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Nitric oxide-induced nuclear GAPDH activates p300/CBP and mediates apoptosis

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Abstract

Besides its role in glycolysis, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) initiates a cell death cascade[1–9]. Diverse apoptotic stimuli activate inducible nitric oxide synthase (iNOS) or neuronal NOS (nNOS), with the generated nitric oxide (NO) *S*-nitrosylating GAPDH, abolishing its catalytic activity and conferring on it the ability to bind to Siah1, an E3-ubiquitin-ligase with a nuclear localization signal (NLS). The GAPDH–Siah1 protein complex, in turn, translocates to the nucleus and mediates cell death; these processes are blocked by procedures that interfere with GAPDH–Siah1 binding. Nuclear events induced by GAPDH to kill cells have been obscure. Here we show that nuclear GAPDH is acetylated at Lys 160 by the acetyltransferase p300/CREB binding protein (CBP) through direct protein interaction, which in turn stimulates the acetylation and catalytic activity of p300/CBP. Consequently, downstream targets of p300/CBP, such as p53 (refs 10–15), are activated and cause cell death. A dominant-negative mutant GAPDH with the substitution of Lys 160 to Arg (GAPDH-K160R) prevents activation of p300/CBP, blocks induction of apoptotic genes and decreases cell death. Our findings reveal a pathway in which NO-induced nuclear GAPDH mediates cell death through p300/CBP.

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AUTHOR CONTRIBUTIONS

Ni.S. and M.R.H. were primarily responsible for experimental design and work, data analysis and preparation of figures, and helped to write the manuscript; M.K., M.C., B.-I.B., Ne.S. and B.T. contributed to data acquisition and analysis; T.D. and V.D. helped with the data analysis, provided technical assistance and material support; S.H.S and A.S. supervised the project and wrote the manuscript.

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p300 and its closely related homologue CBP are the most prominent nuclear protein acetyltransferases[16,17]. In macrophage RAW264.7 cells activated with lipopolysaccharide (LPS) and interferon-γ (IFNγ), the active components of endotoxin, GAPDH translocates to the nucleus in an NO-dependent manner[1]. Under the same conditions, we observed acetylation of GAPDH, which was abolished by treatment with *N*-(3-(aminomethyl)benzyl)acetamidine (1400W), a selective iNOS inhibitor (Fig. 1a). To ascertain whether p300 and/or CBP are physiologically responsible for GAPDH acetylation in intact cells, we depleted p300 and CBP by RNA interference (RNAi). Depletion of either protein decreased GAPDH acetylation, which was abolished following depletion of both CBP and p300 (Fig. 1b). We also observed acetylation of GAPDH by p300 *in vitro* (Supplementary Information, Fig. S1a). Moreover, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometric analysis of nuclear GAPDH in HEK293 cells following apoptotic stress revealed acetylation at Lys 160 (Supplementary Information, Fig. S1b, c). To confirm the site of acetylation in intact cells, we transfected HEK293 cells with GAPDH or a mutant GAPDH (GAPDH-K160R). We observed acetylation of wild-type but not the K160R mutant GAPDH (Fig. 1c). We previously reported that nuclear GAPDH is sulphonated[1]. In HEK293 cells, sulphonated GAPDH in the nucleus was acetylated (Fig. 1d). The K160R mutation selectively abolished acetylation, but did not alter GAPDH catalytic activity, *S*-nitrosylation or nuclear translocation (Supplementary Information, Fig. S1d, e, f).

We previously reported activation of GAPDH–Siah signalling in brains of mice treated with the dopamine neuronal toxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)[18]. Augmented acetylation of GAPDH occurs in MPTP-treated mouse brains and is reversed by pre-treatment with *R*-(−)-deprenyl, which selectively blocks *S*-nitrosylation and nuclear translocation of GAPDH[18] (Fig. 1e; Supplementary Information, Fig. S2a). In dopaminergic neuroblastoma SH-SY5Y cells treated with MPP^+ (1-methyl-4-phenylpyridium), an active metabolite of MPTP, acetylation of GAPDH was increased, an effect blocked by the NOS inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME) (Supplementary Information, Fig. S2b).

p300 binds directly to many of its substrate targets, such as p53 (refs 10^{-12}). We observed binding of p300 to GAPDH *in vitro* (Fig. 2a). In intact RAW264.7 cells, LPS/IFNγ treatment elicited GAPDH–p300 binding, which was prevented by inhibition of iNOS (Fig. 2b). GAPDH also bound to CBP in an NO-dependent manner (Supplementary Information, Fig. S2c). In RAW264.7 cells p300 and GAPDH were colocalized in the nuclei in a granular pattern (Fig. 2c, Supplementary Information, Fig. S2d). Interactions between GAPDH and p300/CBP were augmented in brains of mice treated with MPTP; this was reversed by pretreatment with *R*-(−)-deprenyl (Fig. 2d). Binding of GAPDH to p300/CBP requires intact Lys 160 and is lost in the GAPDH-K160R mutant (Fig. 2e). This suggests that acetylation of GAPDH at Lys 160 is required for binding of GAPDH to p300/CBP, which is consistent with the preserved binding detected when Lys 160 is replaced by Gln (GAPDH-K160Q), which mimics acetylation[19] (Supplementary Information, Fig. S2e).

GAPDH-K160R seems to function as a dominant-negative mutant, as it blocked endogenous GAPDH acetylation and its binding to p300/CBP in HEK293 cells treated with the NO donor *S*-nitroso-glutathione (GSNO; Fig. 2f). Similarly, in activated peritoneal macrophages treated with LPS/IFNγ, the GAPDH-K160R construct prevented acetylation of GAPDH and binding of GAPDH to p300/CBP (Fig. 2g).

To determine the role of GAPDH nuclear translocation in p300/CBP interactions, we examined the disposition of GAPDH with a fused nuclear localization signal (NLS–GAPDH). In LPS/IFNγ-treated macrophages NLS–GAPDH bound to p300/CBP even in iNOS-deleted cells or following mutation at Cys 150, the site of *S*-nitrosylation/sulphonation (Fig. 2g). Thus, NO

does not seem to influence GAPDH–p300/CBP interaction directly but functions principally to facilitate GAPDH nuclear translocation.

