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(54) Titre : NOUVEAUX INHIBITEURS D'ACTIVITE PARP ET LEURS UTILISATIONS
(54) Title: INHIBITORS OF PARP ACTIVITY AND USES THEREOF

(57) **Abrégé/Abstract:**

The present invention relates to new inhibitors of the nucleic enzyme poly(adenosine 5'- diphospho-ribose) polymerase ["poly(ADP-ribose) polymerase" or "PARP", which is also sometimes called "PARS" for poly(ADP-ribose) synthetase]. More particularly, the invention relates to the use of PARP inhibitors to prevent and/or treat tissue damage resulting from cell damage or death due to necrosis or apoptosis; neural tissue damage resulting from ischemia and reperfusion injury; neurological disorders and neurodegenerative diseases; to prevent or treat vascular stroke; to treat or prevent cardiovascular disorders; to treat other conditions and/or disorders such as age-related macular degeneration, AIDS and other immune senescence diseases, arthritis, atherosclerosis, cachexia, cancer, degenerative diseases of skeletal muscle involving replicative senescence, diabetes, head trauma, immune senescence, inflammatory bowel disorders (such as colitis and Crohn's disease), muscular dystrophy, osteoarthritis, osteoporosis, chronic and acute pain (such as neuropathic pain), renal failure, retinal ischemia, septic shock (such as endotoxic shock), and skin aging; to extend the lifespan and proliferative capacity of cells; to alter gene expression of senescent cells; or to radiosensitize hypoxic tumor cells.

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(54) Title: INHIBITORS OF PARP ACTIVITY AND USES THEREOF

(57) Abstract: The present invention relates to new inhibitors of the nucleic enzyme poly(adenosine 5'- diphospho-ribose) polymerase ["poly(ADP-ribose) polymerase" or "PARP", which is also sometimes called "PARS" for poly(ADP-ribose) synthetase]. More particularly, the invention relates to the use of PARP inhibitors to prevent and/or treat tissue damage resulting from cell damage or death due to necrosis or apoptosis; neural tissue damage resulting from ischemia and reperfusion injury; neurological disorders and neurodegenerative diseases; to prevent or treat vascular stroke; to treat or prevent cardiovascular disorders; to treat other conditions and/or disorders such as age-related macular degeneration, AIDS and other immune senescence diseases, arthritis, atherosclerosis, cachexia, cancer, degenerative diseases of skeletal muscle involving replicative senescence, diabetes, head trauma, immune senescence, inflammatory bowel disorders (such as colitis and Crohn's disease), muscular dystrophy, osteoarthritis, osteoporosis, chronic and acute pain (such as neuropathic pain), renal failure, retinal ischemia, septic shock (such as endotoxic shock), and skin aging; to extend the lifespan and proliferative capacity of cells; to alter gene expression of senescent cells; or to radiosensitize hypoxic tumor cells.



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INHIBITORS OF PARP ACTIVITY AND USES THEREOF

Field of the Invention

[0001] The present invention relates to new inhibitors of the nucleic enzyme
5 poly(adenosine 5'-diphospho-ribose) polymerase ["poly(ADP-ribose) polymerase" or
"PARP", which is also sometimes called "PARS" for poly(ADP-ribose) synthetase]. More
particularly, the invention relates to the use of PARP inhibitors to prevent and/or treat
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tissue damage resulting from ischemia and reperfusion injury; neurological disorders and
10 neurodegenerative diseases; to prevent or treat vascular stroke; to treat or prevent
cardiovascular disorders; to treat other conditions and/or disorders such as age-related
macular degeneration, AIDS and other immune senescence diseases, arthritis,
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replicative senescence, diabetes, head trauma, immune senescence, inflammatory bowel
15 disorders (such as colitis and Crohn's disease), muscular dystrophy, osteoarthritis,
osteoporosis, chronic and acute pain (such as neuropathic pain), renal failure, retinal
ischemia, septic shock (such as endotoxic shock), and skin aging; to extend the lifespan and
proliferative capacity of cells; to alter gene expression of senescent cells; or to
radiosensitize hypoxic tumor cells.

20

Background

[0002] Poly(ADP-ribose) polymerase ("PARP") is an enzyme located in the nuclei of cells
of various organs, including muscle, heart and brain cells. PARP plays a physiological role

in the repair of strand breaks in DNA. Once activated by damaged DNA fragments, e.g., after exposure to chemotherapy, ionizing radiation, oxygen free radicals, or nitric oxide (NO), PARP catalyzes the transfer of ADP-ribose units from nicotinamide adenine dinucleotide (NAD⁺) to nuclear acceptor proteins, and is responsible for the formation of
5 protein-bound linear and branched homo-ADP-ribose polymers. The PARP's activation results in the attachment of up to 100 ADP-ribose units to a variety of nuclear proteins, including histones, topoisomerases, DNA and RNA polymerases, DNA ligases, Ca²⁺ - and Mg²⁺ - dependent endonucleases and PARP itself. While the exact range of functions of PARP has not been fully established, this enzyme is thought to play a role in enhancing
10 DNA repair and maintaining DNA integrity.

[0003] During major cellular stresses, however, the extensive activation of PARP can rapidly lead to cell damage or death through depletion of energy stores. Four molecules of ATP are consumed for every molecule of NAD (the source of ADP-ribose) regenerated. Thus, NAD, the substrate of PARP, is depleted by massive PARP activation and, in the
15 efforts to re-synthesize NAD, ATP may also be depleted.

[0004] It has been reported that PARP activation plays a key role in both NMDA- and NO-induced neurotoxicity, as shown by the use of PARP inhibitors to prevent such toxicity in cortical cultures in proportion to their potencies as inhibitors of this enzyme (ZHANG *et al.*, *Science*, vol.263, p:687-89, 1994); and in hippocampal slices (WALLIS *et al.*,
20 *NeuroReport*, vol.5(3), p :245-48, 1993). The potential role of PARP inhibitors in treating neurodegenerative diseases and head trauma has thus been known. Research, however, continues to pinpoint the exact mechanisms of their salutary effect in cerebral ischemia,

(ENDRES *et al.*, *J. Cereb. Blood Flow Metabol.*, vol.17, p:1143-51, 1997) and in traumatic brain injury (WALLIS *et al.*, *Brain Res.*, vol.710, p:169-77, 1996).

[0005] The PARP inhibitors are additionally useful for treating cardiovascular diseases.

Ischemia, a deficiency of oxygen and glucose in a part of the body, can be caused by an
5 obstruction in the blood vessel supplying that area or a massive hemorrhage. Two severe forms, heart attack and stroke, are major killers in the developed world. Cell death results directly and also occurs when the deprived area is reperfused. It has been demonstrated that single injections of PARP inhibitors have reduced the infarct size caused by ischemia and reperfusion of the heart or skeletal muscle in rabbits. In these studies, a single injection of
10 the PARP inhibitor, 3-amino-benzamide (10 mg/kg), either one minute before occlusion or one minute before reperfusion, caused similar reductions in infarct size in the heart (32-42%). Another PARP inhibitor, 1,5-dihydroxyisoquinoline (1 mg/kg), reduced infarct size by a comparable degree (38-48%; THIEMERMANN *et al.*, *Proc. Natl. Acad. Sci. USA*, vol.94, p:679-83, 1997). This finding has suggested that PARP inhibitors might be able to
15 salvage previously ischemic heart or skeletal muscle tissue. Presently, PARP inhibitors are being developed to treat ischemia/reperfusion injuries (ZHANG, *The Prospect for Improved Medicines*, Ashley Publications Ltd, 1999).

[0006] PARP activation has also been shown to provide an index of damage following neurotoxic insults by glutamate (via NMDA receptor stimulation), reactive oxygen
20 intermediates, amyloid .beta.-protein, n-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its active metabolite N-methyl-4-phenylpyridine (MPP⁺), which participate in pathological conditions such as stroke, Alzheimer's disease and Parkinson's disease (ZHANG *et al.*, *J. Neurochem.*, vol.65(3), p:1411-14, 1995). Other studies have continued

to explore the role of PARP activation in cerebellar granule cells in vitro and in MPTP neurotoxicity (COSI *et al.*, *Ann. N. Y. Acad. Sci.*, vol.825, p:366-79, 1997; COSI *et al.*, *Brain Res.*, vol.729, p:264-69, 1996).

[0007] Neural damage following stroke and other neurodegenerative processes is thought
5 to result from a massive release of the excitatory neurotransmitter glutamate, which acts upon the N-methyl-D-aspartate (NMDA) receptors and other subtype receptors. Glutamate serves as the predominate excitatory neurotransmitter in the central nervous system (CNS). Neurons release glutamate in great quantities when they are deprived of oxygen, as may occur during an ischemic brain insult such as a stroke or heart attack. This excess release of
10 glutamate in turn causes over-stimulation (excitotoxicity) of N-methyl-D-aspartate (NMDA), AMPA, Kainate and MGR receptors. When glutamate binds to these receptors, ion channels in the receptors open, permitting flows of ions across their cell membranes, e.g., Ca^{2+} and Na^{+} into the cells and K^{+} out of the cells. These flows of ions, especially the influx of Ca^{2+} , cause overstimulation of the neurons. The over-stimulated neurons secrete
15 more glutamate, creating a feedback loop or domino effect which ultimately results in cell damage or death via the production of proteases, lipases and free radicals. Excessive activation of glutamate receptors has been implicated in various neurological diseases and conditions including epilepsy, stroke, Alzheimer's disease, Parkinson's disease, Amyotrophic Lateral Sclerosis (ALS), Huntington's disease, schizophrenia, chronic pain,
20 ischemia and neuronal loss following hypoxia, hypoglycemia, ischemia, trauma, and nervous insult. Recent studies have also advanced a glutamatergic basis for compulsive disorders, particularly drug dependence. Evidence includes findings in many animal species, as well as, in cerebral cortical cultures treated with glutamate or NMDA, that

glutamate receptor antagonists block neural damage following vascular stroke (DAWSON *et al.*, *H. Hunt Batjer ed.*, p:319-25, 1997). Attempts to prevent excitotoxicity by blocking NMDA, AMPA, Kainate and MGR receptors have proven difficult because each receptor has multiple sites to which glutamate may bind. Many of the compositions that are effective in blocking the receptors are also toxic to animals. As such, there is no known effective treatment for glutamate abnormalities.

[0008] The stimulation of NMDA receptors, in turn, activates the enzyme neuronal nitric oxide synthase (NNOS), which causes the formation of nitric oxide (NO), which more directly mediates neurotoxicity. Protection against NMDA neurotoxicity has occurred following treatment with NOS inhibitors (DAWSON *et al.*, *Proc. Natl. Acad. Sci. USA*, vol.88, p:6368-71, 1991; DAWSON *et al.*, *J. Neurosci.*, vol.13(6), p:2651-61, 1993). Protection against NMDA neurotoxicity can also occur in cortical cultures from mice with targeted disruption of NNOS (DAWSON *et al.*, *J. Neurosci.*, vol.16(8), p:2479-87, 1996).

[0009] It is known that neural damage following vascular stroke is markedly diminished in animals treated with NOS inhibitors or in mice with NNOS gene disruption (IADECOLA, *Trends Neurosci.*, vol.20(3), p:132-39, 1997; HUANG *et al.*, *Science*, vol.265, p:1883-85, 1994; BECKMAN *et al.*, *Biochem. Soc. Trans.*, vol.21, p:330-34, 1993). Either NO or peroxynitrite can cause DNA damage, which activates PARP. Further support for this is provided in SZABO *et al.* (*Proc. Natl. Acad. Sci. USA*, vol.93, p:1753-58, 1996).

[00010] It is also known that PARP inhibitors affect DNA repair generally. CRISTOVAO *et al.* (*Terato., Carcino., and Muta.*, vol.16, p:219-27, 1996) discusses the effect of hydrogen peroxide and .gamma.-radiation on DNA strand breaks in the presence of and in the absence of 3-aminobenzamide, a potent inhibitor of PARP. CRISTOVAO *et al.*

observed a PARP-dependent recovery of DNA strand breaks in leukocytes treated with hydrogen peroxide.

[00011] Evidence also exists that PARP inhibitors are useful for treating inflammatory conditions such as inflammatory bowel disorders (SOUTHAN *et al.*, *Br. J. Pharm.*, vol.117, p:619-32, 1996; SZABO *et al.*, *J. Biol. Chem.*, vol.272, p:9030-36, 1997) or arthritis (SZABO *et al.*, *Portland Press Proc.*, vol.15, p:280-281, 1998; SZABO, *Eur. J. Biochem.*, vol.350(1), p:1-19, 1998; SZABO *et al.*, *Proc. Natl. Acad. Sci. USA*, vol.95(7), p:3667-72, 1998; SZABO *et al.*, *Proc. Natl. Acad. Sci. USA*, vol.93, p:1753-58, 1996; BAUER *et al.*, *Intl. J. Oncol.*, vol.8, p:239-52, 1996; HUGHES *et al.*, *J. Immuno.*, vol.153, p:3319-25, 1994). Thus, SALZMAN *et al.* (*Japanese J. Pharm.*, vol.75 (Supp. I), p:15, 1997) shows that 3-aminobenzamide, a specific inhibitor of PARP activity, reduced the inflammatory response and restored the morphology and the energetic status of the distal colon in rats suffering from colitis induced by intraluminal administration of the hapten trinitrobenzene sulfonic acid in 50% ethanol. As another example, SZABO *et al.* (*Japanese J. Pharm.*, vol.75 (Supp. I), p:102, 1997) discusses the ability of PARP inhibitors to prevent or treat collagen-induced arthritis.

[00012] Further, PARP inhibitors appear to be useful for treating diabetes and have been studied at the clinical level to prevent development of insulin-dependent diabetes mellitus in susceptible individuals (SALDEEN *et al.*, *Mol. Cellular Endocrinol.*, vol.139, p:99-107, 1998). In models of Type I diabetes induced by toxins such as streptozocin and alloxan that destroy pancreatic islet cells, it has been shown that knock-out mice lacking PARP are resistant to cell destruction and diabetes development (PIEPER *et al.*, *Trends Pharmacolog. Sci.*, vol.20, p:171-181, 1999; BURKART *et al.*, *Nature Medicine*, vol.5,

p:314-319, 1999). Administration of nicotinamide, a weak PARP inhibitor and a free-radical scavenger, prevents development of diabetes in a spontaneous autoimmune diabetes model, the non-obese, diabetic mouse (PIEPER *et al.*, 1999, aforementioned). Hence, potent and specific PARP inhibitors may be useful as diabetes-prevention therapeutics.

5 [00013] Further still, PARP inhibitors have been shown to be useful for treating endotoxic shock or septic shock (ZINGARELLI *et al.*, *Shock*, vol.5, p:258-64, 1996; CUZZOCREA, *Brit. J. Pharm.*, vol.122, p:493-503, 1997). ZINGARELLI *et al.* suggests that inhibition of the DNA repair cycle triggered by poly(ADP ribose) synthetase has protective effects against vascular failure in endotoxic shock. ZINGARELLI *et al.* found that nicotinamide
10 protects against delayed, NO-mediated vascular failure in endotoxic shock. ZINGARELLI *et al.* also found that the actions of nicotinamide may be related to inhibition of the NO-mediated activation of the energy-consuming DNA repair cycle, triggered by poly(ADP ribose) synthetase.

[00014] Yet another known use for PARP inhibitors is treating cancer. In fact, the PARP's
15 activity can contribute to the resistance that often develops to various types of cancer therapies, because this cellular ADP-ribose transfer process is associated with the repair of DNA strand breakage in response to DNA damage caused by radiotherapy or chemotherapy. Consequently, inhibition of PARP may retard intracellular DNA repair and enhance the antitumor effects of cancer therapy. Indeed, in vitro and in vivo data show that
20 many PARP inhibitors potentiate the effects of ionizing radiation (U.S. Pat. Nos. 5,032,617; 5,215,738; 5,041,653; 5,177,075) or cytotoxic drugs such as alkylating agents (WELTIN *et al.*, *Oncol. Res.*, vol.6(9), p:399-403, 1994). Thus, inhibitors of the PARP enzyme are useful as adjunct cancer chemotherapeutics.

[00015] Still another use for PARP inhibitors is the treatment of peripheral nerve injuries, and the resultant pathological pain syndrome known as neuropathic pain, such as that induced by chronic constriction injury (CCI) of the common sciatic nerve and in which transsynaptic alteration of spinal cord dorsal horn characterized by hyperchromatosis of cytoplasm and nucleoplasm (so-called "dark" neurons) occurs (MAO *et al.*, *Pain*, vol.72, p:355-366, 1997).

[00016] PARP inhibitors have also been used to extend the lifespan and proliferative capacity of cells including treatment of diseases such as skin aging (U.S. Pat. No. 5,589,483), Alzheimer's disease, atherosclerosis, osteoarthritis, osteoporosis, muscular dystrophy, degenerative diseases of skeletal muscle involving replicative senescence, age-related macular degeneration, immune senescence, AIDS, and other immune senescence diseases; and to alter gene expression of senescent cells.

[00017] Large numbers of PARP inhibitors have been described. For example, BANASIK *et al.* (*J. Biol. Chem.*, vol.267(3), p:1569-75, 1992) examined the PARP-inhibiting activity of over one hundred compounds, the most potent of which were 4-amino-1,8-naphthalimide, 6(5H)-phenanthridone, 2-nitro-6(5H)-phenanthridone, and 1,5-dihydroxyisoquinoline. GRIFFIN *et al.* reported the PARP-inhibiting activity for certain benzamide compounds (*Anti-Cancer Drug Design*, vol.10, p:507-514, 1995; U.S. Pat. No. 5,756,510), benzimidazole compounds (WO 97/04771), and quinalozinone compounds (WO 98/33802). SUTO *et al.* reported PARP inhibition by certain dihydroisoquinoline compounds (*Anti-Cancer Drug Design*, vol.7, p:107-117, 1991). GRIFFIN *et al.* have reported other PARP inhibitors of the quinazoline class (*J. Med. Chem.*, vol.41, p:5247-5256, 1998). Finally, WO 99/11622, WO 99/11623, WO 99/11624, WO 99/11628, WO

99/11644, WO 99/11645, and WO 99/11649 also describe various PARP-inhibiting compounds.

[00018] However, the approach of using these PARP inhibitors in the ways discussed above has been limited in effect. For example, side effects have been observed with some
5 of the best-known PARP inhibitors (MILAM *et al.*, *Science*, vol.223, p:589-91, 1984). Specifically, the PARP inhibitors 3-aminobenzamide and benzamide not only inhibited the action of PARP but also were shown to affect cell viability, glucose metabolism, and DNA synthesis. Thus, it was concluded that the usefulness of these PARP inhibitors may be severely restricted by the difficulty of finding a dose that will inhibit the enzyme without
10 producing additional metabolic effects.

[00019] Accordingly, there remains a need for compounds that inhibit more specifically PARP activity, compositions containing those compounds, and methods utilizing those compounds, wherein the compounds produce more potent and reliable effects with fewer side effects, with respect to inhibiting PARP activity and treating the diseases and
15 conditions discussed herein.

[00020] Macro-H2A1 and macroH2A2 histones are particularly enigmatic histone variants having an N-terminal region with high sequence homology to H2A, and also containing an extensive non histone C-terminal tail that comprises nearly two third of the protein (25 kDa). The human genome contains two genes that code for macroH2A histones. The
20 *MACROH2A1* gene encodes two subtypes MACROH2A1.1 and MACROH2A1.2 produced by alternative splicing. A second gene codes for MACROH2A2 (CHADWICK and WILLARD, *Human. Mol. Genet.*, vol.10, p:1101-1113, 2001). These proteins appear to be enriched in heterochromatin such as the inactive X chromosome (Xi) in female

mammals (COSTANZI and PEHRSON, *Nature*, vol.393, p:599-601, 1998), and discrete heterochromatic loci in senescent and quiescent cells (ZHANG *et al.*, *Dev. Cell*, vol.8, p:19-30, 2005; GRIGORYEV *et al.*, *J. Cell. Sci.*, vol.117, p:6153-6162, 2004). MacroH2A is highly localized in female cells as a distinct nuclear body, referred to as a macro chromatin body (MCB), which is coincident with the Xi and the Barr body (COSTANZI and PEHRSON, *aforementioned*, 1998). The C-terminal region of macroH2A contains a domain termed "macro domain", which is found alone or in multiple copies in a number of otherwise unrelated proteins (PEHRSON and FUJI, *Nuc. Acids Res.*, vol.26, p:2837-2849, 1998), and which is critical for macroH2A MCB formation (CHADWICK *et al.*, *Nuc. Acids Res.*, vol.29(13), p:2699-2705, 2001). It has been suggested that this macro domain defines a superfamily of phosphoesterases that act on ADP ribose derivatives (ALLEN *et al.*, *J. Mol. Biol.*, vol.330, p:503-511, 2003). Recently, KUSTATSCHER *et al.* (*Nat. Struct. Mol. Biol.*, vol.12(7), p:624-5, 2005) shows that macroH2A1.1 binds to monomeric ADP-ribose and to O-acetyl-ADP-ribose (a NAD metabolite). The authors identify Phe348, Asp203, Gly224 and Gly314 as critical residues for O-acetyl-ADP-ribose binding. Nevertheless, the specific macroH2A function is still unknown.

Summary of the Invention

[00021] The inhibitor of the nucleic enzyme poly(adenosine 5'-diphospho-ribose) polymerase ["poly(ADP-ribose) polymerase (PARP) of the invention have a phosphoesterase activity, and comprises an amino acid sequence, which is derived from the C-terminal non-histone domain of a macroH2A histone, optionally fused and/or coupled to at least one heterologous sequence.

[00022] As used therein, a “phosphoesterase activity” means the catalysis of the hydrolysis of one of the two ester bonds in a phosphodiester compound.

[00023] In another embodiment, the composition of the invention comprises (i) an inhibitor of the nucleic enzyme poly(adenosine 5'-diphospho-ribose) polymerase ["poly(ADP-ribose) polymerase (PARP) having a phosphoesterase activity, and comprising an amino acid sequence, which is derived from the C-terminal non-histone domain of a macroH2A histone, optionally fused and/or coupled to at least one heterologous sequence, a nucleic acid coding thereof, or a vector comprising said nucleic acid, and (ii) a pharmaceutically acceptable vehicle.

[00024] In an additional embodiment, a method of prophylactic or therapeutic treatment of a subject suffering from a disease associated with PARP activation comprising the step of administering an effective amount of a composition comprising (i) an inhibitor of the nucleic enzyme poly(adenosine 5'-diphospho-ribose) polymerase ["poly(ADP-ribose) polymerase (PARP) having a phosphoesterase activity, and comprising an amino acid sequence, which is derived from the C-terminal non-histone domain of a macroH2A histone, optionally fused and/or coupled to at least one heterologous sequence, a nucleic acid coding thereof, or a vector comprising said nucleic acid, and (ii) a pharmaceutically acceptable vehicle to said subject.

