. Vojtěšek, J. Vácha, and J. Wiesierska-Gadek. 2001. Segregation of nucleolar components coincides with caspase-3 activation in cisplatin-treated HeLa cells. *J. Cell Sci.* 114:663–670.
- Hu, G., and E.R. Fearon. 1999. Siah-1 N-terminal RING domain is required for proteolysis function, and C-terminal sequences regulate oligomerization and binding to target proteins. *Mol. Cell. Biol.* 19:724–732.
- Itahana, K., K.P. Bhat, A. Jin, Y. Itahana, D. Hawke, R. Kobayashi, and Y. Zhang. 2003. Tumor suppressor ARF degrades B23, a nucleolar protein involved in ribosome biogenesis and cell proliferation. *Mol. Cell.* 12:1151–1164. [http://dx.doi.org/10.1016/S1097-2765\(03\)00431-3](http://dx.doi.org/10.1016/S1097-2765(03)00431-3)
- Jaffrey, S.R., H. Erdjument-Bromage, C.D. Ferris, P. Tempst, and S.H. Snyder. 2001. Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. *Nat. Cell Biol.* 3:193–197. <http://dx.doi.org/10.1038/35055104>
- Korgaonkar, C., J. Hagen, V. Tompkins, A.A. Frazier, C. Allamargot, F.W. Quelle, and D.E. Quelle. 2005. Nucleophosmin (B23) targets ARF to nucleoli and inhibits its function. *Mol. Cell. Biol.* 25:1258–1271. <http://dx.doi.org/10.1128/MCB.25.4.1258-1271.2005>
- Kornberg, M.D., N. Sen, M.R. Hara, K.R. Juluri, J.V. Nguyen, A.M. Snowman, L. Law, L.D. Hester, and S.H. Snyder. 2010. GAPDH mediates nitrosylation of nuclear proteins. *Nat. Cell Biol.* 12:1094–1100. <http://dx.doi.org/10.1038/ncb2114>
- Kurki, S., K. Peltonen, L. Latonen, T.M. Kiviharju, P.M. Ojala, D. MEEK, and M. Laiho. 2004. Nucleolar protein NPM interacts with HDM2 and protects tumor suppressor protein p53 from HDM2-mediated degradation. *Cancer Cell.* 5:465–475. [http://dx.doi.org/10.1016/S1535-6108\(04\)00110-2](http://dx.doi.org/10.1016/S1535-6108(04)00110-2)
- Lee, J.T., T.C. Wheeler, L. Li, and L.S. Chin. 2008a. Ubiquitination of alpha-synuclein by Siah-1 promotes alpha-synuclein aggregation and apoptotic cell death. *Hum. Mol. Genet.* 17:906–917. <http://dx.doi.org/10.1093/hmg/ddm363>
- Lee, S.B., T.L. Xuan Nguyen, J.W. Choi, K.H. Lee, S.W. Cho, Z. Liu, K. Ye, S.S. Bae, and J.Y. Ahn. 2008b. Nuclear Akt interacts with B23/NPM and protects it from proteolytic cleavage, enhancing cell survival. *Proc. Natl. Acad. Sci. USA.* 105:16584–16589. <http://dx.doi.org/10.1073/pnas.0807668105>

- Liu, J., J. Stevens, C.A. Rote, H.J. Yost, Y. Hu, K.L. Neufeld, R.L. White, and N. Matsunami. 2001. Siah-1 mediates a novel beta-catenin degradation pathway linking p53 to the adenomatous polyposis coli protein. *Mol. Cell.* 7:927–936. [http://dx.doi.org/10.1016/S1097-2765\(01\)00241-6](http://dx.doi.org/10.1016/S1097-2765(01)00241-6)
- Liu, X., Z. Liu, S.W. Jang, Z. Ma, K. Shinmura, S. Kang, S. Dong, J. Chen, K. Fukasawa, and K. Ye. 2007. Sumoylation of nucleophosmin/B23 regulates its subcellular localization, mediating cell proliferation and survival. *Proc. Natl. Acad. Sci. USA.* 104:9679–9684. <http://dx.doi.org/10.1073/pnas.0701806104>