Auto-acetylation of p300 augments its acetyltransferase activity[20]. We examined the possibility that NO–GAPDH signalling regulates p300/CBP auto-acetylation. In RAW264.7 cells, LPS/IFNγ treatment enhanced p300 acetylation in an NO-dependent manner (Fig. 3a). In peritoneal macrophages, p300 acetylation and GAPDH–p300 binding were diminished in preparations from *iNOS* knockout mice (Fig. 3b). Acetylation of p300/CBP is dependent on GAPDH, as it was eliminated in RAW264.7 cells depleted of GAPDH by RNAi (Fig. 3c). Acetylation of p300 was also lost in cells overexpressing GAPDH-K160R, confirming a role for this mutant as dominant-negative (Fig. 3d). GAPDH augmented auto-acetylation of p300 *in vitro*, measured by incorporation of ^{14}C isotope from acetyl CoA to a p300 fragment (Fig. 3e) with no alteration by GSNO (Fig. 3f). This is consistent with the primary role of NO being to convey GAPDH to the nucleus where it comes into contact with p300, facilitating auto-acetylation.

Physiological activation of p300/CBP by GAPDH should lead to acetylation of downstream targets. p300/CBP activates several genes, including some associated with apoptosis, of which p53 is particularly well characterized[10–12]. Moreover, a major role for p53 in cell death induced by oxidative stress is well established[21–23]. GSNO treatment of GAPDH-transfected HEK293 cells enhanced p53 acetylation (Fig. 4a). This process may reflect binding of GAPDH to p300/CBP, as it is abolished in the GAPDH-K160R mutant. Acetylation of p53requires NO, being prevented by treatment with 1400W (Supplementary Information, Fig. S2f), and is dependent on GAPDH, being lost following GAPDH depletion by RNAi (Supplementary Information, Fig. S2g). These influences suggest protein interactions between GAPDH, p300 and p53 in intact cells, consistent with the NO-dependent binding of GAPDH and p53 (Fig. 4b). Moreover, in the absence of p300, GAPDH does not bind to p53 *in vitro* (Fig. 4c). In contrast to GAPDH, p53 does not stimulate acetylation of p300 (Supplementary Information, Fig. S2h).

p53 induces apoptosis mainly by transactivating downstream genes among which *PUMA* is particularly prominent[13–15]. To determine whether the GAPDH–p300–p53 cascade acts through such genes, we used a chromatin immunoprecipitation (ChIP) procedure[24] to determine whether these proteins form a complex with the *PUMA* promoter. In U2OS cells, which express endogenous wild-type p53, we observed association of sulphonated GAPDH (the nuclear form of GAPDH), p300 and p53 at the *PUMA* promoter (Fig. 4d). This complex is dependent on the GAPDH–p300 interaction. Thus, GAPDH-K160R abolished association of endogenous sulphonated GAPDH and p300 with the *PUMA* promoter (Fig. 4e; Supplementary Information, Fig. S3a). Depletion of GAPDH by RNAi substantially reduced the interaction of sulphonated GAPDH, p300 and p53 with the *PUMA* promoter (Fig. 4f). Consistent with the ChIP results, augmentation of PUMA protein levels in the presence of LPS/IFNγ was blocked by GAPDH-K160R (Supplementary Information Fig. S3b, c). The induction of PUMA is prevented by either genetic depletion of iNOS or the iNOS inhibitor 1400W, indicating that it is dependent on NO (Supplementary Information, Fig. S3c–e). The influence of NO on PUMA, similarly to its effect on p300/CBP (Fig. 2g), reflects enhanced nuclear translocation of GAPDH. Thus, NLS–GAPDH induces PUMA similarly in both wild-type and iNOS-deleted macrophages. Moreover, NLS–GAPDH with the Cys 150 mutation can induce PUMA (Supplementary Information, Fig. S3c).

Other p53 targets, such as Bax and p21, are also induced by overexpression of wild-type GAPDH and blocked by expression of GAPDH-K160R or RNAi to *GAPDH* (Fig. 4g; Supplementary Information, Fig. S3f, g). Brains of mice treated with MPTP, in which the GAPDH–p300 cascade is activated (Figs 1e, 2d), show augmented p53 acetylation and PUMA

induction (Fig. 4h, i). Consistent with the notion that p53/PUMA is regulated by the GAPDH–p300 cascade, pretreatment with *R*-(−)-deprenyl in the mice blocked augmented p53 acetylation and PUMA induction (Fig. 4h, i).

To determine whether cell death associated with GAPDH involves the p300 cascade, we transfected peritoneal macrophages with GAPDH or GAPDH-K160R (Fig. 5a). Overexpression of wild-type GAPDH doubled cell death in the presence of LPS/IFNγ; this was blocked by expression of GAPDH-K160R. Similar results were seen in the dopaminergic neuroblastoma SH-SY5Y cells (Fig. 5a). The principal role for NO is to facilitate nuclear translocation of GAPDH, as p300/CBP binding and PUMA expression (Fig. 2g; Supplementary Information, Fig.S3c), as well as LPS/IFNγ-induced cell death (Supplementary Information, Fig. S4a) are maintained when NLS–GAPDH is overexpressed in iNOS-deleted macrophages. This GAPDH cascade is mediated by p53 and p300/CBP, because genetic depletion of p53 or RNAi depletion of p300/CBP decreased apoptosis (Supplementary Information, Fig. S4b, c). The GAPDH–p300 cascade also mediates etoposide-triggered apoptosis, as expression of GAPDH-K160R inhibited etoposide-initiated cell death (Supplementary Information, Fig. S4d, e). To further assess a role for p53 in cell death mediated by the GAPDH–p300–CBP pathway, we depleted p53 by RNAi in SH-SY5Y cells stably overexpressing GAPDH or GAPDH-K160R. In these cells we examined levels of PUMA (Fig. 5b) and apoptotic cell death monitored by TUNEL assay (Fig. 5c). GSNO treatment substantially augmented binding of p300/CBP to GAPDH, levels of PUMA, as well as cell death, which was greatly diminished in cells expressing GAPDH-K160R (Fig. 5b; Supplementary Information, Fig. S4f, g). Depletion of p53 virtually eliminated the augmentation of PUMA and cell death. This supports the notion that cell death elicited by this signalling cascade is associated with p53 and its downstream target PUMA.

In summary, nuclear *S*-nitrosylated/sulphonated GAPDH is acetylated by p300/CBP and, in turn, stimulates these proteins to acetylate and activate downstream targets, such as p53 (Supplementary Information, Fig. S4h). This cascade seems to mediate the NO-triggered GAPDH cell death pathway. Evidence for this conclusion includes the anti-apoptotic action of the GAPDH-K160R dominant-negative mutant that selectively blocks GAPDH–p300/CBP signalling. p300/CBP acetylates nuclear GAPDH which enhances the ability of GAPDH to stimulate auto-acetylation of p300/CBP. This suggests a feed-forward activation cycle wherein p300/CBP acetylates and augments the ability of GAPDH to stimulate acetylation of p300/CBP, which further acetylates GAPDH. This cascade is consistent with previous reports that overproduction of NO results in p53-mediated transactivation[25], and that loss of p53 function by inactivating mutations abrogates NO-induced apoptosis in human lymphoblastoid cells[26].