20 Brief Description of the Drawings

[00025] Figure 1 shows the immunofluorescence staining obtained with anti-HA antibody (anti-HA) on Hela cells stably expressing tagged macro-H2A1.1 (e-MH1.1) and H2A (e-H2A) histones, or not (control) compared to the chromatin localization (DAPI).

[00026] Figure 2 shows the signal obtained with anti-Flag antibody after immunoblotting of purified nuclear complexes from HeLa cells expressing the tagged H2A (e-H2A) and macroH2A1.1 (e-MH1.1) histones.

[00027] Figure 3 shows the silver staining of the separating polyacrylamide gel of anti-
5 FLAG/HA immunoprecipitation from nuclear extracts of Hela cells expressing tagged H2A (H2A com), and MacroH2A1.1 (MH1.1 com) histones respectively. The polypeptides identified by mass spectrometric analyses are indicated. M corresponds to the M12 protein molecular weight marker (INVITROGEN).

[00028] Figure 4 shows the Human recombinant PARP-1 protein in bounded (B) and
10 unbounded (U) fraction to the recombinant GST (GST), GST-H2A (H2A), GST-macroH2A1.1 (MH1.1) or GST-NHR (Non Histone Region) proteins.

[00029] Figure 5 described the different steps of the Tap-Chip method. The purified chromatin is digested with micrococcal nuclease (Mnase). Then, the nucleosomes containing the tagged macro-H2A1.1 histone were purified by immunoprecipitation with
15 anti-HA and anti-FLAG antibodies. DNA is then purified and phosphorylated with T4 DNA kinase. An adenosine nucleotide is added to the 3' position of the fragment with Taq polymerase, and the obtained fragment is finally cloned in a plasmid for further sequencing.

[00030] Figure 6A shows the PCR amplification products obtained with *hsp70-1* promoter
20 (P) and coding (C) region primers from one extract of immunoprecipitated chromatin from Hela cells stably expressing tagged macro-H2A1.1 histone. Control amplification have been done with the same primers on chromatin from Hela cells before immuniprecipitation (input). Lane M correspond to a molecular ladder (1,000 pb ladder, INVITROGEN).

[00031] Figure 6B show the relative enrichment of macroH2A1.1 histone on promoter region (gray) versus the coding region (hatched) of *hsp70-1* gene in HeLa cells.

[00032] Figure 6C shows the PCR amplification products obtained with *hsp70-1* promoter (P) and coding (C) region primers from one extract of macroH2A1.1 histone immunoprecipitated chromatin from non-transfected HeLa cells. Lane M correspond to a molecular ladder (1,000 pb ladder, INVITROGEN).

[00033] Figure 7A shows the quantity of H2A or macroH2A1.1 histones, or of PARP-1 on *hsp70-1* promoter versus GAPDH promoter in HeLa cells after a heat shock (HS, +) or without any heat shock (-). Each result corresponds to the mean of three independent experiments.

[00034] Figure 7B shows the quantity of H3 or H3.3 histones, and of PARP-1 on *hsp70-1* promoter versus GAPDH promoter in HeLa cells after a heat shock (HS, +) or without any heat shock (-). Each result corresponds to the mean of three independent experiments.

[00035] Figure 8A shows the quantity of ADP-ribose on *hsp70-1* promoter versus GAPDH promoter in HeLa cells after a heat shock (HS, +) or without any heat shock (-). Each result corresponds to the mean of three independent experiments.

[00036] Figure 8B shows the quantity of acetylated H4 histone on *hsp70-1* promoter versus GAPDH promoter in HeLa cells after a heat shock (HS, +) or without any heat shock (-). Each result corresponds to the mean of three independent experiments.

[00037] Figure 9 shows the relative expression of PARP-1 30 in HeLa cells minutes after a heat shock (HS, +) or without any heat shock (-), and in HeLa cells transfected or not with a PARP-1 specific siRNA or an unrelated siRNA (Scr); and the relative expression of

macroH2A1 in cells transfected or not with a macroH2A1 specific siRNA or an unrelated siRNA (Scr).

[00038] Figure 10A shows the relative expression of hsp70-1 mRNA versus GAPDH mRNA 30 minutes in HeLa cells after a heat shock (HS, +) or without any heat shock (-) by
5 real-time PCR. Said cells have been transfected or not with unrelated siRNA (scrambled), PARP-1 specific siRNA (PARP-1), and macroH2A1 specific siRNA (MacroH2A1).

[00039] Figure 10B shows a kinetic analysis by real-time PCR of hsp70-1 expression versus GAPDH expression in HeLa cells transfected with macroH2A1 specific siRNA (+siRNA macro-H2A1) or without any siRNA (-siRNA macro-H2A1). Said assays were
10 realized on transfected cells 10, 20 or 30 minutes after a heat shock (HS, +) or in the absence of any heat shock (-). The results are the mean of three independent experiments.

[00040] Figure 11 shows the sequence alignment of macroH2A1.1 (NP_613075) macroH2A1.2 (NP_004884) and macroH2A2 (NP_061119) macro domains with some
15 selected macro domains of non-histone proteins. Residues conserved in the macro domain are marked in red. The residues altered by the alternative splicing of the *MacroH2A1* gene are underlined. This altered region is predicted to bind phosphate groups and to hydrolyze the ADP-ribose (GDIT consensus motif). AF1521 is a protein from *Archaeoglobus fulgidus* with homology to the macro domain of MacroH2A. AF1521 is crystallized. NP_598908.1 is an AF1521 orthologue from mouse. YBR022WP is a *S. cerevisiae* protein with
20 exclusively a macro domain. YBR022WP was reported to process the 1''-phosphat group of 1''-phospho-ADP-ribose.

[00041] Figure 12 shows the kinetic analysis of PARP-1 auto-ADP-ribosylation activity of recombinant PARP-1, purified macroH2A1.1 complex (MH1.1), or purified macroH2A1.1 mutant complex (MH1.1-mut.com) in the presence of ^{32}P -NAD $^{+}$.

[00042] Figure 13 shows the amount of DNA immuno-precipitated with Hsp70.1, Hsp70.2
5 or Hsp70.8 promoter's probe as a function of percent input DNA.

[00043] Figure 14 shows the stable association of PARP-1 with the e-mH2A1.1 complex after isolation of said complex on a 15-35% glycerol gradient, fractionation of said gradient and loading of fractions (1 to 9) on a 4-12% SDS PAGE. The positions of the core histones, e-mH2A1.1 and PARP-1 were indicated at the left part of the figure. M,
10 molecular mass marker.

[00044] Figure 15 shows isolated e-mH2A1.1 complex (com) after separation on a 12% PAGE, and silver stained (left panel), and western-blot of the complex revealed by anti-ADP ribose antibody (right panel). M, molecular markers with the molecular masses indicated at the left.

[00045] Figure 16 shows the sequence alignment of macroH2A with known phosphoesterases. The two conserved tetrapeptide signatures in *S. cerevisiae* (P53314, first row), *A. thaliana* (Y11650, second row) and human (BC006392.1, third row) phosphoesterases are shown and aligned with human mH2A1.1 (fourth row), human mH2A1.2 (fifth row) and human mH2A2 (sixth row). These alignments highlight the
15 importance of the HXTX consensus motif (eighth row) located in the active site. This motif
20 was mutated to alanine (AAAA) in mH2A1.1 (mH2A1.1-mut, seventh row).

[00046] Figure 17 shows the altered binding of mono-ADP-ribose to the mutated mH2A1.1. Recombinant wild type (WT) or the mutated (Mut) mH2A1.1 were purified to

homogeneity. . Increasing amounts of both proteins were loaded (in duplicate) on filters. One filter was then incubated with ^{32}P -ADP-ribose (upper panel), whereas the other one was stained with Coomassie blue as a control for equal loading (lower panel).

[00047] Figure 18 shows that increasing the ionic strength releases the mutated, but not the
5 WT e-mH2A1.1 protein, from the nucleosome complex. The wild type (WT) and mutated mH2A1.1 nucleosome complexes were isolated from cell lines stably expressing the WT or the mutated e-mH2A1.1 histone using either 150 mM NaCl (left panel) or at 300 mM NaCl (right panel). The complexes were run on a 4-12% PAGE gradient containing SDS, then silver stained. The positions of emH2A1.1 and PARP-1 are indicated. M, protein molecular
10 mass marker. The right panel presents the quantification of PARP-1 (relative to histone H1) within the WT and mutated emH2A1.1 complexes, isolated in 150 mM and 300 mM NaCl, respectively. Note the drastic decrease of the amount of PARP-1 within the mutated mH2A1.1 complex isolated at 300 mM NaCl.

[00048] Figure 19 shows the amount of PARP-1 associated *in vivo* with the *Hsp70.1*
15 promoter in non-heat-shocked (HS : -) or heat-shocked (for 30 min at 42°C ; HS : +) stable cell lines expressing wild type (WT) or mutated e-mH2A1.1 (mH2A1.1-Mut). The cell lines were treated with formaldehyde to crosslink the proteins to DNA, and ChIP was carried out using anti-PARP-1 antibody. Amounts of the real time PCR amplified *Hsp70.1* promoter DNA fragments are presented as a percent of input DNA.

20 [00049] Figure 20 shows the silver staining of the purified wild type macroH2A1.1 (e-mH2A1.1) and the mutant (e-mH2A1.1-mut) nucleosomes used for measurement of PARP-1 enzymatic activity. The complexes were isolated using 100 mM NaCl. The bands corresponding to PARP-1, e-mH2A1.1 and the conventional core histones are indicated. M, protein molecular mass marker.

[00050] Figure 21 shows the quantification of the data shown in figure 12.

[00051] Figure 22 shows 12% SDS-PAGE of the purified e-mH2A1.1 octamers and associated PARP-1. e-mH2A1.1 nucleosome complexes were loaded on a hydroxyl apatite column and after washing with 0.65M NaCl the remaining proteins were eluted with 2M buffered solution of NaCl. To purify PARP-1 from the histone octamers, the 2M NaCl eluate was supplemented with 1 M urea and passed through an Agarose-nickel column. Lane 3, the protein composition of the 2M NaCl eluate. Lane 4, the purified PARP-1. Lanes 1 and 2, molecular mass marker and conventional histone octamer as a control.

[00052] Figure 23 shows the kinetic analysis of PARP-1 activity associated with *in vitro* reconstituted nucleosomes containing either purified e-mH2A1.1 core histones (lanes, 6-9) or conventional core histones (lanes, 2-5). The samples were incubated with ^{32}P -NAD $^{+}$ and run on 12% PAGE containing SDS. Lane 1, contains ^{32}P -NAD $^{+}$ only.

[00053] Figure 24 shows that the suppression of the expression of mH2A1 with siRNA downregulates the amount of PARP-1 associated with the *Hsp70.1* promoter. Control non-treated (-siRNA-mH2A1) and siRNA-treated (+siRNA-mH2A1) HeLa cells were crosslinked with formaldehyde and were used for ChIP with anti-PARP-1 antibody. DNA was isolated from both ChIP samples and submitted to real time PCR amplification with primers specific for the *Hsp70.1* promoter.

20 Detailed Description

[00054] The inventors have now discovered that macroH2A histone can specifically bind PARP-1 through its C-terminal non-histone domain, and consequently repress the PARP-1 activity through the phosphatase activity of its macro domain.

[00055] This invention provides a new tool to inhibit the nucleic enzyme poly(adenosine 5'-diphospho-ribose) polymerase ["poly(ADP-ribose) polymerase" (PARP) and methods to treat and/or prevent PARP activation associated diseases.

[00056] Consequently, in one aspect the present invention relates to an inhibitor of the
5 nucleic enzyme poly(adenosine 5'-diphospho-ribose) polymerase ["poly(ADP-ribose) polymerase" having a phosphoesterase activity, and comprising an amino acid sequence, which is derived from the C-terminal non-histone domain of a macroH2A histone, optionally fused and/or coupled to at least one heterologous sequence.

[00057] Advantageously, said inhibitor is a synthetic or a recombinant polypeptide.

10 [00058] As used herein the term "macroH2A histone" relates to an unusual histone H2A variant that have a large C-terminal non-histone domain (for a review, see PERCHE *et al.*, *Med. Sci.*, vol.19(11), p:1137-45, 2003). Actually, three human macroH2A histones, having such C-terminal non-histone domain, have been identified: macroH2A1.1 and macroH2A1.2 produced by alternative splicing from a single gene, and macroH2A2.

15 [00059] As used herein the term "C-terminal non-histone domain" relates to the large C-terminal region of macroH2A histone that shares no homology with histone H2A. Said non-histone domain can be identified from one of skills in the art with simple sequence analysis. As an example, said non-histone domain corresponds to residues 121 to 369 of human macroH2A1.1 (SEQ ID NO:1), residues 121 to 371 of human macroH2A1.2 (SEQ
20 ID NO: 2), and residues 121 to 372 of human macroH2A2 (SEQ ID NO: 3).

[00060] Advantageously, the C-terminal non-histone domain of macroH2A histone is selected in the group comprising the C-terminal non-histone domain of macroH2A1.1, macroH2A1.2, and macroH2A2 histones, preferably the C-terminal non-histone domain of

human macroH2A1.1, macroH2A1.2, and macroH2A2 histones. Preferably, said C-terminal non-histone domain corresponds to the C-terminal non-histone domain of macroH2A1.1.

[00061] As used herein an “heterologous sequence” relates to any amino acid sequence
5 which is not derived from macroH2A histone variants, like macroH2A1.1, macroH2A1.2, and macroH2A2. This heterologous sequence can for example consist of an amino acids sequence, which facilitates penetration of the inhibitor of the invention from the outside medium into the intracellular medium, and quite specifically into the nucleus of the cell. Such amino acids sequence are well known from one of skill in the art, and examples of
10 such amino acids sequence are described in EP 1512696, WO 02/10201, EP 15226183, and WO 2004/069279. This heterologous sequence can also for example facilitate the purification of the inhibitor from bacteria. Such amino acids sequence are also well known from one of skill in the art, and examples of such amino acids sequence include His tag, GST protein, FLAG tag, and HA tag.

15 [00062] An amino acid sequence “derived from” or a “derivative of” the C-terminal non-histone domain of macroH2A histone selected in the group comprising the C-terminal non-histone domain of human macroH2A1.1 (amino acid 121 to 367 of SEQ ID NO:1), of human macroH2A1.2 (amino acids 121 to 371 of SEQ ID NO:2), and of human macroH2A2 (amino acids 121 to 372 of SEQ ID NO:3) relates to amino acid sequence
20 sharing an identity of more than 60 % with said C-terminal non-histone domain or fragments thereof, for example of more than 70% or of more than 80%, preferably of more than 85%, most preferably of more than 90% and advantageously of more than 95%.

[00063] The identity differences between the above described C-terminal non-histone domains and the amino acid sequence of the inhibitor of the invention result from amino acids substitution in the amino acid sequence of said inhibitor.

[00064] Preferably, the substituted amino acid(s) in these C-terminal non-histone domains
5 domains preserve or increase the phosphoesterase activity of said domains. Such substitutions can be identified by one of skills in the art in view of its general knowledge and/or with simple experiments. Preferably, said substitutions correspond to amino acid residues having the same charge, hydrophobicity, sterical hindrance, and/or chemical function as related to the corresponding residues in the C-terminal non-histone domains of
10 macroH2A histones.

[00065] According to a specific embodiment, said amino acid sequence have an identity of 100% with said macroH2A C-terminal non-histone domain or fragments thereof.

[00066] Advantageously, said amino acid sequence which is derived from the non-histone domain of a macroH2A histone is less than 350 amino acids in length, preferably less than
15 300 amino acids, as an example less than 250 amino acids or less than 200 amino acids in length, and more preferably less than 150 amino acids.

[00067] According to a preferred embodiment, said amino acid sequence which is derived from the non-histone domain of a macroH2A histone does not comprise the macroH2A histone fold domain having homology with histone H2A (LUGER *et al.*, *Nature*, vol.389
20 (6648), p: 251-260, 1997), herein incorporated by reference), and preferably said amino acid sequence does not comprise any sequence having homology with histone H2A.

[00068] Advantageously, said amino acid sequence does not comprise any histone fold domain.

[00069] Advantageously, said amino acid sequence which is derived from the non-histone domain of a macroH2A histone is more than 20 amino acids in length, preferably more than 25 amino acids, as an example more than 35 amino acids or more than 50 amino acids in length, and more preferably more than 60 amino acids.

5 [00070] According to a preferred embodiment, said amino acid sequence, which is derived from the non-histone domain of a macroH2A histone comprises the macro domain of a macroH2A histone.

[00071] As used herein the term “macro domain” relates to a region present in the C-terminal non-histone domain of macroH2A histones, and which is also found alone or in
10 multiple copies in a number of otherwise unrelated proteins (PEHRSON and FUJI, 1998, aforementioned ; ALLEN *et al.*, 2003, aforementioned). Said macro domain can be also simply identified from one of skills in the art with simple sequence analysis. As an example, said macro domain corresponds to residues 184 to 369, preferably 202 to 369 of human macroH2A1.1 (SEQ ID NO:1), residues 183 to 371, preferably 201 to 371 of
15 human macroH2A1.2 (SEQ ID NO:2), and residues 184 to 372, preferably 202 to 372 of human macroH2A2 (SEQ ID NO:3). Said macro domain includes critical residues for phosphoesterase activity, some of which are identified in the examples below, and in KUSTATSCHER *et al.* (2005, abovementioned, herein incorporated by reference). Some potentially critical residues for phosphoestrerase activity can also be identified as the
20 conserved residues in macro domains of AF1521 family members described in ALLEN *et al.* (2003, abovementioned, herein incorporated by reference). Finally, said macro domain of macroH2A histone is potentially an ADP binding domain as described in KARRAS *et al.* (*EMBO journal*, vol.24(11), p: 1911-1920, 2005: See Figures 6A and B).

[00072] In a second aspect the invention relates to a nucleic acid encoding for an inhibitor as described above.

[00073] Said nucleic acid corresponds to RNA or DNA, preferably to DNA.

[00074] According to a particular embodiment, the nucleic acid encoding the inhibitor is
5 operatively linked to a gene expression sequence, which directs the expression of nucleic acid within a prokaryotic or an eukaryotic cell, preferably an eukaryotic cell. The "gene expression sequence" is any regulatory nucleotide sequence, such as a promoter sequence or promoter-enhancer combination, which facilitates the efficient transcription and translation of the inhibitor to which it is operatively linked. The gene expression sequence
10 may, for example, be a mammalian or viral promoter, such as a constitutive or inducible promoter. Constitutive mammalian promoters include, but are not limited to, the promoters for the following genes: hypoxanthine phosphoribosyl transferase (HPTR), adenosine deaminase, pyruvate kinase, beta.-actin promoter, muscle creatine kinase promoter, human elongation factor promoter and other constitutive promoters. Exemplary viral promoters
15 which function constitutively in eukaryotic cells include, for example, promoters from the simian virus (e.g., SV40), papilloma virus, adenovirus, human immunodeficiency virus (HIV), cytomegalovirus (CMV), Rous sarcoma virus (RSV), hepatitis B virus (HBV), the long terminal repeats (LTR) of Moloney leukemia virus and other retroviruses, and the thymidine kinase promoter of herpes simplex virus. Others constitutive promoters are
20 known to those of ordinary skill in the art. The promoters useful as gene expression sequences of the invention also include inducible promoters, such as promoters inducible under stress conditions. Inducible promoters are expressed in the presence of an inducing agent. For example, the *hsp70-1* promoter is induced to promote transcription and

translation after a heat shock. Others inducible promoters are known to those of ordinary skill in the art.

[00075] In general, the gene expression sequence shall include, as necessary, 5' non-transcribing and 5' non-translating sequences involved with the initiation of transcription and translation, respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5' non-transcribing sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined antigen nucleic acid. The gene expression sequences optionally include enhancer sequences or upstream activator sequences as desired. Preferably, the gene expression sequence include a nuclear localization signal (NLS) fused to the nucleic acid sequence coding for the inhibitor of the invention in order to facilitate translocation of said inhibitor into the nucleus of the cell. NLS sequences are well known from one of skills in the art.

[00076] As used herein, the inhibitor nucleic acid sequence and the gene expression sequence are said to be "operably linked" when they are covalently linked in such a way as to place the expression or transcription and/or translation of the inhibitor coding sequence under the influence or control of the gene expression sequence. Two DNA sequences are said to be operably linked if induction of a promoter in the 5' gene expression sequence results in the transcription of the inhibitor sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the inhibitor sequence, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a gene expression sequence would be operably linked to a inhibitor nucleic acid sequence if the gene expression sequence were capable of effecting

transcription of that nucleic acid sequence such that the resulting transcript is translated into the desired protein or polypeptide.

[00077] The inhibitor nucleic acid may be delivered *in vivo* alone or in association with a vector.

5 [00078] In a third aspect the invention relates to a vector comprising a nucleic acid sequence as previously described.

[00079] In its broadest sense, a "vector" is any vehicle capable of facilitating the transfer of the inhibitor nucleic acid to the cells and preferably cells expressing nucleic enzyme poly(adenosine 5'-diphospho-ribose) polymerase ["poly(ADP-ribose) polymerase" (PARP).

10 Preferably, the vector transports the nucleic acid to cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector. The vector optionally includes the above-described gene expression sequence to enhance expression of the inhibitor nucleic acid in PARP expressing cells. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles
15 derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the inhibitor nucleic acid sequences. Viral vectors are a preferred type of vector and include, but are not limited to nucleic acid sequences from the following viruses: retrovirus, such as moloney murine leukemia virus, harvey murine sarcoma virus, murine mammary tumor virus, and rouse sarcoma virus; adenovirus, adeno-associated
20 virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors not named but known to the art.

[00080] Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses, the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes in vivo. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in KRIEGLER (A Laboratory Manual," W.H. Freeman C.O., New York, 1990) and in MURRY ("Methods in Molecular Biology," vol.7, Humana Press, Inc., Clifton, N.J., 1991).