- Matsumoto, A., K.E. Comatas, L. Liu, and J.S. Stamler. 2003. Screening for nitric oxide-dependent protein-protein interactions. *Science.* 301:657–661. <http://dx.doi.org/10.1126/science.1079319>
- Matsuzawa, S., S. Takayama, B.A. Froesch, J.M. Zapata, and J.C. Reed. 1998. p53-inducible human homologue of *Drosophila* seven in absentia (Siah) inhibits cell growth: suppression by BAG-1. *EMBO J.* 17:2736–2747. <http://dx.doi.org/10.1093/emboj/17.10.2736>
- Moriyoshi, K., K. Iijima, H. Fujii, H. Ito, Y. Cho, and S. Nakanishi. 2004. Seven in absentia homolog 1A mediates ubiquitination and degradation of group 1 metabotropic glutamate receptors. *Proc. Natl. Acad. Sci. USA.* 101:8614–8619. <http://dx.doi.org/10.1073/pnas.0403042101>
- Nott, A., P.M. Watson, J.D. Robinson, L. Crepaldi, and A. Riccio. 2008. S-Nitrosylation of histone deacetylase 2 induces chromatin remodelling in neurons. *Nature.* 455:411–415. <http://dx.doi.org/10.1038/nature07238>
- Polekhina, G., C.M. House, N. Traficante, J.P. Mackay, F. Relaix, D.A. Sassoon, M.W. Parker, and D.D. Bowtell. 2002. Siah ubiquitin ligase is structurally related to TRAF and modulates TNF-alpha signaling. *Nat. Struct. Biol.* 9:68–75. <http://dx.doi.org/10.1038/nsb743>
- Rubbi, C.P., and J. Milner. 2003. Disruption of the nucleolus mediates stabilization of p53 in response to DNA damage and other stresses. *EMBO J.* 22:6068–6077. <http://dx.doi.org/10.1093/emboj/cdg579>
- Schonhoff, C.M., M.C. Daou, S.N. Jones, C.A. Schiffer, and A.H. Ross. 2002. Nitric oxide-mediated inhibition of Hdm2-p53 binding. *Biochemistry.* 41:13570–13574. <http://dx.doi.org/10.1021/bi026262q>
- Sen, N., M.R. Hara, A.S. Ahmad, M.B. Cascio, A. Kamiya, J.T. Ehmsen, N. Agrawal, L. Hester, S. Doré, S.H. Snyder, and A. Sawa. 2009. GOSPEL: a neuroprotective protein that binds to GAPDH upon S-nitrosylation. *Neuron.* 63:81–91. <http://dx.doi.org/10.1016/j.neuron.2009.05.024>
- Wu, M.H., and B.Y. Yung. 2002. UV stimulation of nucleophosmin/B23 expression is an immediate-early gene response induced by damaged DNA. *J. Biol. Chem.* 277:48234–48240. <http://dx.doi.org/10.1074/jbc.M206550200>
- Xu, Z., A. Sproul, W. Wang, N. Kukekov, and L.A. Greene. 2006. Siah1 interacts with the scaffold protein POSH to promote JNK activation and apoptosis. *J. Biol. Chem.* 281:303–312. <http://dx.doi.org/10.1074/jbc.M509060200>
- Yu, Z., T. Kunczewicz, W.P. Dubinsky, and B.C. Kone. 2006. Nitric oxide-dependent negative feedback of PARP-1 trans-activation of the inducible nitric-oxide synthase gene. *J. Biol. Chem.* 281:9101–9109. <http://dx.doi.org/10.1074/jbc.M511049200>
- Zhang, J., M.G. Guenther, R.W. Carthew, and M.A. Lazar. 1998. Proteasomal regulation of nuclear receptor corepressor-mediated repression. *Genes Dev.* 12:1775–1780. <http://dx.doi.org/10.1101/gad.12.12.1775>