Recently Green and collaborators[27] reported that GAPDH diminishes caspase-independent cell death by increasing expression of Atg12, a protein involved in autophagy, and by augmenting autophagy of damaged mitochondria. It is unclear how the caspase-independent cytotoxicity of the HeLa, Jurkat and murine embryonic fibroblast cells used by Green and associates relates to cytotoxicity in the six types of cells we used. In their study the glycolytic activity of GAPDH was important for its role in caspase-independent death, whereas the NO–GAPDH–p300/CBP cascade required a catalytically-inactive *S*-nitrosylated GAPDH.

The NO-triggered GAPDH death pathway acts through downstream genes including *p53* (ref. 28). Besides their role in p53 regulation, p300 and CBP are co-activators for a large number of genes, which may also mediate apoptosis induced by GAPDH–p300/CBP. Furthermore, separate interactions of GAPDH with p300 and CBP have different consequences, and synergy of p300 and CBP is also possible[29–31]. Alterations of p300/CBP have been linked to a broad spectrum of diseases ranging from the immunodeficiency Rubinstein Taybi syndrome to neurological conditions, such as Huntington's disease[32–34]. We have detected a role for the

GAPDH–p300/CBP cascade in the MPTP-induced model of Parkinson's disease[18]. This model affords a specific example in which therapeutic interventions, such as drugs that selectively prevent binding of components of the GAPDH–p300/CBP complex, may be developed.

METHODS

Reagents and animals

Unless otherwise noted, chemicals were purchased from Sigma. Anti-rabbit and anti-mouse IgG agarose were purchased from eBiosciences. 1400W was purchased from Alexis. p300 fragment was purchased from ProteinOne. Two methods for RNAi were used in this study: synthesized small oligonucleotides for siRNA were from Dharmacon RNA Technologies (for GAPDH). The vector system for shRNA (for CBP and p300) is as described previously[1] The following sequences for silencing gene expressions were chosen, according to previous publications[1,35]. GAPDH, 5′-CGGGAAGCTCACTGGCATG-3′; p300, 5′-GTTCAAACGCCGAGTCTTCTT-3′; CBP, 5′-TCAACTCCTGTGTCGTCTTTT-3′; and control 5′-TTCTCCGAACGTGTCACGTTT-3′. Antibodies were obtained from the following companies: anti-GAPDH monoclonal antibody (Biogenesis); anti-PUMA, anti-histone H2B and anti-phospho-histone H2B (Ser 14) antibodies (Upstate); anti-HA antibody (Covance); anti-actin antibody (Chemicon); anti-CBP antibody, anti-p53 antibody, anti-Bax antibody, anti-p300 antibody and normal IgG (SantaCruz); anti-acetyl-CBP/p300 and anti-acetyl-lysine antibodies (Cell Signaling). The polyclonal antibodies against GAPDH and sulphonated-GAPDH were prepared as described[1]. Animal models treated with MPTP were prepared and processed as published[18].

Mass spectrometry

HEK293 cells were treated with staurosporine (1 μM) for 24 h. Cells were lysed in RIPA-A (0.3% Triton X-100, 50 mM Tris pH 7.4 and 1 mM EDTA) with rotation at 4 °C for 30 min. Cell lysates were centrifuged at 14,000 *g* for 10 min. GAPDH was extracted from the pellet with RIPA-B (1% Triton X-100, 1% SDS, 50 mM Tris-Cl, pH 7.4, 500 mM NaCl and 1 mM EDTA), affinity-precipitated with an anti-GAPDH antibody, subjected to SDS–PAGE under non-reducing conditions and visualized by colloidal Coomassie staining. Gel-purified GAPDH was tryptic-digested and analysed by MALDI-TOF mass spectrometry as described[1].

Protein interaction assays

GST-tagged proteins were prepared according to the manufacturer's recommendations (Pharmacia Biotech) and purified through glutathione–sepharose (Amersham Biosciences)[1]. Co-immunoprecipitation studies used anti-acetyl Lys (1:200 dilution) or anti-p300 (1:200 dilution) or anti-CBP (1:200) antibody following the published protocol[1].

Cell culture

RAW264.7, SH-SY5Y, HEK293, HCT116, and U2OS cells were maintained in Dullbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 2 mM l-glutamine at 37 °C with a 5% CO_2 atmosphere in a humidified incubator. Peritoneal macrophages were prepared by intraperitoneal injection of 1.5 ml of 3% sterile thioglycolate medium as described[1].

For RNAi experiments, media for RAW264.7 cells or U2OS was supplemented with 10 mM pyruvate and cells were transfected with 167 nM GAPDH or control siRNA using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. Twenty-four hours after transfection, RAW264.7 cells were incubated in the presence of LPS/IFNγ (LPS 1 μg ml^{-1} and IFNγ 10 ng ml^{-1}) for an additional 24 h. Immunofluorescent staining of cells for

confocal microscopy was carried out as described previously[1]. For transient transfection of expression constructs, we used Polyfect reagent (Invitrogen) for HEK293 cells and Lipofectamine 2000 for SH-SY5Y cells, U2OS cells and peritoneal macrophages.

SH-SY5Y cells stably overexpressing wild-type GAPDH or GAPDH-K160R were generated by transfecting cells with a construct of pcDNA-GAPDH or GAPDH-K160R in the presence of neomycin (600 μg ml^{-1}). The selected cell lines were further maintained in medium containing neomycin (200 μg ml^{-1}; ref. [36]). Silencing of *p53* in these stable cell lines was by using MISSION shRNA lentiviral particles (Sigma), according to the manufacturer's protocol.

S-nitrosylation biotin switch assay and glycolytic assay for GAPDH

These assays were performed as described[1].

Quantification of colocalization

The correlation coefficient for colocalization of GAPDH and p300 was determined using the colocalization function of the Zeiss LSM software, restricting the analysis to cell nuclei. The reported values represent the cumulative average of five cells from each of three separate plates.

In vitro acetylation assay

In 30 μl of reaction buffer (20 mM Tris-Cl, pH 8.0, 20% glycerol, 100 mM KCl, 1 mM DTT and 0.2 mM EDTA), 20 μM of acetyl-CoA or 1 μl of ^{14}C-acetyl-CoA (60 mCi $mmol^{-1}$), and 100 ng of a p300 fragment were added. After incubation at 30 °C for 1 h, the reaction was stopped by the addition of 10 μl of SDS sample buffer. The samples were subjected to SDS–PAGE gels and western blotting or autoradiography.