[00081] Preferred viruses for certain applications are the adeno-viruses and adeno-associated viruses, which are double-stranded DNA viruses that have already been approved for human use in gene therapy. The adeno-associated virus can be engineered to be replication deficient and is capable of infecting a wide range of cell types and species. It further has advantages such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hemopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner,

thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

[00082] Other vectors include plasmid vectors. Plasmid vectors have been extensively described in the art and are well known to those of skill in the art. See e.g., SANBROOK *et al.*, "Molecular Cloning: A Laboratory Manual," Second Edition, Cold Spring Harbor Laboratory Press, 1989. In the last few years, plasmid vectors have been used as DNA vaccines for delivering antigen-encoding genes to cells *in vivo*. They are particularly advantageous for this because they do not have the same safety concerns as with many of the viral vectors. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operatively encoded within the plasmid. Some commonly used plasmids include pBR322, pUC18, pUC19, pRC/CMV, SV40, and pBlueScript. Other plasmids are well known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using restriction enzymes and ligation reactions to remove and add specific fragments of DNA. Plasmids may be delivered by a variety of parenteral, mucosal and topical routes. For example, the DNA plasmid can be injected by intramuscular, intradermal, subcutaneous, or other routes. It may also be administered by intranasal sprays or drops, rectal suppository and orally. It may also be administered into the epidermis or a mucosal surface using a gene-gun. The plasmids may be given in an aqueous solution, dried onto gold particles or in association with another

DNA delivery system including but not limited to liposomes, dendrimers, cochleate and microencapsulation.

[00083] The nucleic acid vector can include selectable markers that are active both in bacteria and in mammalian cells.

5 [00084] According to a preferred embodiment, the nucleic acid vector of the present invention corresponds to “naked DNA” like plasmids, cosmids or phagemids. Such naked DNA can be associated with non-lipid cationic polymers (WU and WU, *J. Biol. Chem.*, vol.263, p: 14621-4, 1988) or liposomes (BRIGHMAN *et al.*, *Am. J. Med. Sci.*, vol.298, p: 278-81, 1989) to form complexes enhancing cellular uptake.

10 [00085] According to another preferred embodiment, the nucleic acid vector is a viral vector adapted for *in vivo* gene therapy protocols. Examples of appropriate viral vectors includes retroviral vectors as described in EP 0871459, EP 0386882 and EP 1222300 and adenovirus vectors as described in US 2004/ 265273 and US 6,638,502. In this case, the internalization of virus occurs through the specific interaction of the viral envelope with a
15 cell surface receptor, followed by receptor-mediated endocytosis of the virus/receptor complex.

[00086] According to a fourth aspect the present invention relates to a composition, preferably a pharmaceutical composition, comprising (i) an inhibitor as described previously, a nucleic acid coding thereof, or a vector comprising said nucleic acid, and (ii)
20 a pharmaceutically acceptable vehicle.

[00087] The composition of the invention inhibits PARP activity, and can be used to prevent and/or to treat diseases associated with PARP activation. For example, the composition of the invention can be used to treat or prevent neural tissue damage resulting

from cell damage or death due to necrosis or apoptosis, like cardiovascular tissue damage resulting from cardiac ischemia or reperfusion injury or neurodegenerative diseases. The composition of the invention can be used to extend or increase the lifespan or proliferation of cells and thus to treat or prevent diseases associated therewith and induced or
5 exacerbated by cellular senescence including skin aging, atherosclerosis, osteoarthritis, osteoporosis, muscular dystrophy, degenerative diseases of skeletal muscle involving replicative senescence, age-related macular degeneration, immune senescence, AIDS and other immune senescence diseases, and other diseases associated with cellular senescence and aging, as well as to alter the gene expression of senescent cells. The composition of the
10 invention can further be used to treat cancer and to radiosensitize hypoxic tumor cells to render the tumor cells more susceptible to radiation therapy and to prevent the tumor cells from recovering from potentially lethal damage of DNA after radiation therapy, presumably by their ability to prevent DNA repair.

[00088] Pharmaceutically acceptable vehicles are well known from one of skills in the art.
15 As an example of pharmaceutically acceptable vehicle, the composition may comprise emulsions, microemulsions, oil-in-water emulsions, anhydrous lipids and oil-in-water emulsions, other types of emulsions. The composition may also comprise one or more additives (e.g., diluents, excipients, stabilizers, preservatives). See, generally, *Ullmann's Encyclopedia of Industrial Chemistry*, 6th Ed. (various editors, 1989-1998, Marcel Dekker);
20 and *Pharmaceutical Dosage Forms and Drug Delivery Systems* (ANSEL *et al.*, 1994, WILLIAMS & WILKINS).

[00089] Inhibitor of the invention, nucleic acids encoding thereof or nucleic acid vectors comprising such nucleic acid may be solubilized in a buffer or water or incorporated in

emulsions and microemulsions. Suitable buffers include, but are not limited to, phosphate buffered saline $\text{Ca}^{++}/\text{Mg}^{++}$ free (PBS), phosphate buffered saline (PBS), normal saline (150 mM NaCl in water), Tris buffer and surfactants.

[00090] There are numerous causes of peptide instability or degradation, including
5 hydrolysis and denaturation. Hydrophobic interaction may cause clumping of molecules together (i.e. aggregation). This result may entail diminution of the PARP repression. Stabilizers may be added to lessen or prevent such problems.

[00091] Stabilizers include cyclodextrine and derivatives thereof (see U.S. Pat. No.5,730,969). Suitable preservatives such as sucrose, mannitol, sorbitol, trehalose,
10 dextran and glycerin can also be added to stabilize the final formulation. A stabilizer selected from ionic and non-ionic surfactants, D-glucose, D-galactose, D-xylose, D-galacturonic acid, trehalose, dextrans, hydroxyethyl starches, and mixtures thereof may be added to the formulation. Addition of alkali metal salt or magnesium chloride may stabilize a peptide. The peptide may also be stabilized by contacting it with a saccharide selected
15 from the group consisting of dextran, chondroitin sulphuric acid, starch, glycogen, dextrin, and alginic acid salt. Other sugars that can be added include monosaccharides, disaccharides, sugar alcohols, and mixtures thereof (e.g., glucose, mannose, galactose, fructose, sucrose, maltose, lactose, mannitol, xylitol). Polyols may stabilize a peptide, and are water-miscible or water-soluble. Suitable polyols may be polyhydroxy alcohols,
20 monosaccharides and disaccharides including mannitol, glycerol, ethylene glycol, propylene glycol, trimethyl glycol, vinyl pyrrolidone, glucose, fructose, arabinose, mannose, maltose, sucrose, and polymers thereof. Various excipients may also stabilize peptides, including serum albumin, amino acids, heparin, fatty acids and phospholipids, surfactants, metals,

polyols, reducing agents, metal chelating agents, polyvinyl pyrrolidone, hydrolysed gelatin, and ammonium sulfate.

[00092] Advantageously, said composition comprises the inhibitor of the invention, a nucleic acid coding thereof, or a nucleic acid vector in an amount sufficient to inhibit
5 PARP activity.

[00093] As an example, said composition can comprise a concentration of said inhibitor of more than 10^{-12} M, preferably more than 10^{-9} M, as an example more than 10^{-8} M or more than 10^{-6} M, and most preferably more than 10^{-3} M.

[00094] In a fifth aspect the present invention relates to a method of prophylactic or
10 therapeutic treatment of a subject suffering from a disease associated with PARP activation comprising the step of administering an effective amount of a composition as described above to said subject.

[00095] As used herein, the term "subject" denotes a Mammal, such as a rodent, a feline, a canine and a primate. The subject is an animal such as cow, pig, horse, chicken, cat, dog
15 and most preferably a human.

[00096] The inhibitors of the invention inhibit PARP activity and, thus, are believed to be useful for treating tissue damage resulting from cell damage or death due to necrosis or apoptosis, neural tissue damage resulting from ischemia and reperfusion injury, neurological disorders and neurodegenerative diseases; to prevent or treat vascular stroke;
20 to treat or prevent cardiovascular disorders; to treat other conditions and/or disorders such as age-related macular degeneration, AIDS and other immune senescence diseases, arthritis, atherosclerosis, cachexia, cancer, degenerative diseases of skeletal muscle involving replicative senescence, diabetes, head trauma, immune senescence, inflammatory

disorders like inflammatory bowel disorders such as colitis and Crohn's disease, muscular dystrophy, osteoarthritis, osteoporosis, chronic and/or acute pain (such as neuropathic pain), renal failure, retinal ischemia, septic shock (such as endotoxic shock), and skin aging; to extend the lifespan and proliferative capacity of cells; to alter gene expression of
5 senescent cells; or to radiosensitize tumor cells.

[00097] Examples of neurodegenerative diseases that are treatable by the method of the present invention include, without limitation, trigeminal neuralgia; glossopharyngeal neuralgia; Bell's Palsy; myasthenia gravis; muscular dystrophy; amyotrophic lateral sclerosis; progressive muscular atrophy; progressive bulbar inherited muscular atrophy;
10 herniated, ruptured or prolapsed invertebrate disk syndromes; cervical spondylosis; plexus disorders; thoracic outlet destruction syndromes; peripheral neuropathies such as those caused by lead, dapsone, ticks, porphyria, or Guillain-Barre syndrome; Alzheimer's disease; Huntington's Disease and Parkinson's disease.

[00098] Examples of cardiovascular disorders that can either cause ischemia or are caused
15 by reperfusion of the heart include, but are not limited to, coronary artery disease, angina pectoris, myocardial infarction, cardiovascular tissue damage caused by cardiac arrest, cardiovascular tissue damage caused by cardiac bypass, cardiogenic shock, and related conditions that would be known by those of ordinary skill in the art or which involve dysfunction of or tissue damage to the heart or vasculature, especially, tissue damage
20 related to PARP activation.

[00099] For example, the methods of the invention are useful for treating cancers and radiosensitizing tumor cells in cancers such as ACTH-producing tumors, acute lymphocytic leukemia, acute nonlymphocytic leukemia, cancer of the adrenal cortex,

bladder cancer, brain cancer, breast cancer, cervical cancer, chronic lymphocytic leukemia, chronic myelocytic leukemia, colorectal cancer, cutaneous T-cell lymphoma, endometrial cancer, esophageal cancer, Ewing's sarcoma, gallbladder cancer, hairy cell leukemia, head & neck cancer, Hodgkin's lymphoma, Kaposi's sarcoma, kidney cancer, liver cancer, lung cancer (small and/or non-small cell), malignant peritoneal effusion, malignant pleural effusion, melanoma, mesothelioma, multiple myeloma, neuroblastoma, non-Hodgkin's lymphoma, osteosarcoma, ovarian cancer, ovary (germ cell) cancer, prostate cancer, pancreatic cancer, penile cancer, retinoblastoma, skin cancer, soft-tissue sarcoma, squamous cell carcinomas, stomach cancer, testicular cancer, thyroid cancer, trophoblastic neoplasms, uterine cancer, vaginal cancer, cancer of the vulva and Wilm's tumor. Examples of additional therapeutic agents that may be used in conjunction with the inhibitor of the invention include, but are not limited to: 5-fluorouracil, leucovorin, 5'-amino-5'deoxythymidine, oxygen, carbogen, red cell transfusions, perfluorocarbons (e.g., Fluosol-DA), 2,3-DPG, BW12C, calcium channel blockers, pentoxifylline, antiangiogenesis compounds, hydralazine, L-BSO, adriamycin, camptothecin, carboplatin, cisplatin, daunorubicin, docetaxel, doxorubicin, interferon (alpha, beta, gamma), interleukin 2, irinotecan, paclitaxel, topotecan, and therapeutically effective analogs and derivatives of the same.

[000100] For medical use, the amount required of the inhibitor of the invention to achieve a therapeutic effect will vary according to the particular inhibitor administered, the route of administration, the mammal under treatment, and the particular disorder or disease concerned. A suitable systemic dose of the inhibitor of the invention for a mammal suffering from, or likely to suffer from, any condition as described herein is typically in the

range of about 0.001 mg/kg to about 50 mg/kg. Typically, dosage levels on the order of about 0.1 mg to about 10,000 mg of the inhibitor are useful in the treatment of the above conditions, with preferred levels being about 0.1 mg to about 1,000 mg. The specific dose level for any particular patient will vary depending upon a variety of factors, including the activity of the specific compound employed; the age, body weight, general health, sex, and diet of the patient; the time of administration; the rate of excretion; any combination of the compound with other drugs; the severity of the particular disease being treated; and the form and route of administration. Typically, in vitro dosage-effect results provide useful guidance on the proper doses for patient administration. Studies in animal models can also be helpful. The considerations for determining the proper dose levels are well-known in the art.

[000101] In the methods of the present invention, the composition may be administered, for example, orally, parenterally, by inhalation spray, topically, rectally, nasally, buccally, sublingually, vaginally, intraventricularly, or via an implanted reservoir in dosage formulations containing conventional non-toxic pharmaceutically-acceptable carriers, adjuvants and vehicles.

[000102] The compositions used in the methods of the present invention may be administered by a single dose, multiple discrete doses or continuous infusion. Any administration regimen regulating the timing and sequence of delivery of the compound can be used and repeated as necessary to effect treatment. Such regimen may include pretreatment and/or co-administration with additional therapeutic agents.

[000103] To maximize protection of tissue damage, the composition of the invention should be administered to the affected cells as soon as possible.

[000104] In a sixth aspect the present invention relates to the use of an inhibitor as described above, a nucleic acid encoding thereof, or a nucleic acid vector comprising said nucleic acid for the manufacture of a medicament for the prevention or treatment of a subject suffering of from a disease associated with PARP activation.

5 [000105] The inhibitors of the invention inhibit PARP activity and, thus, are believed to be useful for treating tissue damage resulting from cell damage or death due to necrosis or apoptosis, neural tissue damage resulting from ischemia and reperfusion injury, neurological disorders and neurodegenerative diseases; to prevent or treat vascular stroke; to treat or prevent cardiovascular disorders; to treat other conditions and/or disorders such
10 as age-related macular degeneration, AIDS and other immune senescence diseases, arthritis, atherosclerosis, cachexia, cancer, degenerative diseases of skeletal muscle involving replicative senescence, diabetes, head trauma, immune senescence, inflammatory disorders like inflammatory bowel disorders such as colitis and Crohn's disease, muscular dystrophy, osteoarthritis, osteoporosis, chronic and/or acute pain (such as neuropathic
15 pain), renal failure, retinal ischemia, septic shock (such as endotoxic shock), and skin aging; to extend the lifespan and proliferative capacity of cells; to alter gene expression of senescent cells; or to radiosensitize tumor cells.

[000106] The present invention will be understood more clearly on reading the description of the experimental studies performed in the context of the research carried out by the
20 applicant, which should not be interpreted as being limiting in nature.

EXAMPLES

1) Plasmid construction

[000107] The cDNA clones corresponding to the complete coding sequence from H2A (NM_138609; nucleic acid 186 to 1295 of SEQ ID NO: 4), macroH2A1.1 (NM_004893; nucleic acid 174 to 1289 of SEQ ID NO: 5) and macroH2A1.2 (NM_018649; nucleic acid 214 to 1332 of SEQ ID NO: 6) histones were PCR-amplified from image clones purchased from INVITROGEN with primers incorporating restriction enzyme recognition sites. The amplified PCR fragments were then subcloned into the Xho I-Not I sites of pREV-HTF retroviral vector or pGEX-5X.1 vector (AMERSHAM) according to the manufacturer's instruction. Constructs were sequenced to ensure sequence integrity.

10 2) Expression and localization of macroH2A1.1

[000108] MacroH2A1.1 and H2A histones were each stably expressed as fusion proteins with N-terminal double HA and double Flag epitope tags (e-MH1.1, and e-H2A) in HeLa cells by retroviral transduction according to standard protocols. Immunofluorescence experiments were performed on stably transfected cells using Rat anti-HA antibody (ROCHE, 1:300 dilution), and a goat anti-rat IgG coupled to Alexa Fluor 488 (MOLECULAR PROBES, 1:400 dilution) as a secondary antibody according to the manufacturers' instruction. The localization of chromatin was assessed in the same cells with DAPI coloration.

[000109] The results reveal that the tagged histones colocalize with chromatin (see Figure 1), indicating that the presence of the tag epitopes do not interfere with their deposition. In contrast to tagged H2A histone (e-H2A), that shows a broad staining in the nucleus, tagged macroH2A1.1 histone (e-MH1.1) shows a localized staining restricted mainly to condensed

chromatin. We conclude from these data that tagged macroH2A1.1 histone (e-MH1.1) and tagged H2A histone (e-H2A) are both functionally deposited into nucleosomes *in vivo*.

3) Purification of macroH2A1.1 associated nucleosomes

[000110] The nuclear pellets prepared from the above described HeLa cells expressing the H2A and macroH2A1.1 proteins fused with N-terminal double-HA and double-FLAG epitope tags (e-H2A/e-MH1.1) were digested with micrococcal nuclease to give predominantly mononucleosomes as described in SOLLNER-WEBB and FELSENFELD (*Biochemistry*, vol.14(3), p: 2915-20, 1975). Mononucleosomes containing e-H2A or e-MH1.1 were purified from the resulting material by immunoprecipitation on anti-FLAG antibody-conjugated agarose (SIGMA). The bound nucleosomes were eluted with the FLAG peptide (DYKDDDDK; SEQ ID NO: 7), and were further affinity purified by anti-HA antibody-conjugated agarose (SIGMA) and eluted with the HA peptide (YPYDVPDYA; SEQ ID NO: 8). A small fraction of the purified complexes has been analyzed with an anti-FLAG antibody (ROCHE) on SDS-PAGE gel to assess for its enrichment in tagged macroH2A1.1 and H2A histones.

[000111] The immunoblotting with anti-flag antibody revealed one specific band at the expected size for tagged macroH2A1.1 or H2A histones (see Figure 2). The biochemical fractionation has revealed that only a small fraction of macroH2A1.1 histone was found to be present as a soluble protein in the nuclear extract, and that most of the protein was found to be present in the nuclear pellet tightly associated to chromatin. We have found that tagged macroH2A1.1 histone can be efficiently solubilized together with mononucleosomes by digesting chromatin with controlled amount of micrococcal nuclease.

[000112] Then, the purified complexes containing tagged macroH2A1.1 or H2A histones were run on 12 % denaturing polyacrylamide gel, which was silver stained using SilverQuest® kit (INVITROGEN) according to the manufacturer's instruction. Finally, the different gel separated polypeptides were identified by mass spectrometry. As a control, we

5 performed the same purification on untransduced HeLa cells.

[000113] Numerous polypeptides were found associated with e-mH2A1.1 and e-H2A nucleosomes (figure 3).

[000114] Mass spectrometric analyses, identified histones H3, H4, H2A, H2B, mH2A1.1 Ku 80, KU 70, Hsp70, HDAC1, the two subtypes of H1 (H1.1 and H1.2), Hp1 α , Hp1 β and

10 poly(ADP-ribose) polymerase I (PARP-1) as common components in both e-mH2A1.1 and e-H2A complexes (figure 3). A comprehensive list of the identified proteins, with their accession numbers, is presented in the following table.

[000115] List of proteins associated with H2A and mH2A1.1 nucleosome complexes.

H2A	mH2A1.1	Accession numbers
	PHF-14	O94880
PARP-1	PARP-1	P09874
	Topo I	P11387
	DDX 21	Q9NR30
KU 80	KU 80	P13010
KU 70	KU 70	P12956
	PRMT5	AAH25979
Hsp 70	Hsp 70	P17066
HDAC1	HDAC1	Q13547
	Hp1- bp 74	CAI12528
	MEP 50	Q9BQA1
	RCC 1	P18754

	DEK	P35659
mH2A1	mH2A1	O75367
H1.2	H1.2	P16403
H1.1	H1.1	Q02539
	Ran	P62826
Hp1 α	Hp1 α	P45973
Hp1 β	Hp1 β	P83916
H3	H3	AAI08702
H2B	H2B	CAB02542
H2A	H2A	CAG33360
H4	H4	CAG46986

[000116] Although, both complexes were found to contain PARP-1, only the e-mH2A1.1 complex had a significant amount of PARP-1.

[000117] A part of the purified complex was run on a 15-35% glycerol gradient, the gradient was fractionated and the different fractions were loaded on a 4-12% SDS-PAGE. The positions of the core histones, e-mH2A1.1 and PARP-1 are indicated at the left part of the figure 14.

[000118] Finally, the amount of PARP-1 present in the complex was found to be proportional to the amount of e-mH2A1.1 and histone H4 (figure 3 and 14) suggesting a direct interaction between mH2A1.1 and PARP-1. In contrast, the H2A complex contained ~10 times less PARP-1 (the ratio between PARP-1 and histone H4 was found to be close to 0.1, Figure 3, right panel). No polypeptides were detected by silver staining from the mock purification of untransduced HeLa cells (data not shown), indicating that all detectable polypeptides in these complexes are specific to macroH2A1.1 or H2A histones.

4) macroH2A1.1 interacts with PARP-1 through its C-terminal non-histone region

[000119] To confirm the interaction between macro-H2A1.1 histone and PARP-1, we performed a GST-pull down experiment using a recombinant macroH2A1.1 as bait. Histone H2A and GST were used as controls. GST fusion histones were expressed in BL21 (pLysS) *E. Coli* strain grown at 25°C. The soluble proteins were purified on glutathione
5 Sepharose 4B beads (AMERSHAM) according to the manufacturer's instruction. Human recombinant PARP-1 (ALEXIS) was incubated for 1 h with the recombinant GST fused or not with H2A histone (GST-H2A), macroH2A1.1 histone (GST-macroH2A1.1), or to the non histone region (NHR; amnion acids 121 to 369 of SEQ ID NO:1) of macroH2A1.1 (GST-NHR), at 30°C with gentle mix in PBS with 100 mM KCl and 0.02 % NP40
10 (SIGMA). After a washing step, the bounded proteins were eluted in LAEMMLI's buffer, fractionated on a 12 % SDS-PAGE protein gel, blotted and then revealed by a human anti-PARP-1 antibody (ALEXIS).

[000120] The results show that the recombinant macroH2A1.1 histone interacts specifically with PARP-1 through its non histone region (NHR), and that this interaction
15 did not depend on the presence of DNA (see Figure 4). The C-terminal non-histone region macroH2A1.1 comprises a C-terminal region without any homology with other proteins (residue 121 to 201) and the macro domain. Thus, these results suggest that this C-terminal region, which if macroH2A1.1 specific is a good candidate as PARP-1 binding site.

20 5) Identification of macroH2A1.1 target genes

[000121] A number of studies suggest a general involvement of macroH2A histone in heterochromatin establishment or maintenance. These data strongly support an involvement of macroH2A histone in transcription repression, but the mechanism by which this

repression is achieved is unknown. These studies used mainly the immunofluorescence approach and failed to identify specific macroH2A histone target genes. To address this question, we have developed a highly specific chromatin immunoprecipitation assay that uses the tandem-affinity purification method to identify specific target DNA sequences
5 associated with macroH2A1.1 nucleosomes. We called this approach “Tap-Chip” (see Figure 5), for “Tandem Affinity Purification and Chromatin ImmunoPrecipitation assay”. Our approach did not use formaldehyde crosslinking or PCR amplification, which have the inherent risk of artifacts.