Chromatin immnoprecipitation (ChIP) assay

This was performed as described previously[24]. In brief, intact cells were treated with 2 mM disuccinimidyl glutarate (Pierce) to crosslink protein complexes, then were treated with formaldehyde to link protein to DNA covalently. Cells were lysed, the nucleoprotein complexes were sonicated and the crosslinked DNA–protein complexes were enriched by immuno-precipitation. The retrieved complexes were then analysed by PCR amplification to detect and quantify specific DNA targets. *PUMA* promoter elements were detected by PCR using the forward primer, 5′-CTGTGGCCTTGTGTCTGTGAGTAC-3′, and the reverse primer, 5′-CCTAGCCCAAGGCAAGGAGGAC-3′ (ref. 37). For real-time PCR we used Brilliant SYBR green master mix (Stratagene) according to the manufacturer's protocol.

RT–PCR analysis

Total RNA was extracted from cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription as well as PCR procedures used Superscript One Step RT–PCR with Platinum Taq (Invitrogen) for p21, Bax, PUMA and actin genes. The sequences are as follows: p21 forward, 5′-CTGGAGACTCTCAGGGTCGAAA-3′; p21 reverse, 5′-GATTAGGGCTTCCTCTTGGAGAA-3′; PUMA forward, 5′-AGAGGGAGGAGTCTGGGAGTG-3′; PUMA reverse; 5′-GCAGCGCATAT ACAGTATCTTACAGG-3′; Bax forward, 5′-TGCAGAGGATGATGCTGAC-3′; Bax reverse, 5′-CCTGTGACCTGAAGGAGG-3′

Apoptosis detection

Peritoneal macrophages and SH-SY5Y cells were transfected with HA-tagged wild-type GAPDH or GAPDH-K160R and treated with LPS/IFNγ or GSNO, respectively. Cells were permeabilized for 15 min in 0.1% Triton X-100 in PBS and incubated with a monoclonal anti-HA antibody followed by a secondary antibody conjugated with FITC (green). Cells were co-

stained with the DNA-binding dye, 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI). Nuclear morphology of transfected cells stained green was assessed by visualization of DAPI-stained cells with a Zeiss fluorescence microscope. The proportion of condensed nucleus was used as a semi-quantitative indicator of apoptosis, for example, apoptosis (percentage).

HEK 293 cells were transfected with HA–GAPDH or HA–GAPDH-K160R separately and treated with 200 μM GSNO or 100 μM etoposide for 16 h in the presence of 10% FBS. Apoptosis, indicated by externalization of phosphatidylserine from the inner to the outer leaflet of the plasma membrane, was monitored by measurement of annexin V-FITC binding cells (Roche Biosciences) with flow cytometry (FACScalibur, Becton Dickinson)[27].

Apoptosis was also quantified in peritoneal macrophages from wild-type or *iNOS* knockout mice, wild-type or *p53* knockout HCT116 and U2OS cells using an enzyme immunoassay (Roche Applied Science, cat no. 11544675001) based on a combination of antibodies that recognize histones and DNA, as described previously[38]. Briefly, primary macrophages were transfected with various GAPDH constructs and treated with LPS/IFNγ for 16 h. HCT116 and U2OS cells were treated with GSNO for 16 h. Cells were lysed in buffer (supplied by the manufacturer) and an aliquot of the cytosolic fraction was used to quantify proteins using standard methods. Cytoplasmic fractions containing fragmented DNA were transferred to streptavidin-coated microtitre plates that had been incubated with a biotinylated monoclonal anti-histone antibody. The amount of fragmented DNA bound to the anti-histone antibody was evaluated by a peroxidase-conjugated monoclonal anti-DNA antibody using ABTS (2,2′-azino-di(3 ethylbezthiazoline sulfonic acid)) as a substrate at 405 nm. Absorbance values were normalized by the values obtained from control cell lysates and is expressed as arbitrary units.

We also used the levels of phosphorylated histone H2B at Ser 14 in western blotting, as another marker associated with apoptosis.

Cell death of SH-SY5Y cells stably expressing either GAPDH or GAPDH-K160R was assayed 16 h after exposure to GSH or GSNO (200 μM). Numbers of total and apoptotic cells were determined by counting nuclei stained with 100 ng ml^{-1} DAPI and TUNEL, respectively. Cells were fixed in 4% paraformaldehyde and then stained using the *In situ* cell death detection kit (Roche) according to protocols provided by the manufacturer. The cells were examined under a confocal microscope (Zeiss) and cell death was evaluated as the ratio of TUNEL-positive cells to total cells.

Statistical analysis

P values were calculated by one-way ANOVA by using MINITAB 13 (Minitab, State College, PA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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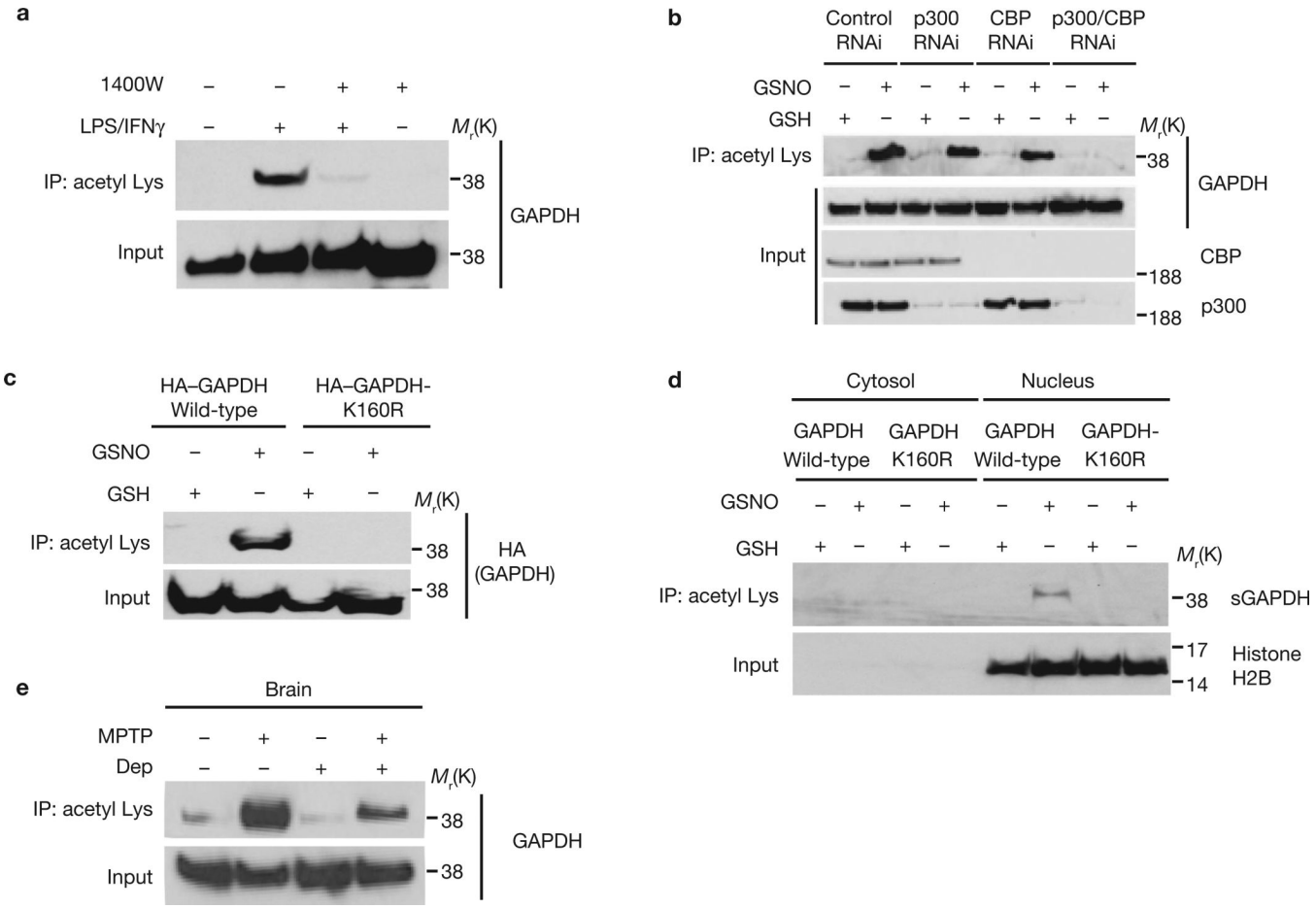

Figure 1.

GAPDH is acetylated in the nucleus at Lys 160 following NO stimulation. (**a**) GAPDH acetylation in RAW264.7 cells treated with LPS/IFNγ for 16 h is abolished by the iNOS inhibitor 1400W. Cell lysates were immunoprecipitated with an anti-acetyl Lys antibody, and the immunoprecipitates were analysed by western blotting with an anti-GAPDH antibody. (**b**) Both p300 and CBP contribute to acetylation of GAPDH in U2OS cells treated with the NO donor, GSNO. Depletion of p300 or CBP by RNAi leads to diminished acetylation of GAPDH. (**c**) GAPDH mutation at Lys 160 abolishes its acetylation in the presence of 200 μM GSNO in HEK293 cells. (**d**) Acetylation of sulphonated GAPDH (sGAPDH) is observed only in the nucleus where it requires intact Lys 160. Cytosolic or nuclear fractions of HEK293 cells were immnoprecipiated with an anti-acetyl Lys antibody, and the immunoprecipitates were analysed by western blotting with a specific anti-sulphonated-GAPDH antibody. (**e**), *R*-(−)-Deprenyl (Dep) inhibits the acetylation of GAPDH in brains of mice treated with MPTP.

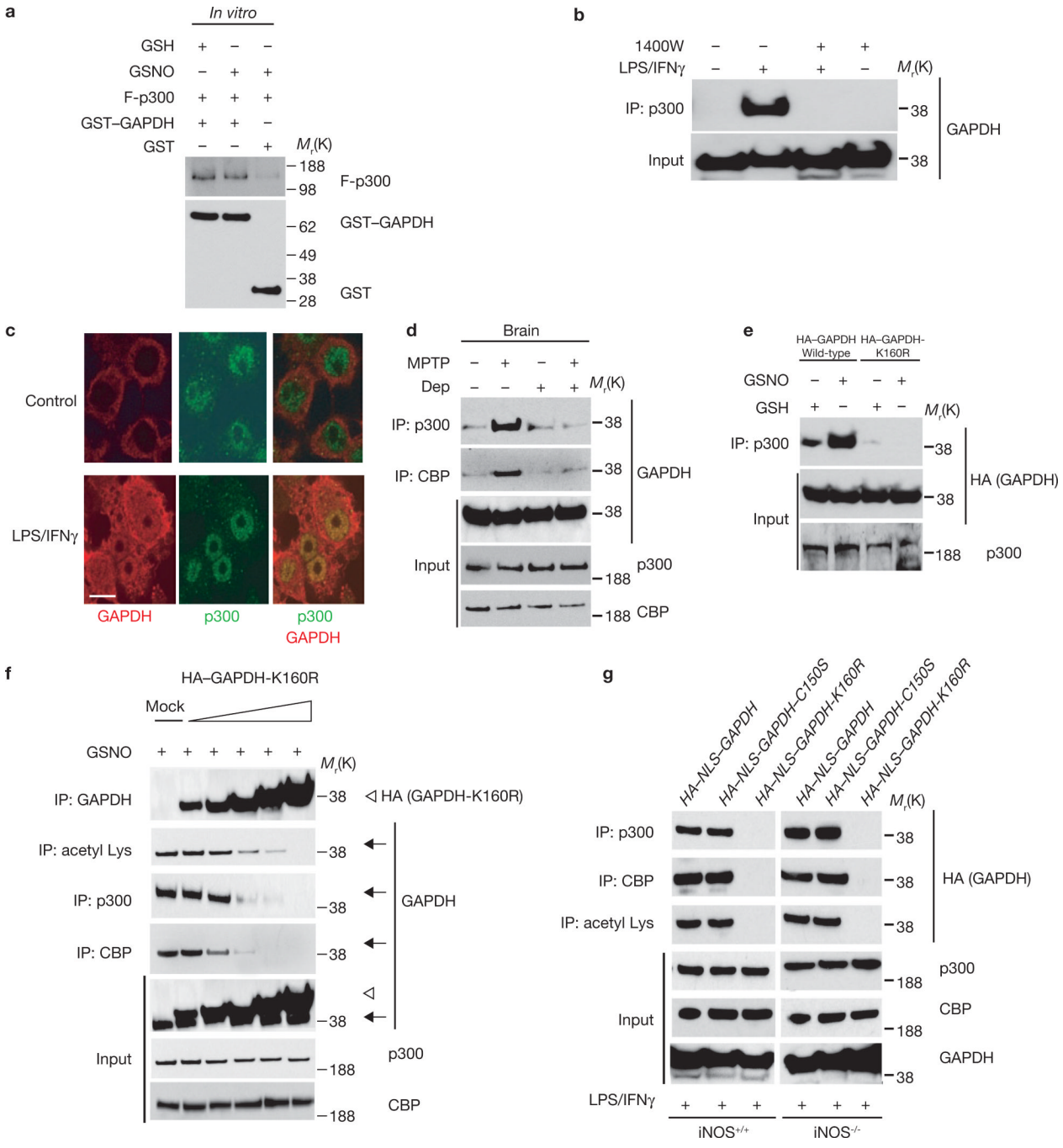

Figure 2.