[000122] We purified the nuclei from HeLa cell line stably expressing tagged
10 macroH2A1.1 histone according to standard methods. The purified nuclei were digested with controlled amount of micrococcal nuclease to give predominantly mono- and dinucleosomes (data not shown). The digested chromatin was found to contain a significant amount of the tagged macroH2A1.1 histone (data not shown). MacroH2A1.1 mono- and dinucleosomes were immunopurified with (i) an anti-Flag antibody, and then with (ii) an
15 anti-HA antibody as above described. A small fraction of the purified mono- and dinucleosomes has been analyzed on SDS-PAGE gel to assess for its enrichment in tagged macroH2A1.1 histone (data not shown). As a control, we performed mock purification from a non-tagged HeLa cell line, and no polypeptides can be detected by silver staining (data not shown).

20 [000123] Immunopurified macroH2A1.1 mono- and dinucleosomes were digested with proteinase K, and phenol extracted according to standard methods. The purified DNA was treated with T4 DNA kinase (BIOLABS) and a 3' A-overhangs were added using Taq DNA polymerase (AMERSHAM). DNA Fragments corresponding to macroH2A1.1 mono-

and dinucleosomes were cloned into pcDNA3.1-Topo vector using TA cloning technology (INVITROGEN). The transformed clones were checked for inserts and sequenced by using vector-specific primers. The sequences obtained were then identified by Blast search of the human genome database (<http://www.ncbi.nlm.nih.gov/>).

5 [000124] The results in the literature show that MacroH2A is associated to gene repression and localize essentially to the inactive X-chromosome where it contributes to X inactivation. Interestingly, our results show that that macroH2A is not restricted to the X-chromosome but it is widespread along the genome. 45% of the 60 identified genes are enzymes, and the others are involved in different biological pathways. Moreover, the
10 analysis of these identified genomic sequences revealed that macroH2A1.1 histone associates mainly with promoter regions of several highly regulated genes, and then could be involved in the general regulation of inducible or early responsive genes.

[000125] One of the identified genomic sequence corresponds to *hsp70-1* promoter gene. Hsp70-1 (or hsp70i) is one of the most prominent and best characterized member of the
15 family hsp70 family, which encompasses at least 11 genes, and encodes a group of highly related proteins (TAVARIA *et al.*, 1996). Hsp70-1 is inducible in response to heat shock and chemical stresses and the probe used here picks up specifically this gene.

6) MacroH2A1.1 targets specifically the *hsp70* promoter

20 [000126] In order to confirm the association of macroH2A1.1 histone with gene promoters, we analyzed its distribution on the *hsp70-1* locus using semi-quantitative and real time PCR. The chromatin from Hela cells expressing tagged macroH2A1.1 histone (e-MH1.1) was precipitated as described bellow, with anti-flag and anti-HA antibodies. The chromatin

was cross-linked with formaldehyde solution for 10 min at 37°C, and then sonicated to obtain an average length of 300 to 800 bp DNA fragments. The extracts were standardized by non-denaturing gel electrophoresis and each sample was analyzed independently by semi-quantitative PCR with the following primers:

- 5 - *hsp70-1* promoter (forward): 5'-GGCGAAACCCCTGGAATATTCCCGA-3'
 (SEQ ID NO: 9);
- *hsp70-1* promoter (reverse): 5'-AGCCTTGGGACAACGGGAG-3' (SEQ ID NO:
 10);
- *hsp70-1* coding region (forward): 5'-CAGGTGATCAACGACGGAGACA-3'
10 (SEQ ID NO: 11);
- *hsp70-1* coding region (reverse): 5'-GTCGATCGTCAGGATGGACACG-3'
 (SEQ ID NO: 12).

[000127] Semi-quantitative PCR were performed with Taq DNA polymerase (PROMEGA)
15 on the *hsp70.1* promoter and coding region using the oligonucleotide pairs described
 below. Samples were amplified for 25 cycles (93°C for 30 sec, 58°C for 30 sec, and 72°C
 for 1 min), and run on 2 % agarose gels, visualized with ethidium bromide and quantified
 by densitometry.

[000128] The results show that the DNA fragment spanning the *hsp70-1* promoter (P, 191
20 bp) was enriched, whereas the fragment located in the coding region (C, 363 bp) was not
 (see Figure 6A). These results demonstrate that macroH2A1.1 is present at the *hsp70-1*
 promoter.

[000129] We then used a real-time PCR to quantify the relative enrichment of macroH2A1.1 histone in the promoter region using a LightCycler (ROCHE DIAGNOSTICS). Two different dilutions of each sample were analyzed independently by Q-PCR with the aforementioned primers and GAPDH primers for normalization (GAPDH (forward): 5'-
5 GGA CCT GAC CTG CCG TCT AGA A-3' (SEQ ID NO: 13); GAPDH (reverse): 5'-
GGTG TCG CTG TTG AAG TCA GAG-3' (SEQ ID NO: 14). The copy numbers were calculated as described in FERREIRA *et al.* (2001), and the results from Q-PCR were presented as the ratio between Hsp70 mRNA versus GAPDH mRNA. Finally, the PCR values were normalized against values obtained with chromatin from non-transfected cells.

10 [000130] The results show that MacroH2A1.1 was found to be enriched 450 times on the promoter region of the *hsp70-1* gene compared to the coding region (see Figure 6B).

[000131] We then asked if the ectopically expressed macroH2A1.1 histone adequately reflects the distribution of native protein throughout the genome and hence the target genes identified by our TAP-ChIP approach are indeed the real *in vivo* targets of macroH2A1.1
15 histone. To determine whether the *hsp70-1* promoter is a bona fide target of macroH2A1.1, we examined the *in vivo* distribution of the endogenous macroH2A1.1 histone on the *hsp70-1* promoter using a non-transfected HeLa cell line. *In vivo* chromatin immunoprecipitation (ChIP) assays were performed as described previously using a polyclonal antibody directed against the non-histone region of histone macroH2A1.1. The
20 extracts were standardized by non-denaturing gel electrophoresis and each sample was analyzed independently by semi-quantitative PCR as described previously.

[000132] The results show that the DNA fragment spanning the *hsp70-1* promoter (P) was enriched after coimmunoprecipitation of chromatin with a macroH2A1.1 specific antibody

in three independent ChIP assays, whereas a fragment located in the coding region (C) was not (see Figure 6C). These results demonstrate that macroH2A1.1 is naturally present *in vivo* at the *hsp70-1* promoter.

[000133] We next asked if mH2A1.1 was associated with the promoters of other inducible
5 genes of the *Hsp70* family as well as with constitutively expressed *Hsp70* genes. We used a candidate approach, focusing on the heat shock-inducible *Hsp70.2* and the constitutively expressed *Hsp70.8* (Hsc70) genes (Dworniczak and Mirault 1987).

[000134] A ChIP assay using the anti-FLAG and anti-HA antibodies was carried out with formaldehyde cross-linked chromatin isolated from HeLa cell lines stably expressing e-
10 mH2A1.1 as described previously.

[000135] Finally, Real-time PCR quantification was realized as described previously using primers specific for the respective promoters, which were as follows :

- *Hsp70.2* promoter (forward) : 5'-GGCCGAGAGTCAGGGAGGAACC-3'
(SEQ ID N0 :24);
- 15 - *Hsp70.2* promoter (reverse): 5'-ACTCTTCCAGCTCCACCACAG-3' (SEQ ID N0 :25);
- *Hsp70.8* promoter (forward): 5'-TGTGGCTTCCTTCGTTATTGGA-3' (SEQ ID N0 :26);
- *Hsp70.8* promoter (reverse): 5'-AAATACCGCTGCCATCCCACCG-3' (SEQ
20 ID N0 :27).

[000136] The results clearly show that the promoter region of the *Hsp70.2* gene was enriched in e-mH2A1.1 to an extent similar to that of the *Hsp70.1* gene, whereas the presence of e-mH2A1.1 was barely detectable at the promoter of the *Hsp70.8* gene (Fig 13). These data

suggest that mH2A1.1 may in general be preferentially associated with inducible heat shock genes.

7) Heat shock induced macroH2A1.1 and PARP-1 displacement from the *hsp70-1*
5 promoter

[000137] Intending to comprehensively describe the function of macroH2A1.1 in gene regulation and its relationship with PARP-1, we have examined the order of events occurring at the *hsp70-1* promoter during heat shock dependent transcriptional activation. Different HeLa cell lines expressing a Flag-HA tagged versions of macro-H2A1.1, H2A,
10 H3 or H3.3 histones were established. A non-tagged HeLa cell line was used as a negative control. The expression of *hsp70-1* gene was induced by heat shocking HeLa cells at 42°C for 30 min and a ten fold induction was routinely observed (data not shown). The cells were left to recover for 30 min and immediately treated with formaldehyde to cross-link protein-protein and protein-DNA complexes. Sheared chromatin was then precipitated as
15 described previously using anti-flag antibody for macroH2A1.1, H2A, H3 and H3.3 histones. In parallel, we also examined with specific antibodies the presence of the histone-modifying enzyme PARP-1 (ALEXIS), the ADP-ribose polymers (ALEXIS) and pan-acetylated histone H4 (ALEXIS). As a control, chromatin was immunoprecipitated in the absence of specific antibodies. Standardization of the chromatin inputs for
20 immunoprecipitation was assessed in each experiment using real time PCR. Levels of GAPDH, which is constitutively expressed, were also evaluated as a negative control, and as expected the sequence was found to be barely detectable in the immunoprecipitates. The

hsp70-1 promoter fragment in the immunoprecipitates was quantified using real time PCR as described previously.

[000138] The results show that macroH2A1.1 and PARP-1 are present on the *hsp70-1* promoter before activation and that heat shock induced their displacement from the promoter (see Figure 7A). The association of macro-H2A1.1 with *hsp70-1* promoter dropped by 90%, while the canonical H2A histone was only slightly affected (less than 30% drop). Interestingly, PARP-1 was almost not detectable on the *hsp70-1* promoter after heat shock (see Figure 7A) since a high level of poly(ADP-ribose) polymers appeared at the time of activation (see Figure 8A)). Consistent with recent findings, H3.3 histone was found to replace H3 histone in the transcriptionally active *hsp70-1* promoter (see Figure 7B). In contrast, we found no changes in global H4 histone acetylation (see Figure 8B). These observations suggest that PARP-1 ADP-ribosylation activity is inhibited, while it is sequestered by macro-H2A1.1 on the promoter. PARP-1 becomes active when it is released from the promoter, and modifies local proteins, leading to an accumulation of ADP-ribose moieties.

[000139] Since ADP-ribosylation is essential for the release of chromatin bound proteins (for a recent review see (Kim et al. 2005), we hypothesized that both macroH2A1 and PARP-1 could be ADP-ribosylated.

[000140] To visualize the association of the polymer with the proteins of the e-mH2A1.1 nucleosome complex in the absence of heat shock, we have isolated the e-mH2A1.1 complex from stable HeLa cell lines, separated on a 12% PAGE containing SDS, and silver stained (figure 15, left panel). Then, the gel was blotted and revealed by an anti-ADP ribose antibody (figure 15, right panel).

[000141] The Western blot showed that both PARP-1 and e-mH2A1.1 as well as the core histones H3 and H2B were ADP-ribosylated (Figure 15, right panel), a result in agreement with the literature (Abbott et al., 2005). These results suggested that upon heat shock activation not only mH2A1.1 and PARP-1, but also the core histones should be heavily
 5 ribosylated and consequently released from *Hsp70.1* promoter in an ADP-ribosylation dependent manner.

8) Down regulation of macroH2A1.1 or PARP-1 delays heat shock response

[000142] In order to address more precisely the role of macroH2A1.1 and PARP-1 in the
 10 *hsp70-1* gene regulation, we designed two siRNAs corresponding to their specific mRNA sequences. The siRNA directed against macroH2A1 is located in the macro domain and it is shared by the two isoforms of macro-H2A1, i.e. macro-H2A1.1 and macro-H2A1.2. The siRNA directed against PARP-1 is located in the catalytic domain of PARP-1 (KAMEOKA et al., 2004). A scrambled sequence was used as a negative control (control siRNA). A
 15 search in sequence libraries indicated that our macro-H2A1 and PARP-1 siRNA are restricted to macro-H2A1 and PARP-1 respectively, and that the control siRNA sequence is absent altogether. The sequence of these siRNAs were as follow:

- MacroH2A1 siRNA (MH1 siRNA, SEQ ID NO:15; 5' AAGCAGGGUGAAGUCAGUAA 3'),
- 20 - PARP1 siRNA (PARP-1 siRNA, SEQ ID NO:16 ; AAGCCUCCGCUCCUGAACAAU) ;
- Scrambled control siRNA (Scrambled siRNA, SEQ ID NO:17 ; 5'- CAUGUCAUGUUCACAUCUCTT-3').

[000143] For siRNA transfection, HeLa cells in exponential growth were seeded onto 6-well plates and grown overnight at 37°C in DMEM/10% FBS (INVITROGEN). HeLa cells were transfected with Lipofectamine® (INVITROGEN) according to the manufacturer's instruction, and with 1 µg of each aforementioned siRNA, or no siRNA. 48 hours post-
5 transfection, cells were heat-shocked at 42°C for 60 minutes and left to recover at 37°C for 10, 20 or 30 min. Cells were then harvested and assayed for hsp70.1 expression by RT-PCR. As a control, cells were also assayed for macro-H2A1 and PARP-1 silencing by immunoblotting.

[000144] The results reveal that the expression of endogenous macroH2A1 and PARP-1
10 was almost completely inhibited (as revealed by western blot using specific antibodies against macroH2A1 and PARP-1, see Figure 9). The results also reveal that the down regulation of macro-H2A1.1 or PARP-1 with specific siRNAs delayed the heat shock response respectively by 3 and 10 folds (see Figure 10A). At the same time, HeLa cells transfected with a scrambled siRNA behaved as the control and did not show any delay in
15 hsp70-1 activation.

[000145] In order to confirm this result, we performed a kinetic analysis of hsp70-1 activation in the presence of macroH2A1 siRNAs. HeLa cells transfected or not with macroH2A1 siRNA were heat shocked at 42°C for 30 min and the expression of hsp70-1 was monitored by real-time RT-PCR after 10, 20 and 30 min recovery as previously.

20 [000146] The results clearly show that down regulation of macroH2A1.1 with a specific siRNA results in a delayed heat shock response by a three fold factor (see Figure 10B). Since mH2A1 appeared to be involved in the association of PARP-1 with the *Hsp70.1* promoter, one would expect that the depletion of mH2A1 by siRNA treatment would severely reduce the amount of *Hsp70.1* promoter associated PARP-1, which, in turn, would interfere

with the heat shock response. The ChIP data (Figure 24) demonstrated that the depletion of mH2A1 resulted, as expected, in a strong decrease of the amount of PARP-1 interacting with the *Hsp70.1* promoter.

[000147] Consistent with our results, PARP-1 was found to be required for heat shock-induced puffing and hsp70 expression in drosophila larvae (TULIN and SPRADLING, *Science*, vol.299(5606), p:560-2, 2003). Consequently, our results show that the macroH2A association with *hsp70-1* promoter was clearly linked to its inactive state. The association with PARP-1 supports this view and suggests a role of macroH2A in the regulation of inducible genes that need to respond rapidly to hormones, cytokines, or heat shock. The identification of a large number of genes potentially regulated by macroH2A1.1 supports the notion that macroH2A1.1 is a central regulator that represses diverse cellular processes.

9) MacroH2A1.1 regulates PARP-1 enzymatic activity

[000148] The physical association of macro-H2A1.1 with PARP-1 and their coordinated release from the hsp70-1 promoter after a heat-shock induced activation prompted us to examine the possible regulation of PARP-1 enzymatic activity by macro-H2A1.1. PARP-1 is known to auto-ADP-ribosylates itself and mechanisms controlling this auto-modification are not known.

[000149] Our results suggest that macroH2A is implicated in PARP-1 activity regulation. In order to elucidate this regulation process, we compared macroH2A sequences with proteins having a macro domain, and more specifically with phosphoesterases. In fact, ALLEN *et al.*, (2003, aforementioned) suggests that the macro domain defines a superfamiliy of phosphoesterases that act on ADP ribose derivatives.

[000150] Our analysis shows that macroH2A1.1, macroH2A1.2, and macroH2A2 share an important homology with the catalytic domain of known phosphoesterases (see Figure 11). Moreover, macroH2A1.1 and macroH2A2 share a phylogenetically invariant HXTX motif (in bold in Figure 11 and Figure 16) associated with the phosphoesterase activity (NASR and FILIPOWICZ, *Nucleic Acids Res.*, vol.28(8), p: 1676-83, 2000; HOFMAN *et al.*, *EMBO J.*, vol.19(22), p:6207-17, 2000), wherein the histidine residue is critical for activity. The corresponding residue in macroH2A1.2 is asparagine (N).

[000151] To test if a perturbation in the folding of mH2A1.1 may affect PARP-1 activity and facilitate the release of the otherwise mH2A1.1 nucleosome associated PARP-1, we have generated point mutation in the mH2A1.1 gene that convert at the conserved HXTX motif at positions 213-216 to Alanines (AAAA), and is referred hereafter as MH1.1mut (Figure 16). Indeed, this HXTX motif is present in other macro-domains that possess catalytic activity towards ADP-ribosylated substrates (Figure 16 and Allen et al. 2003). It has previously been shown that the mutation of an analogous motif in a yeast phosphoesterase abolishes its enzymatic activity (NASR and FILIPOWICZ, 2000, abovementioned; HOFMAN *et al.*, 2000, abovementioned). This motif is also in close proximity to the mH2A1.1 pocket (Allen et al. 2003; Chakravarthy et al. 2005), which binds ADP ribose (and possibly mono-ADP ribosylated PARP-1) and its derivative O-acetyl-ADPribose (Karras et al. 2005; Kustatscher et al. 2005), so that mutations in the motif may be expected to affect the binding.

[000152] To test if the above mutations affect the binding of mono-ADP-ribose, increasing amounts of recombinant wild type (WT) and mutated (Mut) mH2A1.1 macro domains were purified to homogeneity, incubated with ³²P-ADP-ribose, and blotted (in duplicate) onto PVDF membranes to detect the labeled proteins (Figure 17, upper panel). The duplicate

membrane was Coomassie stained as a control for equal loading (Figure 17, lower panel). The obtained results confirmed the weaker binding of mono-ADP-ribose to the mutated emH2A1.1 compared to its binding to the WT e-mH2A1.1 (Figure 17, upper panel). This provides evidence that the structure of the e-mH2A1.1 pocket that binds ADP-ribose (Karras
5 et al. 2005; Kustatscher et al. 2005) was perturbed in the mutated protein.

[000153] If the folding of mH2A1.1 is important for the affinity of PARP-1 binding within the e-mH2A1.1 nucleosome complex, alterations in the folding of e-mH2A1.1 should affect PARP-1 binding. To address this, we have purified wild type (WT) and mutated (Mut) emH2A1.1 complexes under different conditions of stringency, i.e. at 150 mM (Figure
10 18, left panel) and 300 mM NaCl (Figure 18, middle panel).

[000154] The results show that remarkably, the amount of PARP-1 associated with the WT and Mut complexes isolated at 150 mM NaCl differed only slightly (see the quantification at Figure 18, right panel). However, the picture was completely different for the complexes isolated at 300 mM NaCl (Figure 18, middle panel and quantification). In this case the
15 amount of PARP-1 that remained associated with the mutated e-mH2A1.1 nucleosome complex did not exceed 8-10% of that associated with the WT emH2A1.1 nucleosome complex (Figure 18, quantification). Therefore, 300 mM NaCl was able to strongly perturb the binding of PARP-1 to the mutated e-mH2A1.1 within the nucleosome complex, arguing for weaker interaction between PARP-1 and the mutated emH2A1.1. This suggests that, *in*
20 *vivo* at the *Hsp70.1* promoter, the amount of PARP-1 associated with mutated e-mH2A1.1 would be smaller compared to that associated with the WT e-mH2A1.1. We found this indeed to be the case (Figure 19). Briefly, we have performed ChIP experiments with anti-PARP-1 by using chromatin isolated from HeLa cell lines stably expressing either WT or mutated e-mH2A1.1, and Q-PCR to quantify the amount of PARP-1- associated *Hsp70.1*
25 promoter. The quantification showed that no more than 15% of *Hsp70.1* promoter was

associated with PARP-1 in the cells expressing the mutated e-mH2A1.1 as compared to that found in the cells expressing the WT protein. Taken together, all the above data demonstrate, both *in vitro* and *in vivo*, that the folding of mH2A1.1 is crucial for the binding of PARP-1 to chromatin.

5 [000155] We have next measured the auto-ADP ribosylation activity of the PARP-1 associated with the mutant and wild type e-mH2A1.1 nucleosome complexes. The two complexes were isolated under the same conditions by double immunoaffinity purification in a buffered solution containing 100 mM NaCl (Figure 20). Under these conditions, the relative amount of PARP-1 associated with the mH2A1.1-mut complex was the same as
10 that associated with the wild type mH2A1.1 complex (Figure 20).

[000156] To measure the auto-ADP ribosylation activity of PARP-1, wild type and mutant complexes containing the same amount of associated PARP-1 were incubated in presence of mononucleosomes and ^{32}P - α -NAD $^{+}$. The poly(ADP-ribosyl)ation was performed *in vitro* in a 20 μ l reaction mixture containing 20 mM Tris-HCl, pH 7.8, 50 mM NaCl, 3mM MgCl $_2$,
15 0.5 mM DTT, 10 μ M $\{^{32}\text{P}\}$ NAD (10 μ Ci/nmol) (AMERSHAM), 100 ng mononucleosomes prepared as described in DUBAND-GOULET *et al.* (*Methods*, vol.33(1), p:12-7, 2004) and 100 ng recombinant PARP-1 (ALEXIS), native macroH2A1.1 associated PARP-1 or native macro-H2A1.1 mutant associated PARP-1. The ADP ribosylation reactions were incubated at 37°C for 1 to 30 min and stopped with 1% SDS and directly loaded on a 12% SDS-
20 PAGE gel.

[000157] The results show that the incubation of the wild type macro-H2A1.1 complex with mononucleosomes in the presence of ^{32}P -NAD $^{+}$ results in a very poor labeling of PARP-1 (see Figure 12), and we could hardly detect labeling after 30 min incubation. The control containing the same amount of recombinant PARP-1 (50 ng) and

mononucleosomes showed a very strong labeling of PARP-1 after only 1 min incubation, suggesting that macro-H2A1.1 interferes with PARP-1 auto-ADP-ribosylation activity. The same experiment performed with macroH2A1.1 mutant complex results in a detectable labeling of PARP-1 after just 1 min incubation with ^{32}P -NAD⁺ (see Figure 12), and this
5 labeling increases linearly until all the ^{32}P -NAD⁺ is converted to ADP-ribose polymer.

[000158] In addition, the extent of PARP-1 labeling during the first minute was at least 37 times much higher than that of the PARP-1 associated with the wild type e-mH2A1.1 nucleosomes (Figure 21, quantification).

[000159] These results strongly suggest that the interaction of mH2A1.1 with PARP-1
10 interferes with the PARP-1 auto-ADP-ribosylation activity. Indeed, the folding of the mutated mH2A1.1 being perturbed (Figure 17 and 18), this would result in a perturbation of the specific interaction between the mutated mH2A1.1 and PARP-1 (figure 18), which in turn would allow PARP-1 to adopt a conformation close to that of the enzyme free in solution, with higher enzymatic activity. Our data on the auto ADP-ribosylation of
15 recombinant PARP-1 are in agreement with this, since both the kinetics and the degree of auto-ADP ribosylation of recombinant PARP-1 were similar to these of the PARP-1 associated with the mutant emH2A1.1 (Figure 12, compare the middle and the right panel). Finally, the NAD concentration used here are close to the physiological concentrations and do not allow the formation of a highly branched PARP-1 (data not shown).

20 [000160] Since the e-mH2A1.1 mononucleosome complex contains, in addition to PARP-1, a number of other proteins it is difficult to completely exclude the possibility that some of these proteins might, as e-mH2A1.1, interact with PARP-1 and interfere also with its enzymatic activity. To rule out this we sought to purify the emH2A1.1 octamers and the

associated PARP-1, to reconstitute e-mH2A1.1 nucleosome-PARP-1 complex from highly purified components and to measure the enzymatic activity of the associated with e-mH2A1.1 nucleosome PARP-1. To this end, we have generated a new HeLa cell line stably expressing a triple tagged version of mH2A1.1 (FLAG-HA-HIS). This has allowed the

5 purification of the e-mH2A1.1 histone octamer and PARP-1 from the emH2A1.1 nucleosome complex. Briefly, the FLAG-HA purified mononucleosomes were adsorbed on a hydroxyl apatite column and washed with 0.65 M NaCl. The washing of the column with 0.65 M NaCl released all of the associated proteins, with the exception of PARP-1, (Figure 22, lane 3, and data not shown), evidencing for a very strong binding of PARP-1 to e-

10 mH2A1.1 nucleosomes (to note is that the remaining on the column e-mH2A1.1 and PARP-1 are in roughly stoichiometric amount, suggesting that one molecule of nucleosomal emH2A1.1 might be complexed with one molecule of PARP-1).

[000161] The e-mH2A1.1 octamer and PARP-1 were released from the column with a buffered solution containing 2M NaCl (Figure 22, lane 3) and were used for reconstitution

15 of emH2A1.1-PARP-1 nucleosome complex. The auto-ADP ribosylation activity of the PARP-1 associated with the reconstituted e-mH2A1.1 nucleosomes was then tested (Figure 23). A solution containing conventional nucleosomes and native PARP-1 (purified from the 2M NaCl eluate of the hydroxyl apatite column immobilized e-mH2A1.1 nucleosome complex (Figure 22, lane 4), was used as a positive control (Figure 23). The reaction was

20 arrested at the indicated times and the samples loaded on a 12% SDS-PAGE gel. After completion of electrophoresis, the auto-ADP-ribosylated PARP-1 was visualized by autoradiography (Figure 23). Incubation of the *in vitro* reconstituted emH2A1.1 nucleosomes with PARP-1 in presence of ^{32}P - α -NAD $^{+}$ resulted in a complete inactivation of

its enzymatic activity (Figure 23, lanes 2-5), while the same PARP-1, when incubated with conventional mononucleosomes, showed a strong ADP-ribosylation activity, which increased with time and reached completion within 10-20 minutes (Figure 23, lanes 6-9). In addition, incubation of the *in vitro* reconstituted mH2A1.1 nucleosomes containing PARP-1 in the presence of a 100 fold excess of conventional nucleosomes failed to reactivate PARP-1 (data not shown), further confirming that the specific interaction between PARP-1 and mH2A1.1 determines the “inactivation” of the enzyme.

10) Identification of the PARP-1 binding site in the C-terminal non histone region of macroH2A1.1

[000162] To identify the domain of macroH2A1.1 specifically interacting with PARP-1, we perform GST-pull down experiments using recombinant macroH2A1.1 non-histone region with different deletions as bait. The experiments are realized as described previously. As control experiments, we use recombinant macroH2A1.1 non-histone region with G224E, which abolish binding of ADP ribose (KUSTATSCHER *et al.*, 2005, abovementioned), and recombinant macro domain of YBR022w (from *S. Cerevisiae*; NP_009578) as baits.

[000163] In order to identify corresponding binding region in macroH2A1.2 and in macroH2A2, we also perform GST-pull down experiments using recombinant macroH2A1.2 or macroH2A2 non-histone region with different deletions as bait.

11) Recombinant inhibitors of PARP-1 activity

[000164] In order to identify new inhibitor of PARP-1 activity, we elaborate different constructions encoding for GST fusion proteins containing the macroH2A1.1 binding site to PARP-1 fused to the macro domain of macroH2A1.2, macroH2A2, or YBR022w (from *S. Cerivisiae* ; NP_009578).

5 [000165] The recombinant proteins are produced as described previously, and purified according to standard protocols.

[000166] We perform poly(ADP-ribosyl)ation assay in the presence of recombinant PARP-1 (ALEXIS) with or without the different GST fusion proteins containing the macroH2A1.1 binding site to PARP-1.

DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 56

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JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME 1 OF 2
CONTAINING PAGES 1 TO 56

NOTE: For additional volumes, please contact the Canadian Patent Office

NOM DU FICHER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

We claim:

1. An inhibitor of the nucleic enzyme poly(adenosine 5'-diphospho-ribose) polymerase ["poly(ADP-ribose) polymerase (PARP) having a phosphoesterase activity, and comprising an amino acid sequence, which is derived from the C-terminal non-histone
5 domain of a macroH2A histone, optionally fused and/or coupled to at least one heterologous sequence.
2. The inhibitor according to claim 1, wherein the C-terminal non-histone domain of macroH2A histone is selected in the group comprising the C-terminal non-histone domain of human macroH2A1.1 (amino acids 121 to 369 of SEQ ID NO:1),
10 macroH2A1.2 (amino acids 120 to 371 of SEQ ID NO:2), and macroH2A2 (amino acids 121 to 372 of SEQ ID NO:3) histones.
3. The inhibitor according to claim 2, wherein preferably the C-terminal non-histone domain of macroH2A histone is the C-terminal non-histone domain of macroH2A1.1.
- 15 4. The inhibitor according to any of claims 1 to 3, wherein said inhibitor comprises an amino acid sequence, which is derived from the C-terminal non-histone domain of a macroH2A histone, and less is than 350 amino acids in length, preferably less than 150 amino acids in length.
5. The inhibitor according to any of claims 1 to 4, wherein said amino acid
20 sequence, which is derived from the C-terminal non-histone domain of a macroH2A histone does not comprise the macroH2A histone fold domain having homology with histone H2A.

6. The inhibitor according to any of claims 1 to 5, wherein the amino acid sequence, which is derived from the non-histone domain of a macroH2A histone comprises the macro domain of a macroH2A histone.

7. The inhibitor according to claim 6, wherein the macro domain of a
5 macroH2A histone is selected in the group comprising the residues 184 to 369, preferably 202 to 369 of human macroH2A1.1 (SEQ ID NO:1), the residues 183 to 371, preferably 201 to 371 of human macroH2A1.2 (SEQ ID NO:2), and the residues 184 to 372, preferably 202 to 372 of human macroH2A2 (SEQ ID NO:3).

8. A nucleic acid encoding for an inhibitor according to any of claims 1 to 7.

10 9. A vector comprising a nucleic acid according to claim 8.

10. A composition comprising (i) an inhibitor according to any of claims 1 to 7, a nucleic acid according to claim 8 or a vector according to claim 9, and (ii) a pharmaceutically acceptable vehicle.

11. Use of an inhibitor according to any of claims 1 to 7, of a nucleic acid
15 according to claim 8 or of a vector according to claim 9 for the manufacture of a medicament for the prevention or treatment of a subject suffering from a disease associated with PARP activation.

12. The use according to claim 11, wherein the disease associated with PARP activation is selected in the group comprising tissue damage resulting from cell damage or
20 death due to necrosis or apoptosis, neural tissue damage resulting from ischemia and reperfusion injury, neurological disorders and neurodegenerative diseases, vascular stroke, cardiovascular disorders, age-related macular degeneration, AIDS, immune senescence diseases, arthritis, atherosclerosis, cachexia, cancer, degenerative diseases of skeletal

muscle involving replicative senescence, diabetes, head trauma, immune senescence, inflammatory disorders, muscular dystrophy, osteoarthritis, osteoporosis, chronic and/or acute pain, renal failure, retinal ischemia, septic shock, skin aging.

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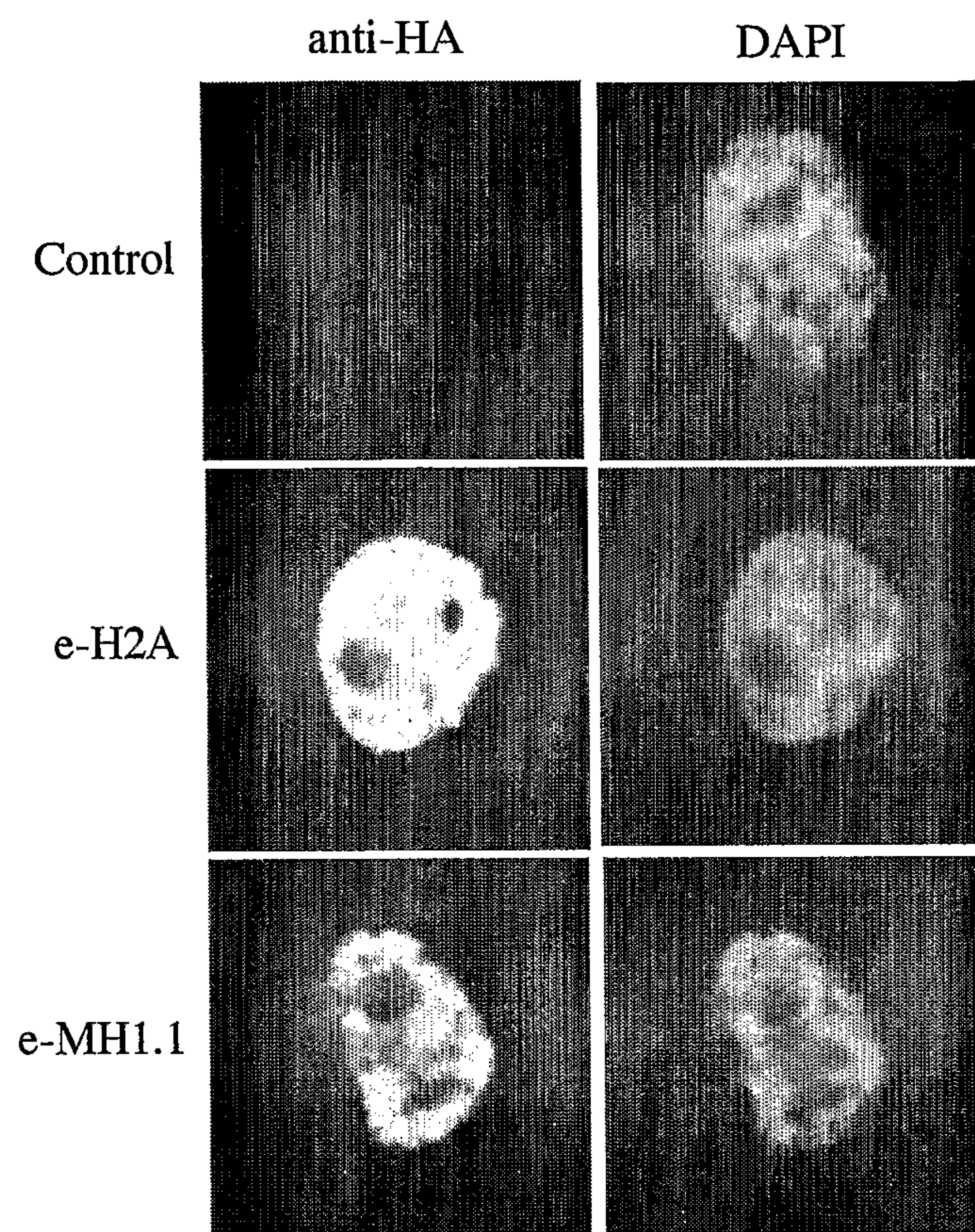


Figure 1

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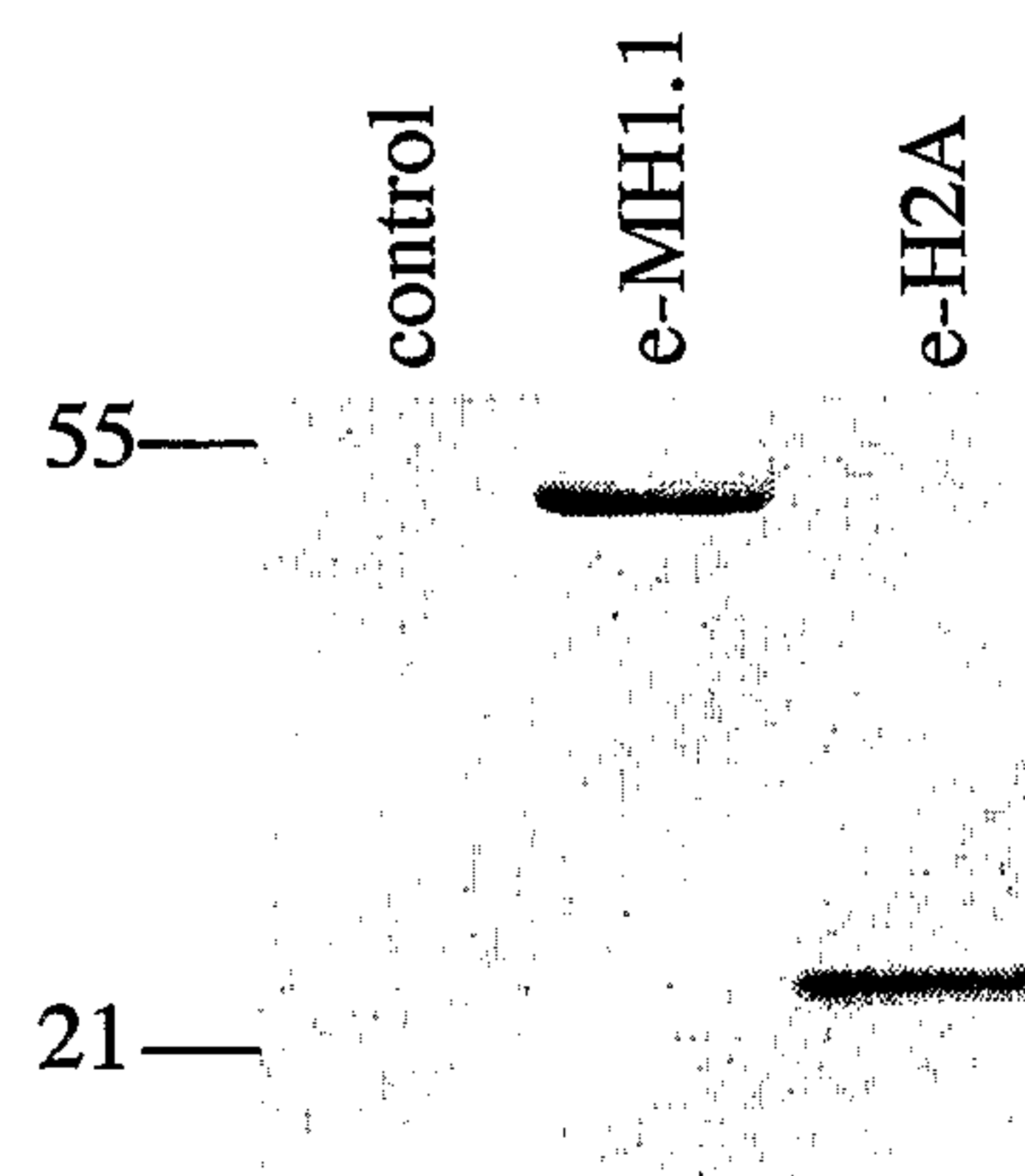


Figure 2

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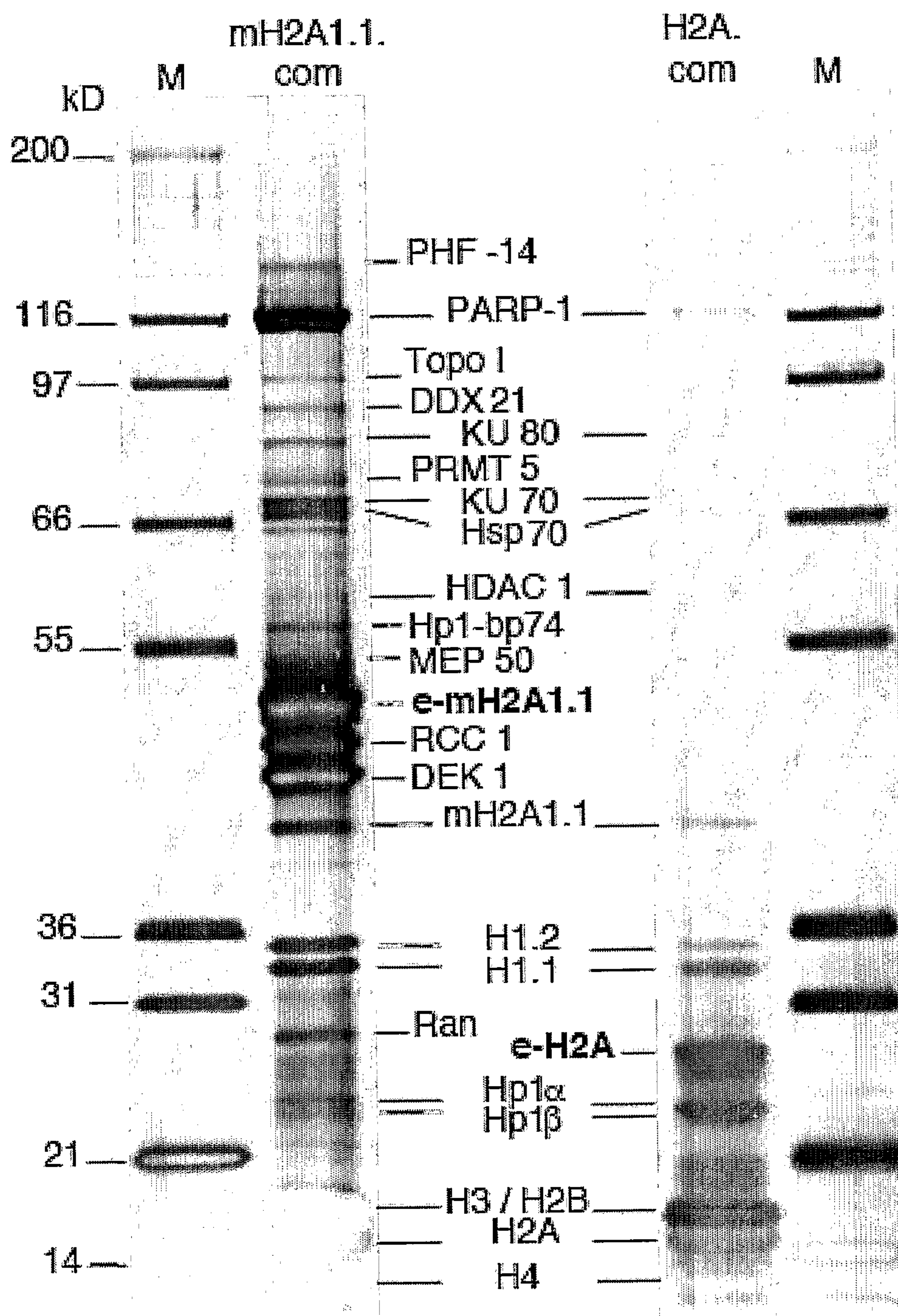


Figure 3

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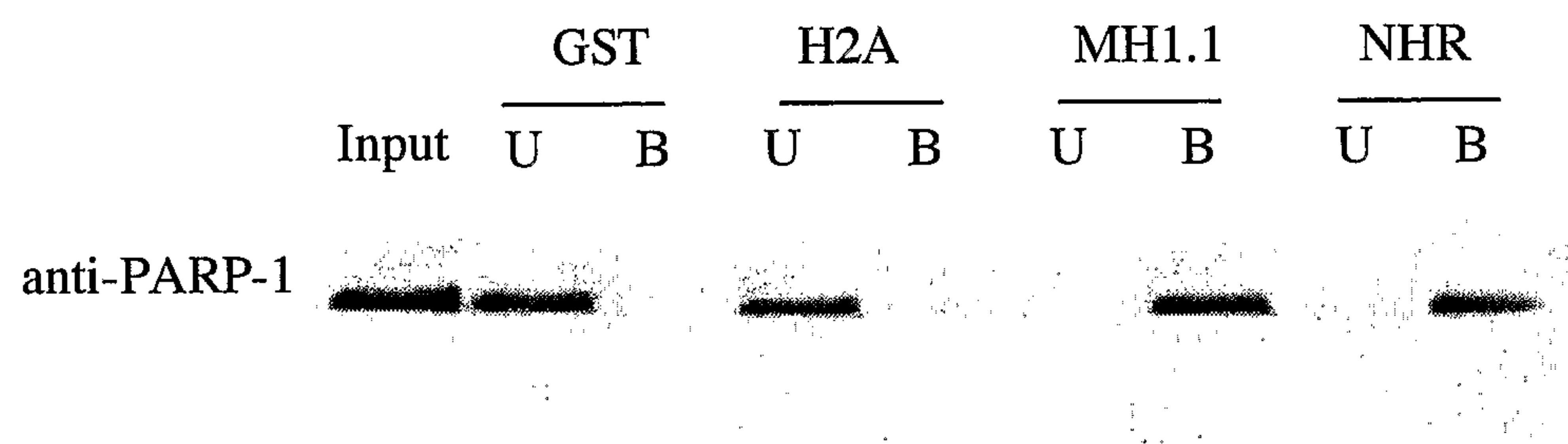