GAPDH interacts with p300/CBP and GAPDH-K160R acts as a dominant-negative mutant. (**a**) GAPDH, native or NO-modified, binds similarly *in vitro* to a fragment of human p300 (F-p300) (amino acids 1135−2414). GST or GST–GAPDH was pre-treated with 50 μM GSH or GSNO for 30 min at 37 °C. F-p300 was added and binding assessed by a GSH-agarose pulldown assay. (**b**) GAPDH–p300 binding occurs in RAW264.7 cells treated with LPS/IFNγ for 16 h and is abolished by the iNOS inhibitor 1400W (100 μM). Cell lysates were immunoprecipitated with an anti-p300 antibody and the immunoprecipitates were analysed by western blotting with an anti-GAPDH antibody. (**c**) GAPDH and p300 are colocalized in the nucleus of RAW264.7 cells after exposure to LPS/IFNγ. Cells were stained with

immunofluorescent anti-GAPDH and anti-p300 antibodies (green, p300; red, GAPDH). Scale bar, 10 μm. (**d**) *R*-(−)-Deprenyl (Dep) inhibits the enhancement of GAPDH–p300/CBP binding in mouse brain elicited by MPTP treatment. (**e**) K160R mutation of GAPDH abolishes GAPDH–p300 interactions in HEK293 cells. (**f**) GAPDH-K160R expression prevents acetylation of endogenous GAPDH and its binding to p300/CBP in a concentration-dependent manner, suggesting that GAPDH-K160R functions as a dominant-negative mutant. Forty-eight hours after transfection with 0.1, 0.5, 2, 3, or 4.5 μg of HA–GAPDH-K160R, HEK293 cells were treated with 200 μM GSNO for 24 h. Cell lysates were immunoprecipitated with anti-GAPDH, anti-p300, anti-CBP or anti-acetyl Lys antibody and the immunoprecipitates were analysed by western blotting with anti-HA or anti-GAPDH antibodies. Arrows indicate endogenous GAPDH and arrowheads indicate exogenous HA–GAPDH-K160R. (**g**) Nuclear localization of GAPDH augments its binding to p300/CBP and acetylation at Lys 160. Peritoneal macrophages from wild-type and *iNOS* knockout mice were transfected with various GAPDH constructs. Cell lysates were immunoprecipitated with anti-p300, anti-CBP or anti-acetyl Lys antibody and the immunoprecipitates were analysed by western blotting with anti-HA antibody. Both HA–NLS–GAPDH and HA–NLS–GAPDH–C150S, but not HA–NLS–GAPDH-K160R, augmented GAPDH–p300/CBP binding, as well as GAPDH acetylation in both iNOS knockout and wild-type cells.

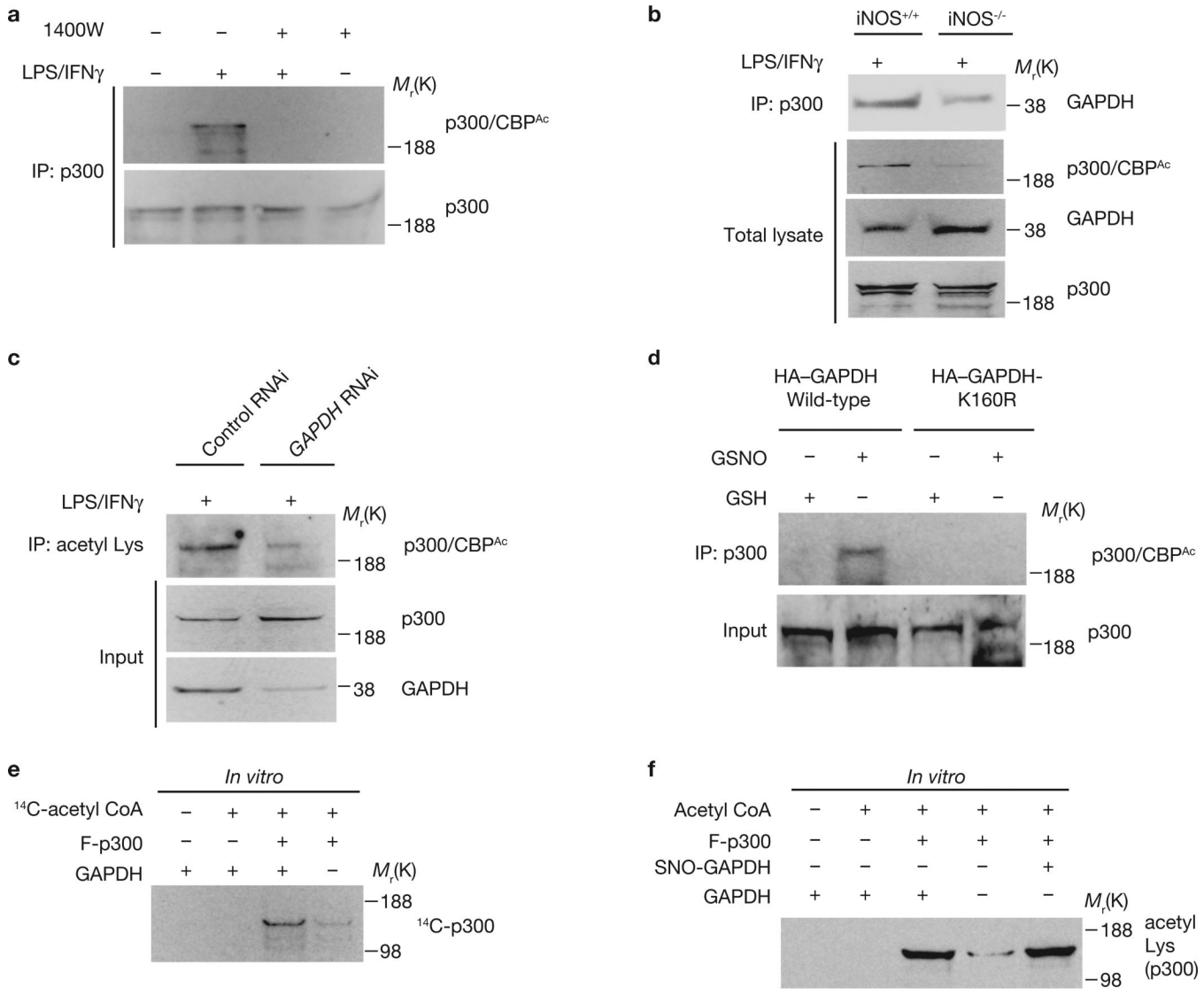

Figure 3.