Figure 4

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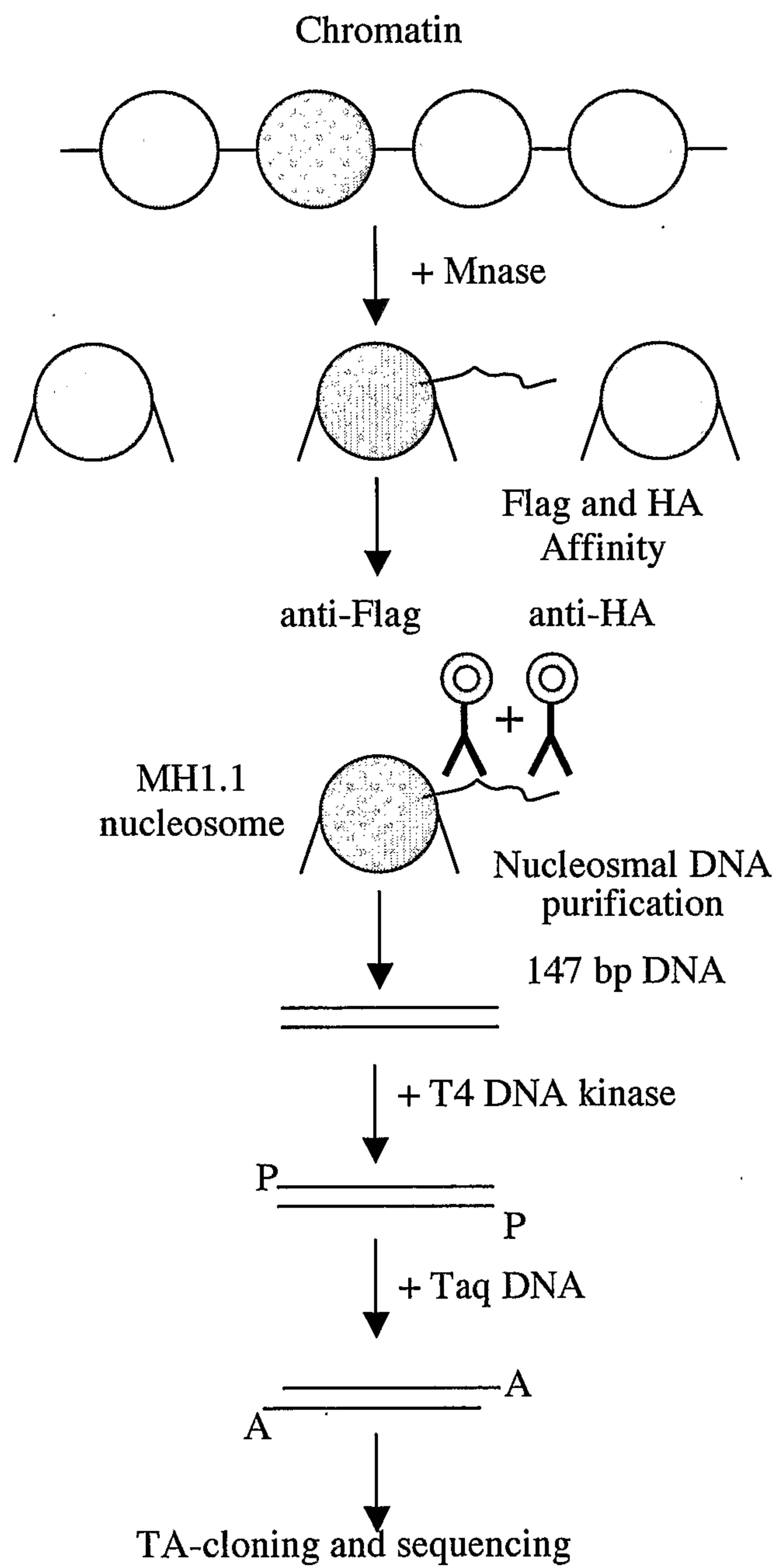


Figure 5

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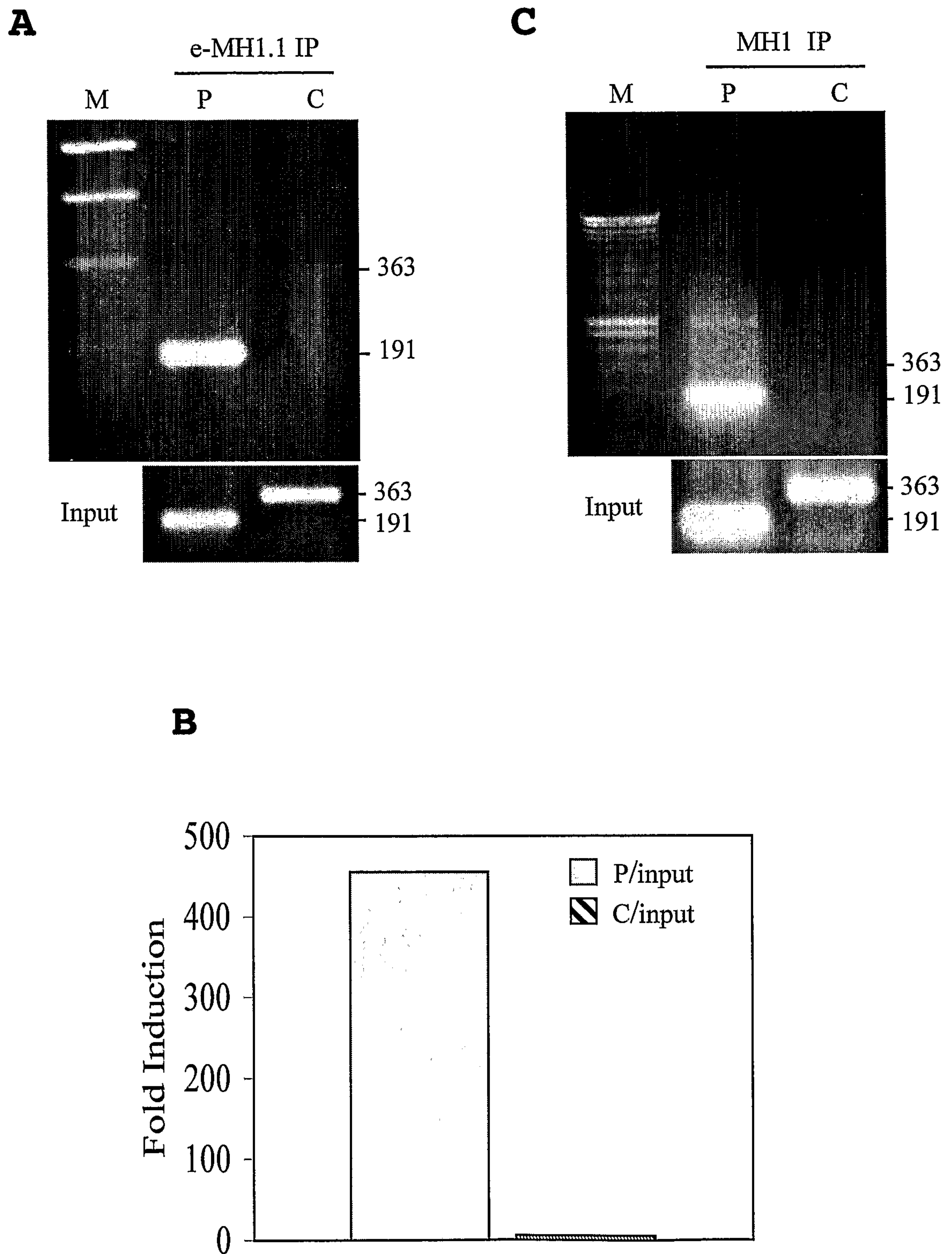


Figure 6

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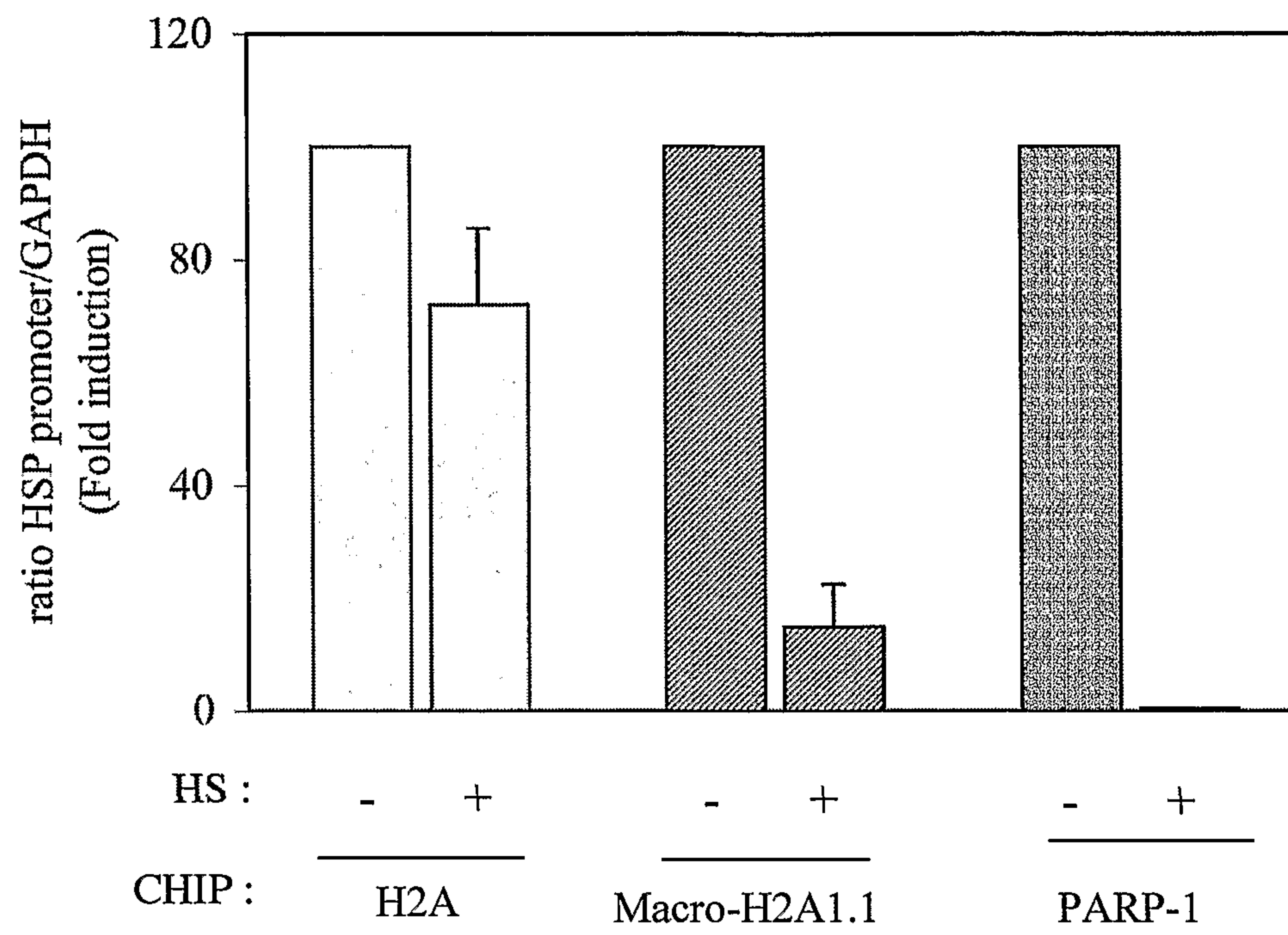
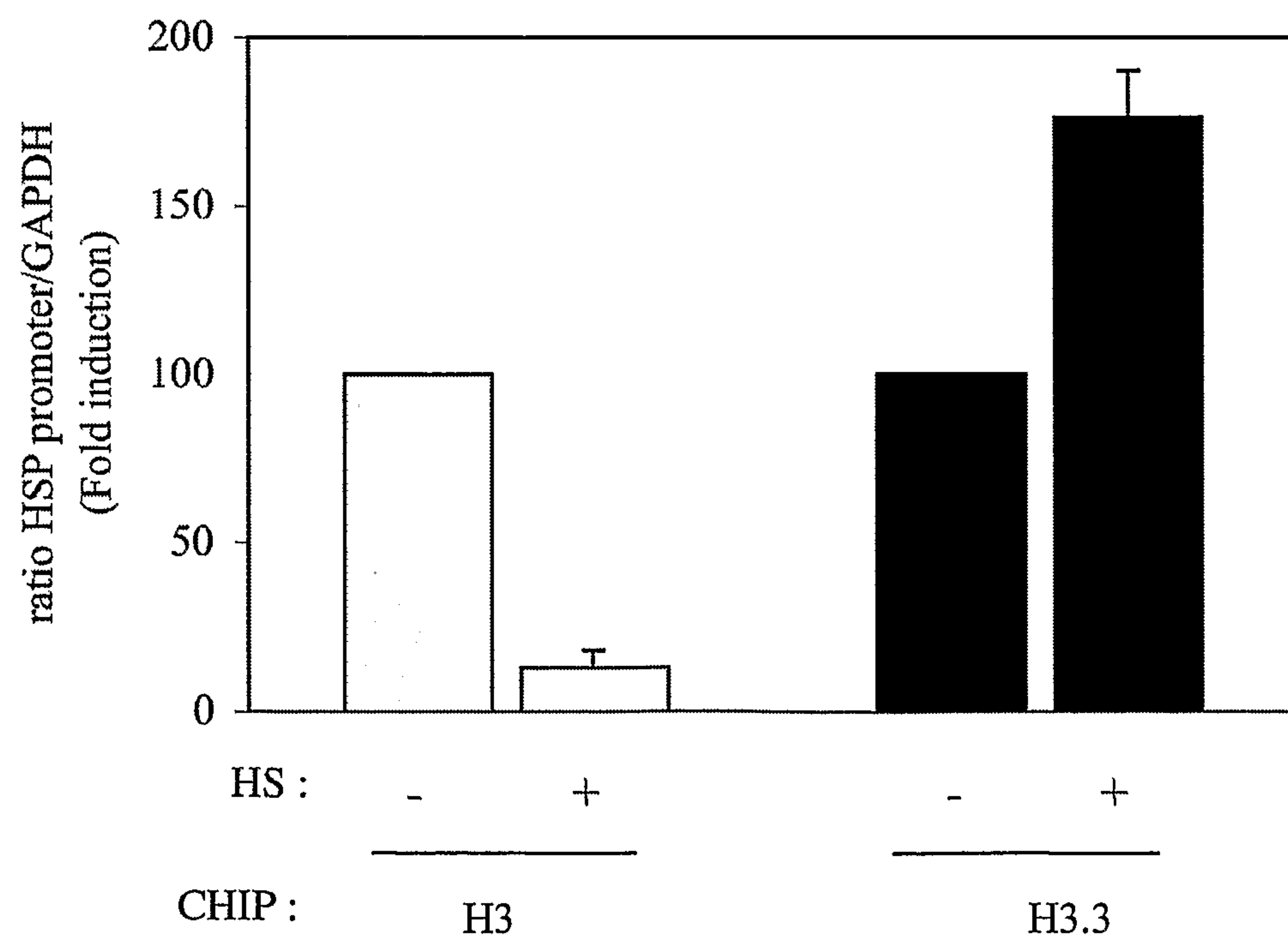
A**B**

Figure 7

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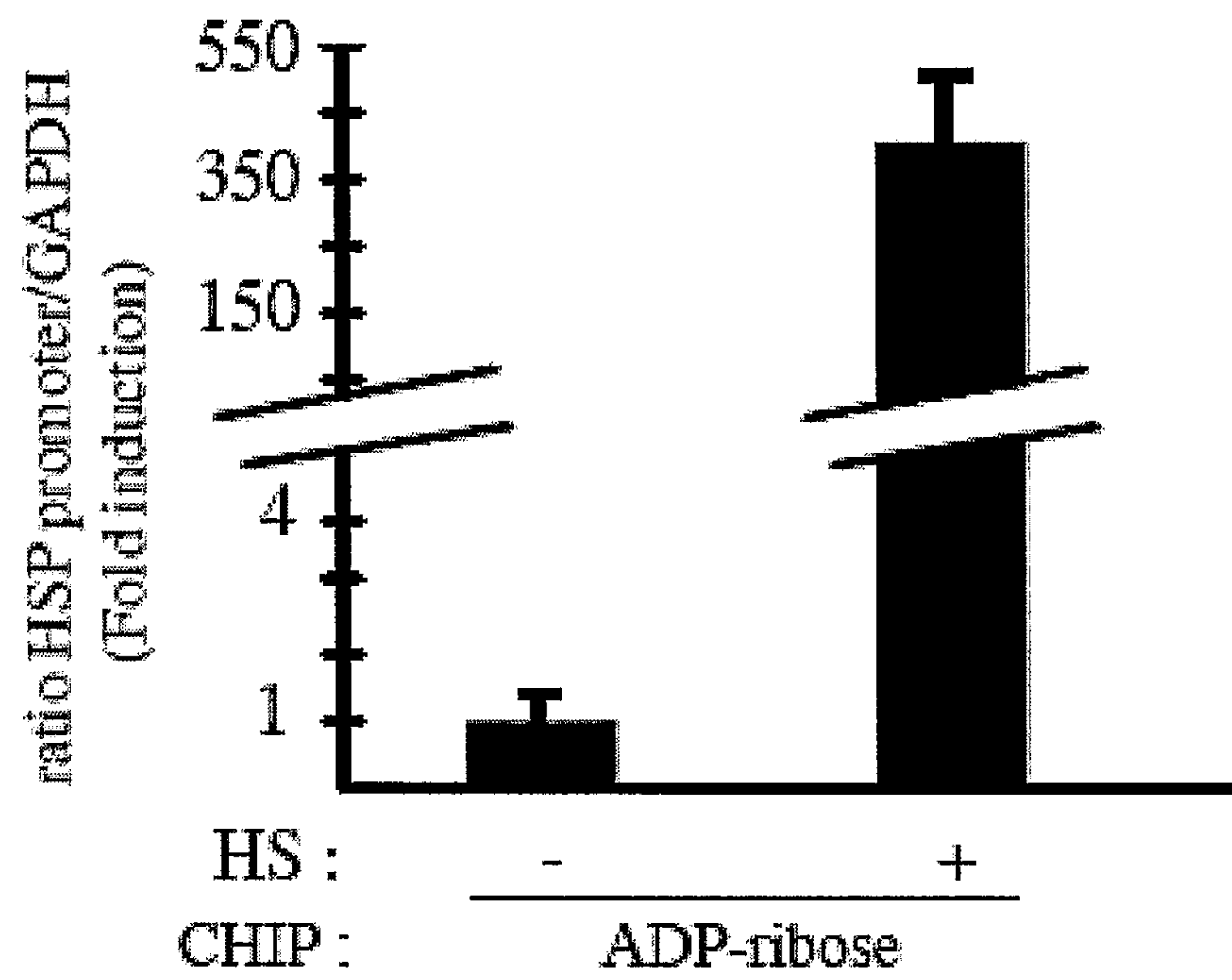
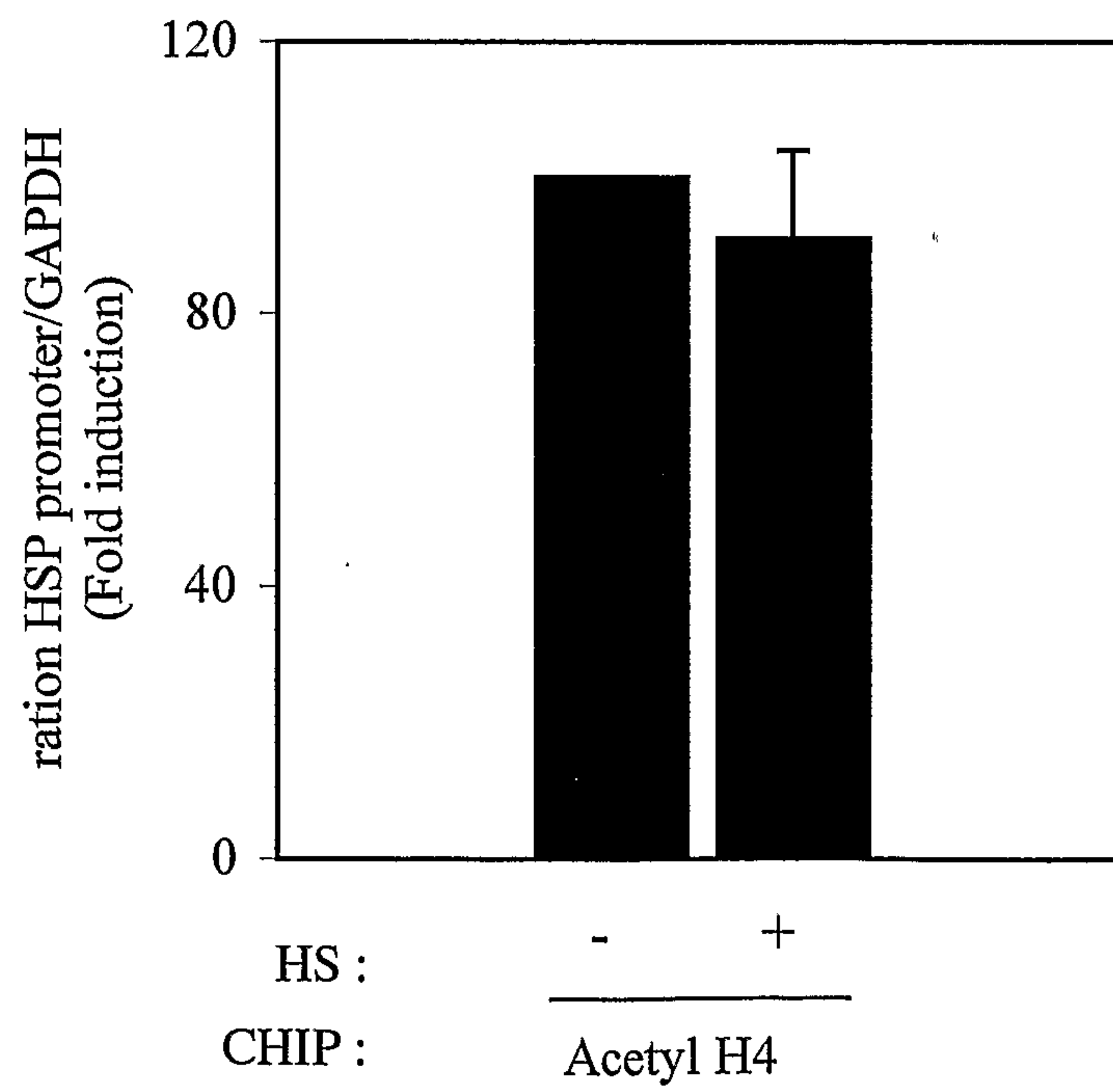
A**B**

Figure 8

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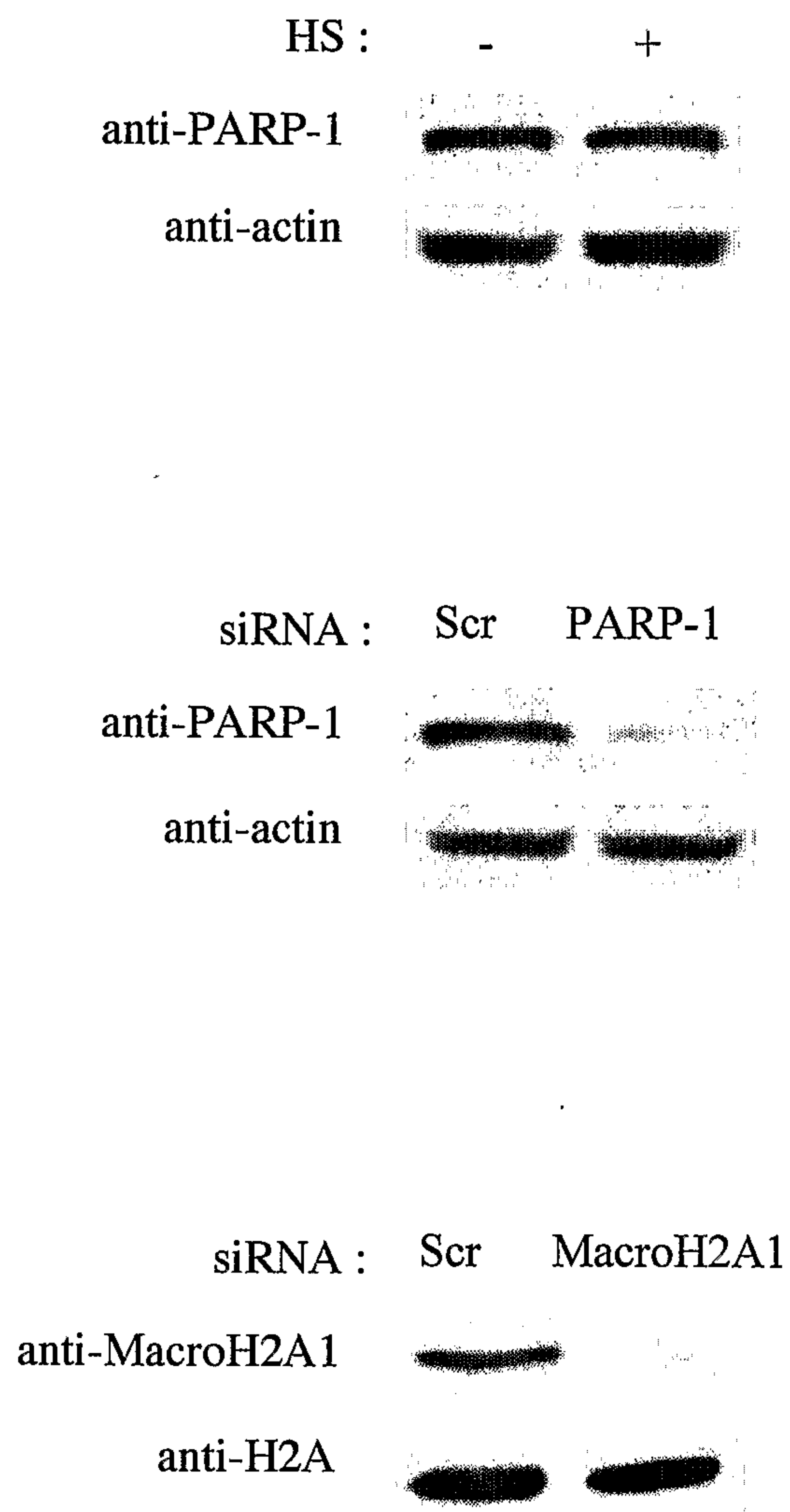


Figure 9

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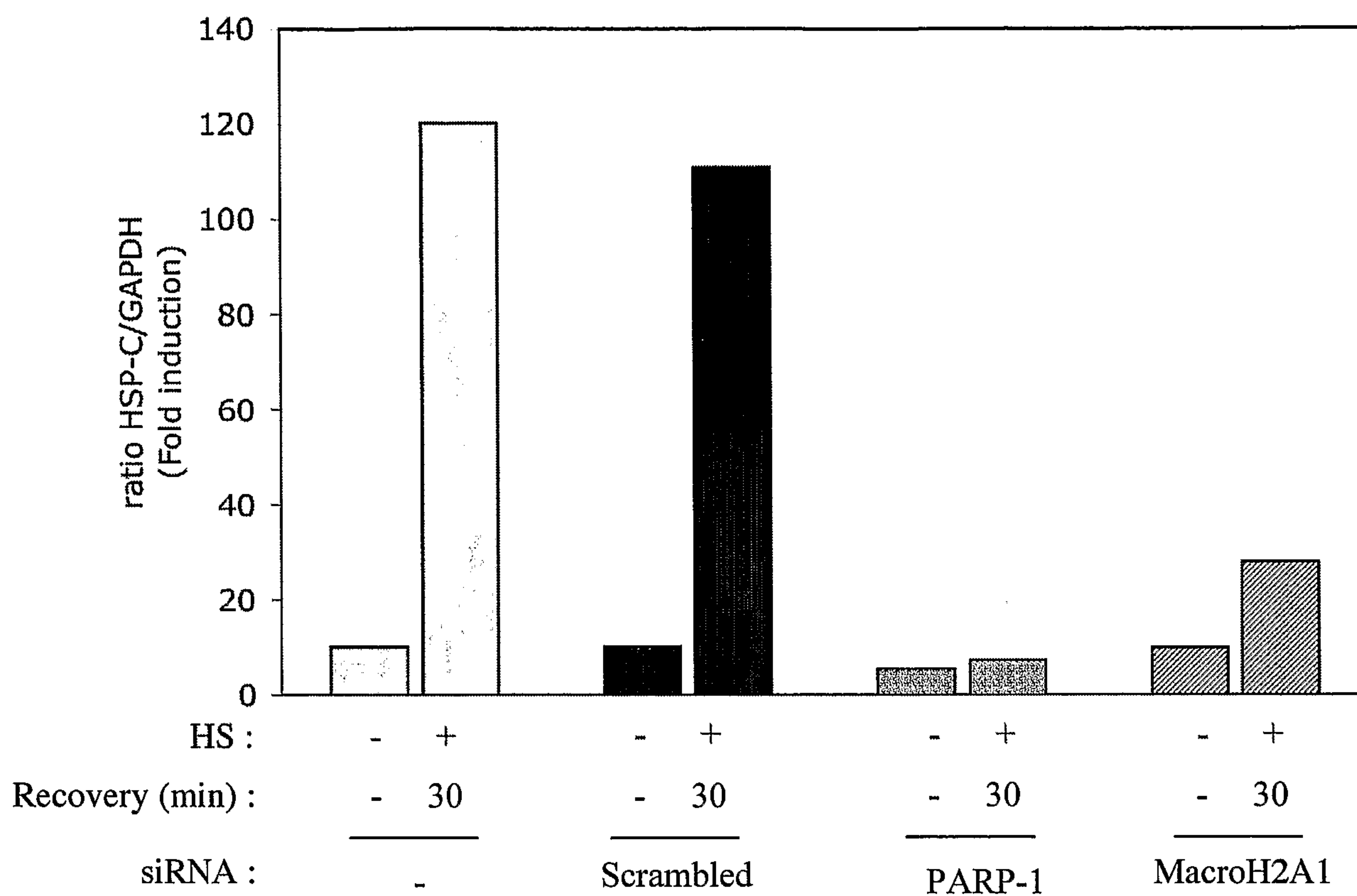
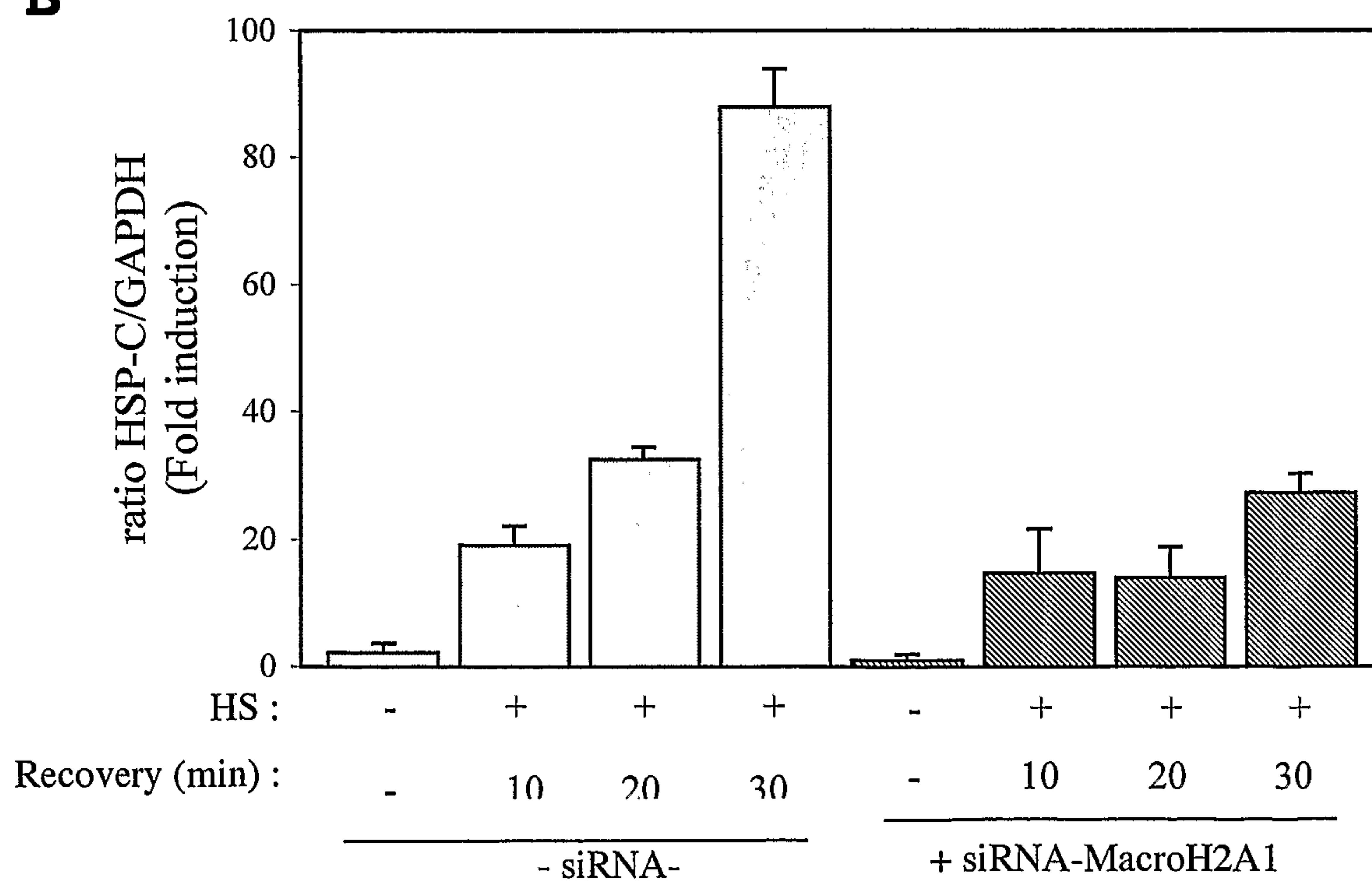
A**B**

Figure 10

macroH2A1.1 macroH2A1.2 macroH2A2 AF1521 NP_598908.1 Ybr022wp	121	220	KGKLEAIIITP	PPAKKAKSPS	QKKPVSKKAG	GKKGARKSKK	KQGEVSKAAS	ADSTTEGTPA	DGFTVLSTKS	LFLGQKLQVV	QASL...	DSDAV	HPTN
			KGKLEAIIITP	PPAKKAKSPS	QKKPVSKKAG	GKKGARKSKK	.QGEVSKAAS	ADSTTEGTPA	DGFTVLSTKS	LFLGQKLNLI	HSE	SNLAGF	EVEAI
			KGKSETILSP	PPEKRGKAT	SGKKGKKSK	AAKPRTSKKS	KPKDSDKEGT	SNSTSEDCPG	DGFTIISKS	LVLGQKLSLT	QSS	SHIGSM	RVEGI
			-----	-----	-----	-----	-----	-----	-----	-----	QG	TQY...	PAKAI
macroH2A1.1 macroH2A1.2 macroH2A2.2 AF1521 NP_598908.1 Ybr022wp	221	320	ADIDLKDDLG	NTLEKKGGKE	FVEAVLELRK	KNGPLEVAGA	AVSAGHGLPA	KFVI	WGADKCEELL	EKTVKNCIAL	ADDKKLKSI	SIGS	RNG
			AEIDLKEDIG	KALEKKAGGKE	FVEAVLELRK	KNGPLEVAGA	AVSAGHGLPA	KFVI	WGADKCEELL	EKTVKNCIAL	ADDKKLKSI	SIGS	RNG
			KRLEHGGGVA	-----	-----	-----	-----	KYVF	-----	-----	-----	AEEMGVESI	PPFPS
			SSLGGGGVD	-----	-----	-----	-----	KYVH	-----	-----	-----	LEHRLRSV	AVSA
macroH2A1.1 macroH2A1.2 macroH2A2.2 AF1521 NP_598908.1 Ybr022wp	321	372	CNGSWGGA	-----	-----	-----	-----	LLICCLFTSS	-----	-----	-----	KLKTFREAKD	KTRTSEDSIG
			FPKQTAAQLT	LKAISSVFS	TMSSSIKTVM	FVLFDSSESIG	IYVQEMAKLD	AN	-----	-----	-----	-----	-----
			FPKQTAAQLT	LKAISSVFS	TMSSSIKTVM	FVLFDSSESIG	IYVQEMAKLD	AN	-----	-----	-----	-----	-----
			FPKQTAAQVT	LKAISAHFDD	SSASSLKNVM	FLLFDSSESIG	IYVQEMAKLD	AK	-----	-----	-----	-----	-----

Figure 11

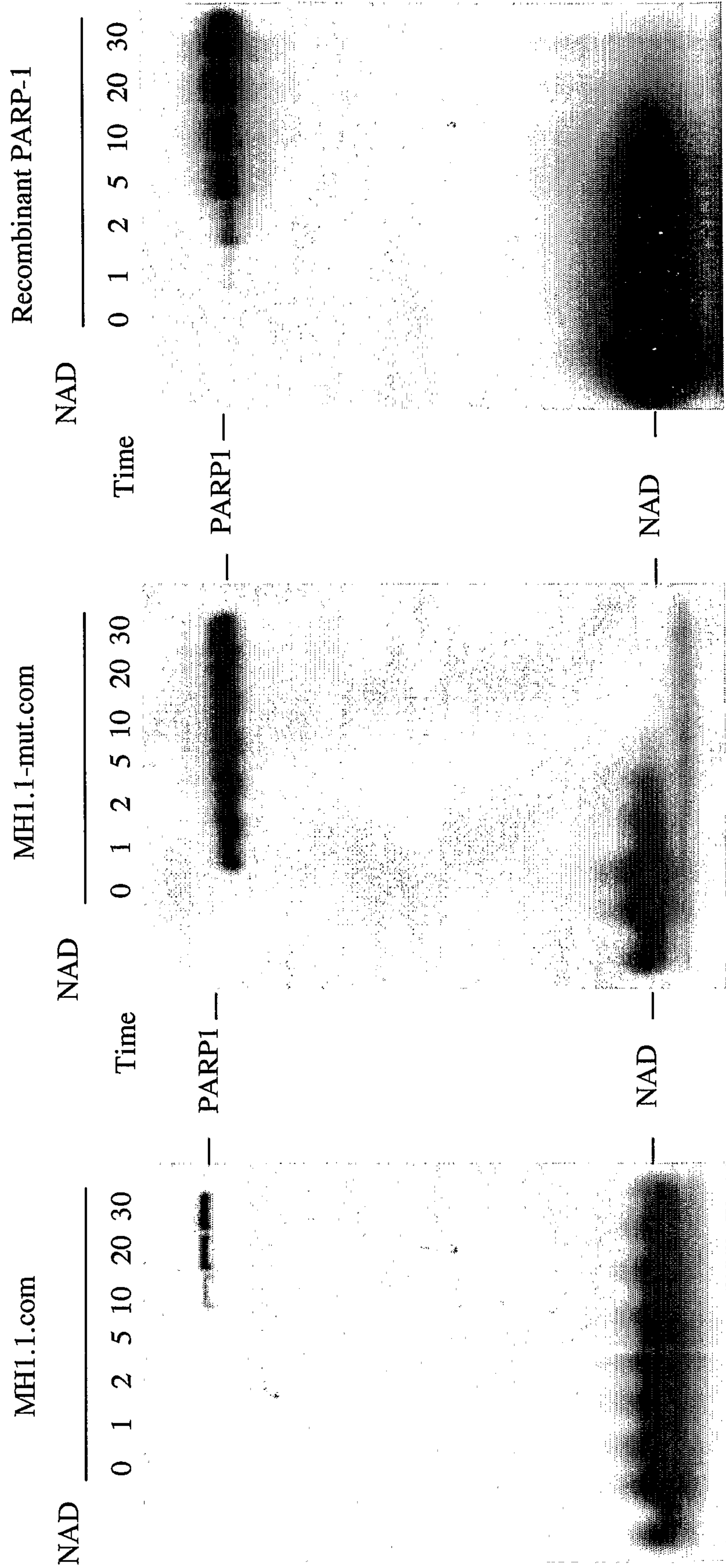


Figure 12

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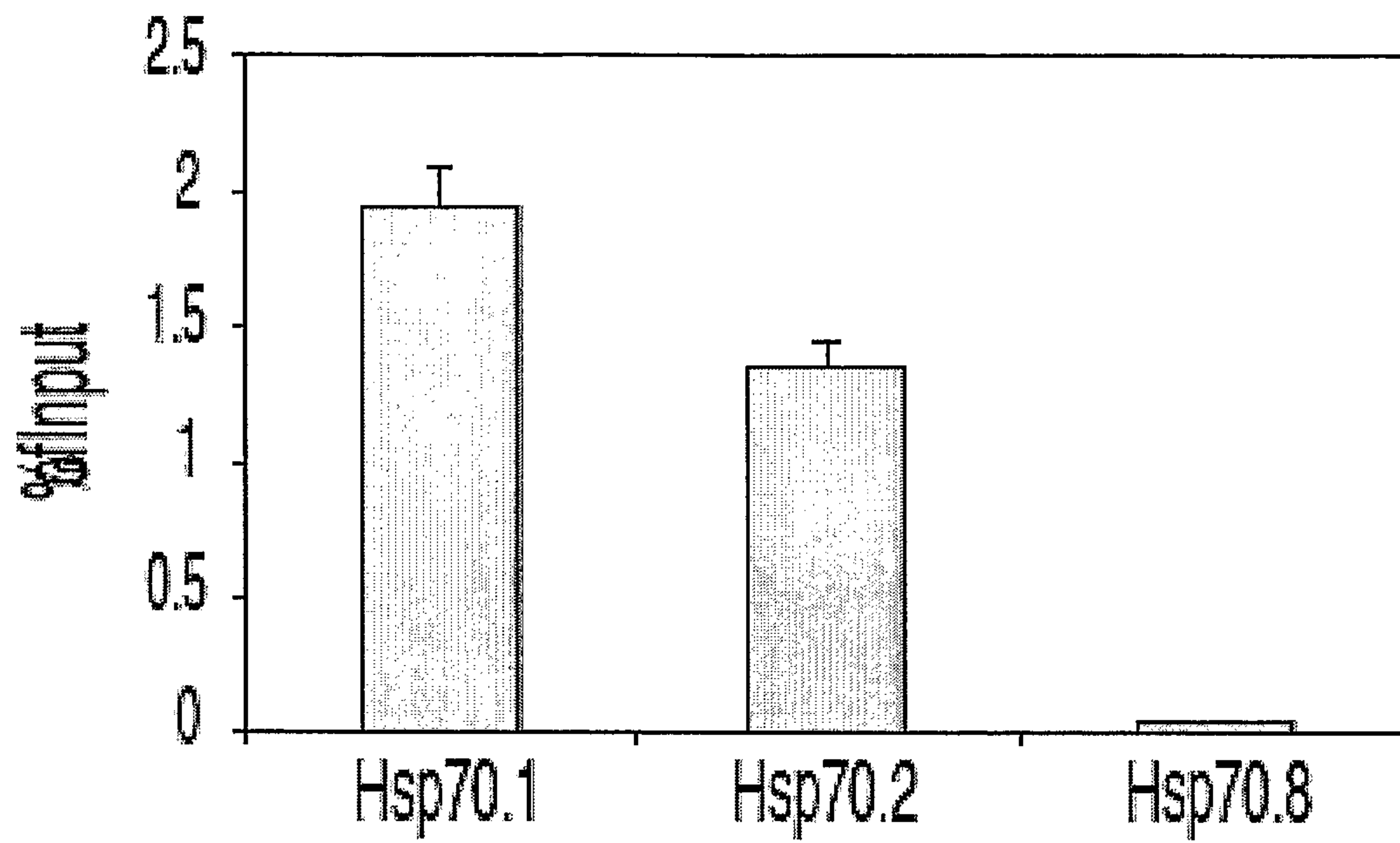