GAPDH increases the catalytic activity of p300. (**a**) Increased acetylation of p300 occurs in RAW264.7 cells treated with LPS/IFNγ for 16 h; this was abolished by the iNOS inhibitor 1400W (100 μM). Cell lysates were immunoprecipitated with an anti-p300 antibody, and the immunoprecipitates were analysed by western blotting with an acetylation-specific p300/CBP antibody. (**b**) Genetic deletion of *iNOS* abolished the increased acetylation of p300/CBP and diminishes p300–GAPDH binding in peritoneal macrophages treated with LPS/IFNγ for 16 h. (**c**) Increased acetylation of p300 in RAW264.7 cells treated with LPS/IFNγ is abolished with depletion of GAPDH by RNAi. (**d**) GAPDH-K160R dominant-negative mutant diminished the increased acetylation of p300 elicited by GSNO in HEK293 cells. Forty-eight hours after transfection with wild-type HA–GAPDH or HA–GAPDH-K160R construct, cells were treated with 200 μM GSH or GSNO. (**e**) GAPDH augments auto-acetylation of a fragment of p300 (F-p300) *in vitro*. Auto-acetylation of F-p300 was assessed with ^{14}C-acetyl-CoA in the presence or absence of purified GAPDH. (**f**) GAPDH, native or NO-modified, augments auto-acetylation of a fragment of p300 (F-p300) *in vitro*. Auto-acetylation of p300 was analysed by western blotting with anti-acetyl Lys antibody. SNO-GAPDH, *S*-nitrosylated GAPDH.

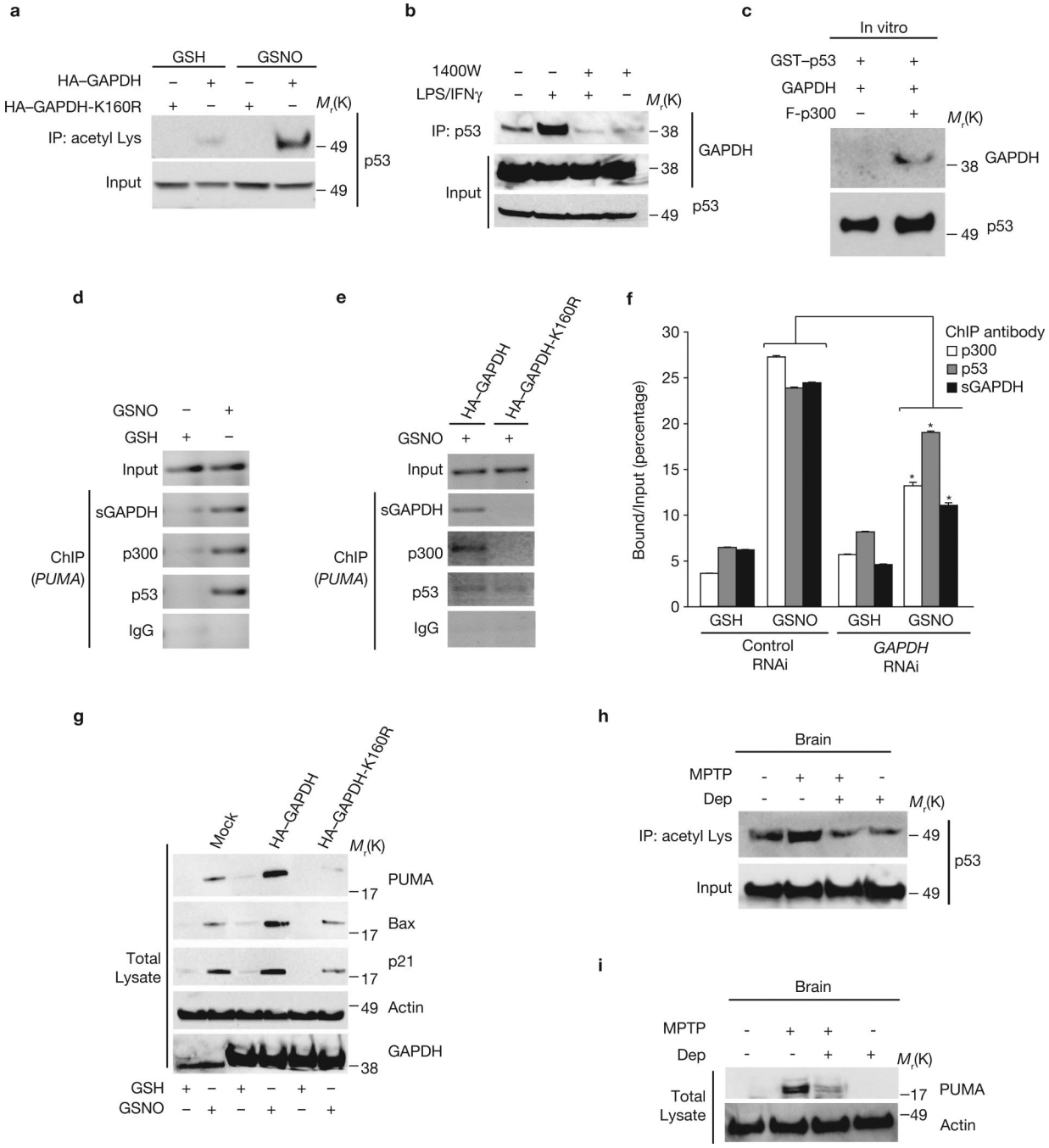

Figure 4.

GAPDH–p300 activates downstream targets, such as p53 and PUMA. (**a**) p53 is acetylated in GSNO-treated HEK293 cells; this is abolished by expression of GAPDH-K160R. Cell lysates were immunoprecipitated with anti-acetyl Lys antibody and the immunoprecipitates were analysed by western blotting with an anti-p53 antibody. (**b**) GAPDH–p53 binding was augmented by treatment with LPS/IFNγ for 16 h in RAW 264.7 cells. This was blocked by 1400W (100 μM). Cell lysates were immunoprecipitated with an anti-p53 antibody and the immunoprecipitates were analysed by western blotting with an anti-GAPDH antibody. (**c**) GAPDH–p300–p53 forms a complex *in vitro*, with p300 required for the interaction. Binding was examined by GSH-agarose pulldown assay. **(d)** Formation of a p53–p300-sulphonated

GAPDH (sGAPDH) complex at the *PUMA* promoter region in U2OS cells treated with GSNO, assayed by ChIP assay. Cells were treated with 200 μM GSH or GSNO for 24 h. (**e**) Formation of p53–p300–sGAPDH complex at the *PUMA* promoter region in U2OS cells with GSNO was blocked by expression of GAPDH-K160R. Forty-eight hours after transfection with HA–GAPDH or HA–GAPDH-K160R, cells were treated with 200 μM GSNO for 24 h. (**f**) RNAi depletion of GAPDH diminishes the formation of the p53–p300– sGAPDH complex at the *PUMA* promoter in U2OS cells. Forty-eight hours after transfection with control or *GAPDH* siRNA, cells were treated with 200 μM GSH or GSNO for 24 h. **P* < 0.001, *n* = 3, mean ± s.e.m., one-way ANOVA. (**g**) Overexpression of GAPDH, but not of GAPDH-K160R, in U2OS cells increases PUMA, BAX and p21 protein levels in the presence of GSNO. Forty-eight hours after transfection with mock, HA–GAPDH or HA–GAPDH-K160R constructs, cells were treated with 200 μM GSH or GSNO. (**h**) Acetylation of p53 was increased in brains of mice treated with MPTP, which was decreased by pre-treatment with *R*-(−)-deprenyl (Dep). (**i**) PUMA levels were increased in brains of mice treated with MPTP, effects prevented by pre-treatment with *R*-(−)-deprenyl (Dep).

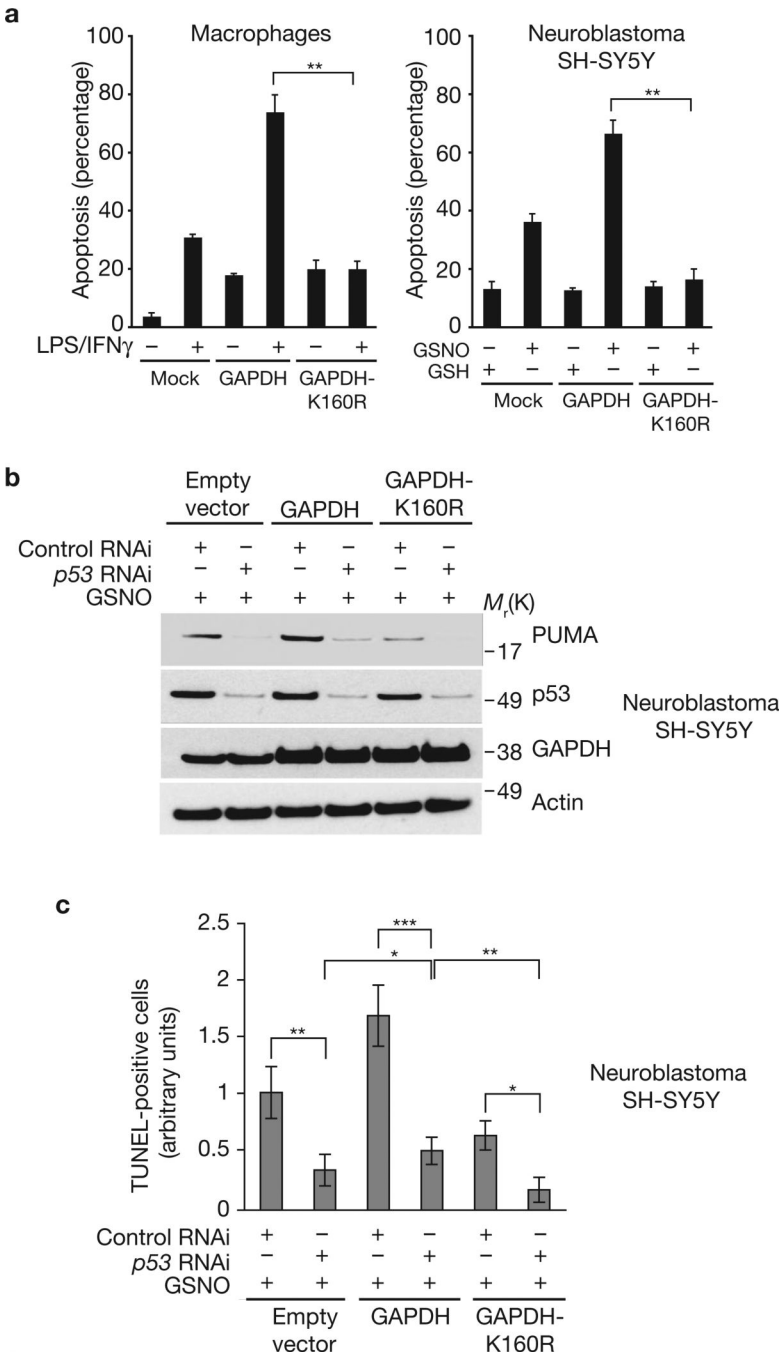

Figure 5.

Influence of GAPDH and GAPDH-K160R on cell death. (**a**) Increased apoptosis in peritoneal macrophages with LPS/IFNγ or in dopaminergic neuroblastoma SH-SY5Y cells with GSNO is blocked by GAPDH-K160R. Forty-eight hours after transfection with either mock, HA–GAPDH or HA–GAPDH-K160R, peritoneal macrophages isolated from wild-type mice were treated with LPS/IFNγ for 16 h. SH-SY5Y cells were treated with 200 μM GSH or GSNO for 24 h, after transfection with mock, HA–GAPDH- or HA–GAPDH-K160R. Apoptosis was assessed by counting condensed nuclei in transfected cells. $^{**}P < 0.001$, $n = 3$, mean ± s.e.m., one-way ANOVA. (**b**) PUMA levels are increased in SH-SY5Y cells stably overexpressing GAPDH but decreased in cells overexpressing GAPDH-K160R, in comparision with cells with

the empty vector, in the presence of 200 μM GSNO for 24 h. Depletion of endogenous p53 by RNAi significantly decreased the levels of PUMA. (**c**) Knockdown of p53 expression in SH-SY5Y cells stably overexpressing wild-type GAPDH or GAPDH-K160R in the presence of 200 μM GSNO for 24 h leads to decrease apoptosis, suggesting a major role of p53 in NO–GAPDH–p300/CBP death cascade. Cell death was measured by TUNEL assay. *$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$, $n = 4$, mean ± s.e.m., one-way ANOVA.