Figure 13

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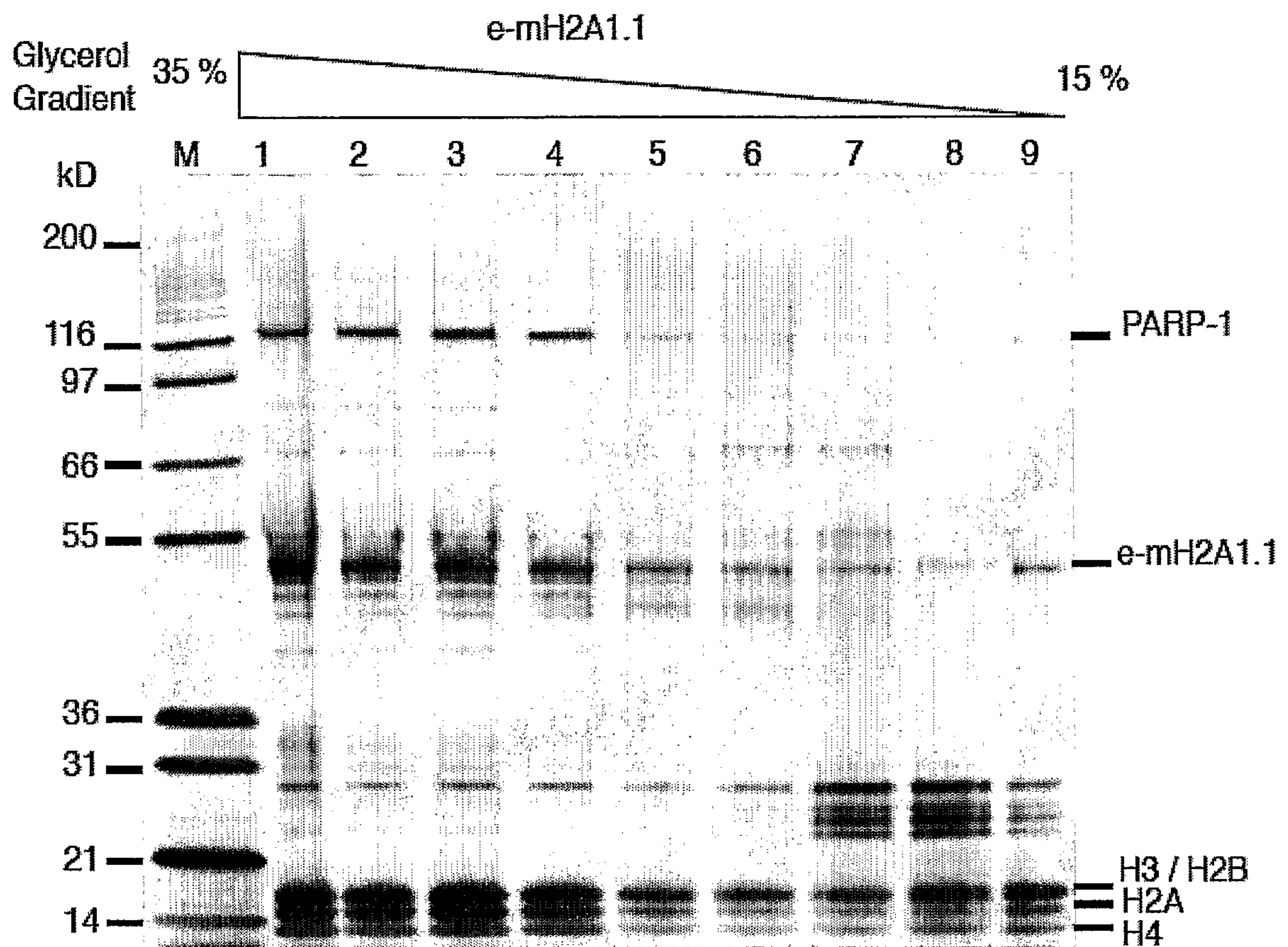


Figure 14

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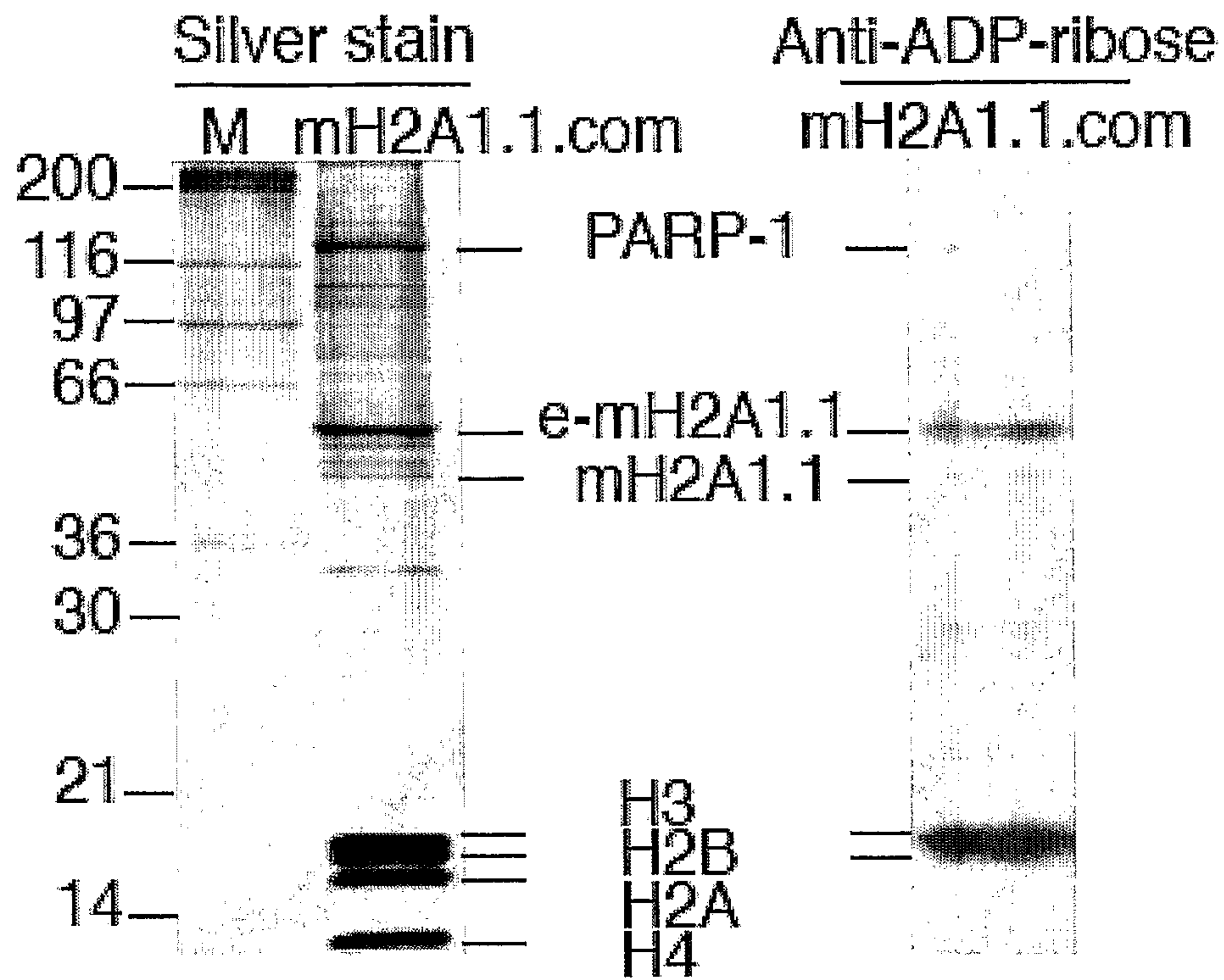


Figure 15

SC. CPDase	1	39	HVTV	150	HVSL	240
At. CPDase	1	43	HVTV	119	HLSL	182
Hs. CPDase	1	252	HCTT	331	HITL	422
Hs. mH2A1.1	1	213	HPTN	271	HCNS	370
Hs. mH2A1.2	1	216	NPTN	274	HCNS	373
Hs. mH2A2	1	216	HPTT	274	HCHI	373
Hs. mH2A1.1-mut	1	213	AAAA	273	HCNS	370
Consensus			HXTX		HXTX	
					S	
					N	
					H	

Figure 16

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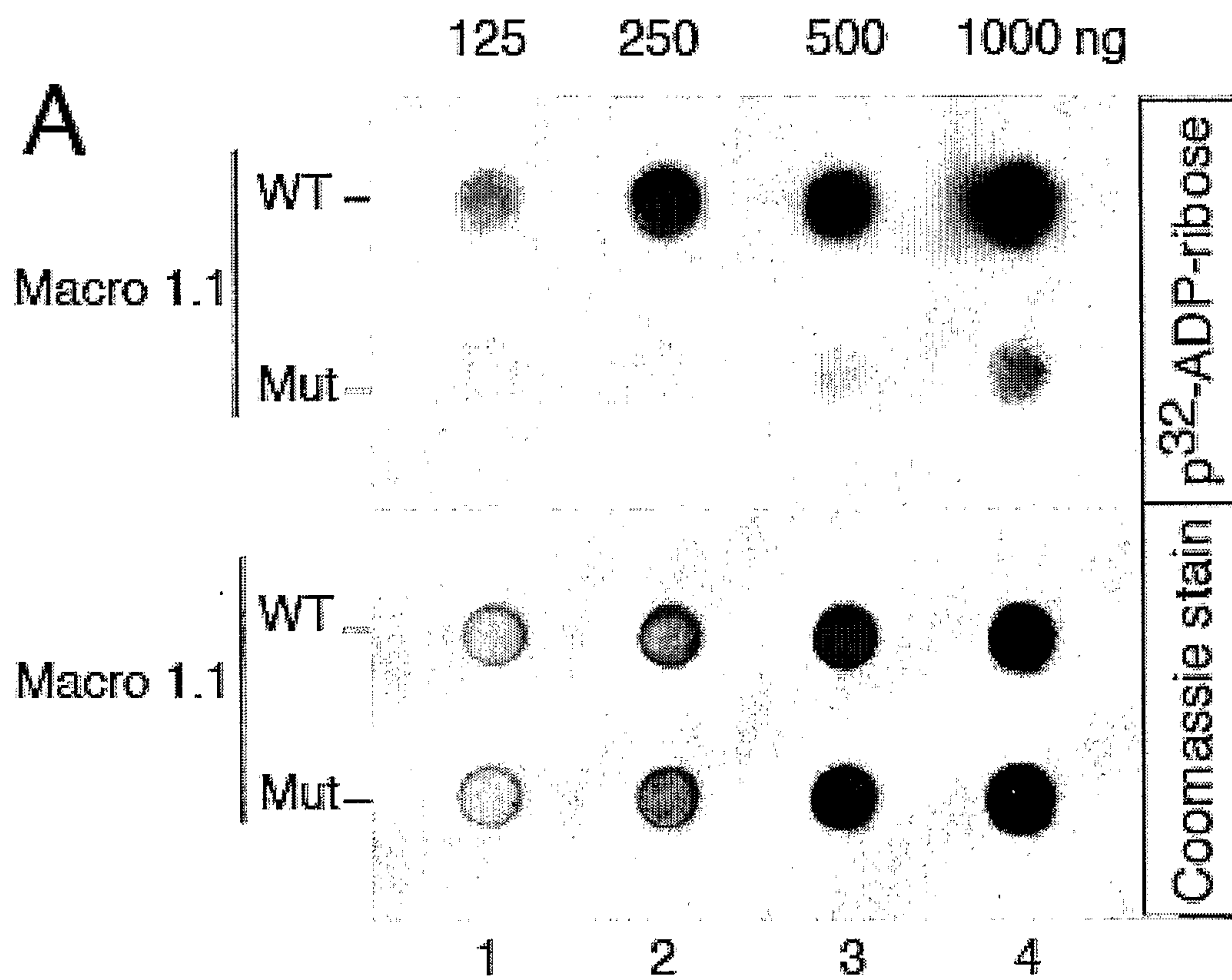


Figure 17

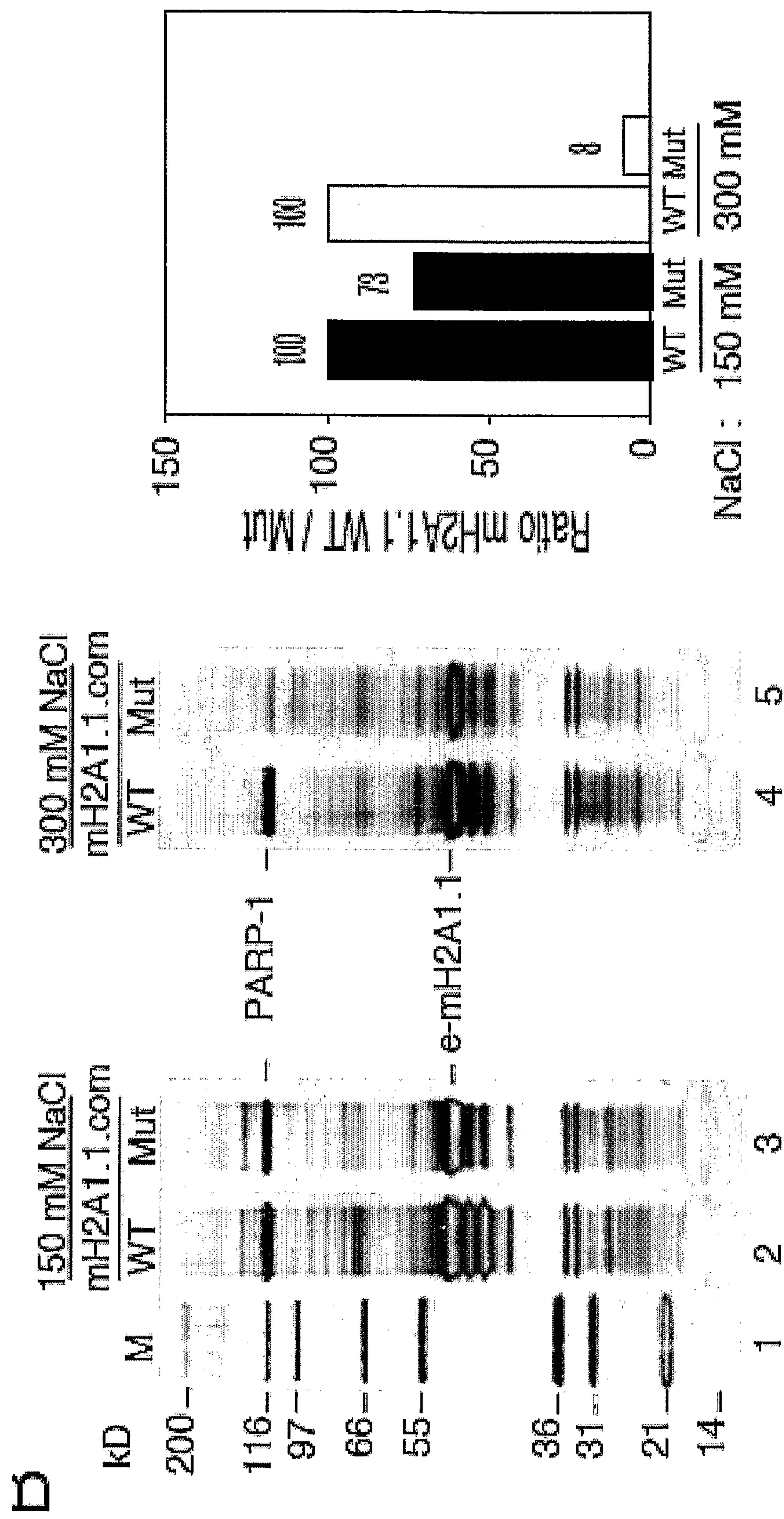


Figure 18

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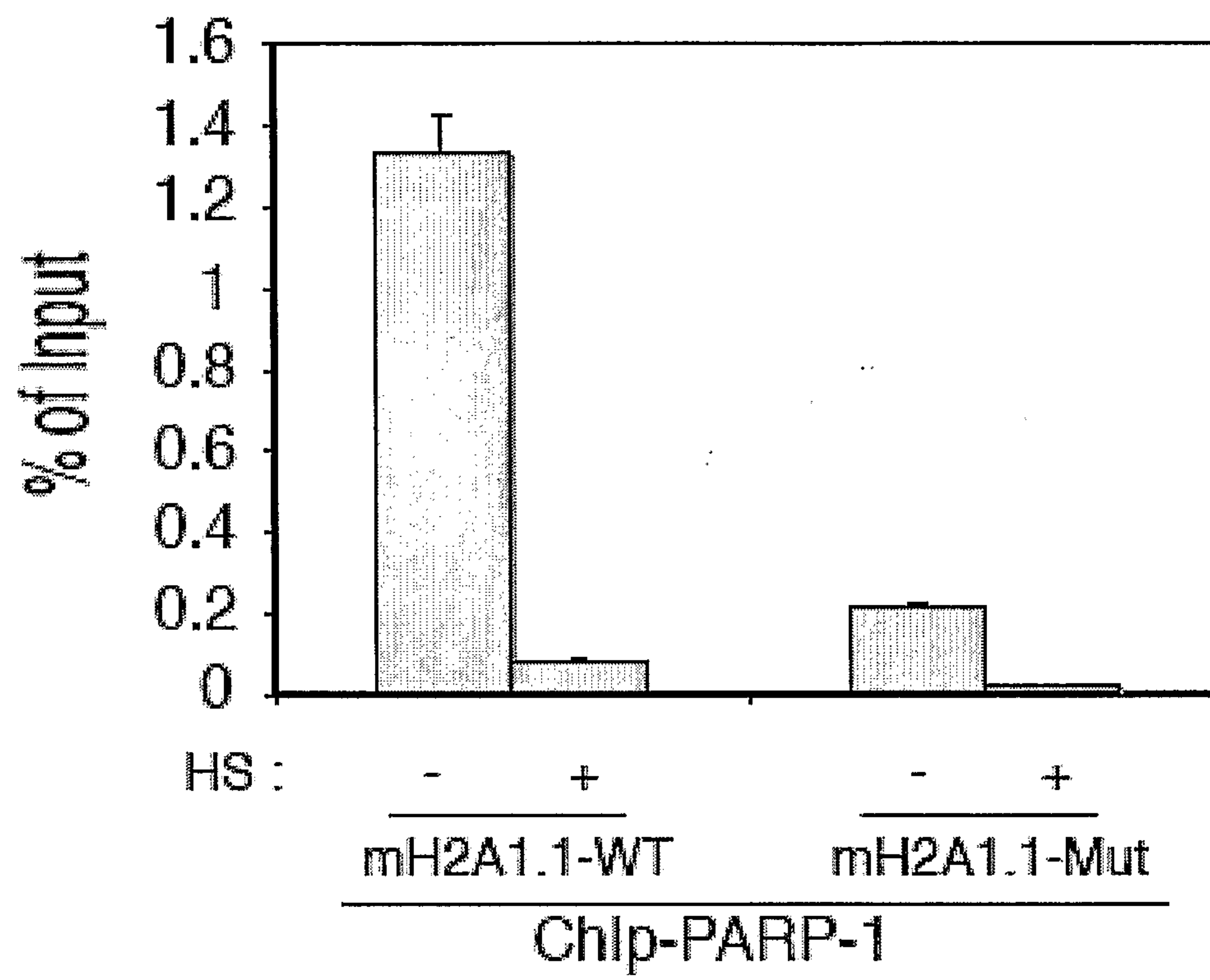


Figure 19

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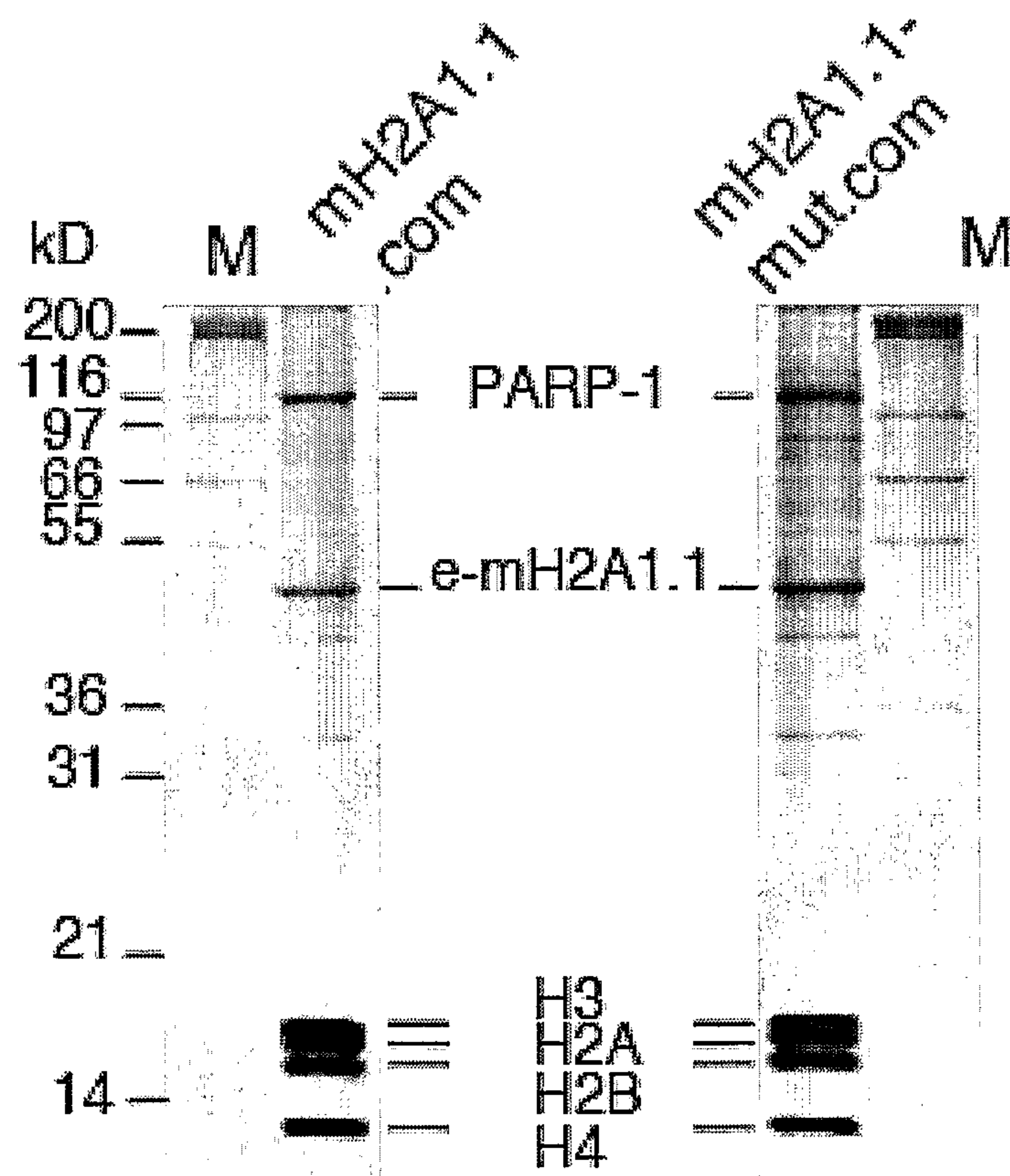


Figure 20

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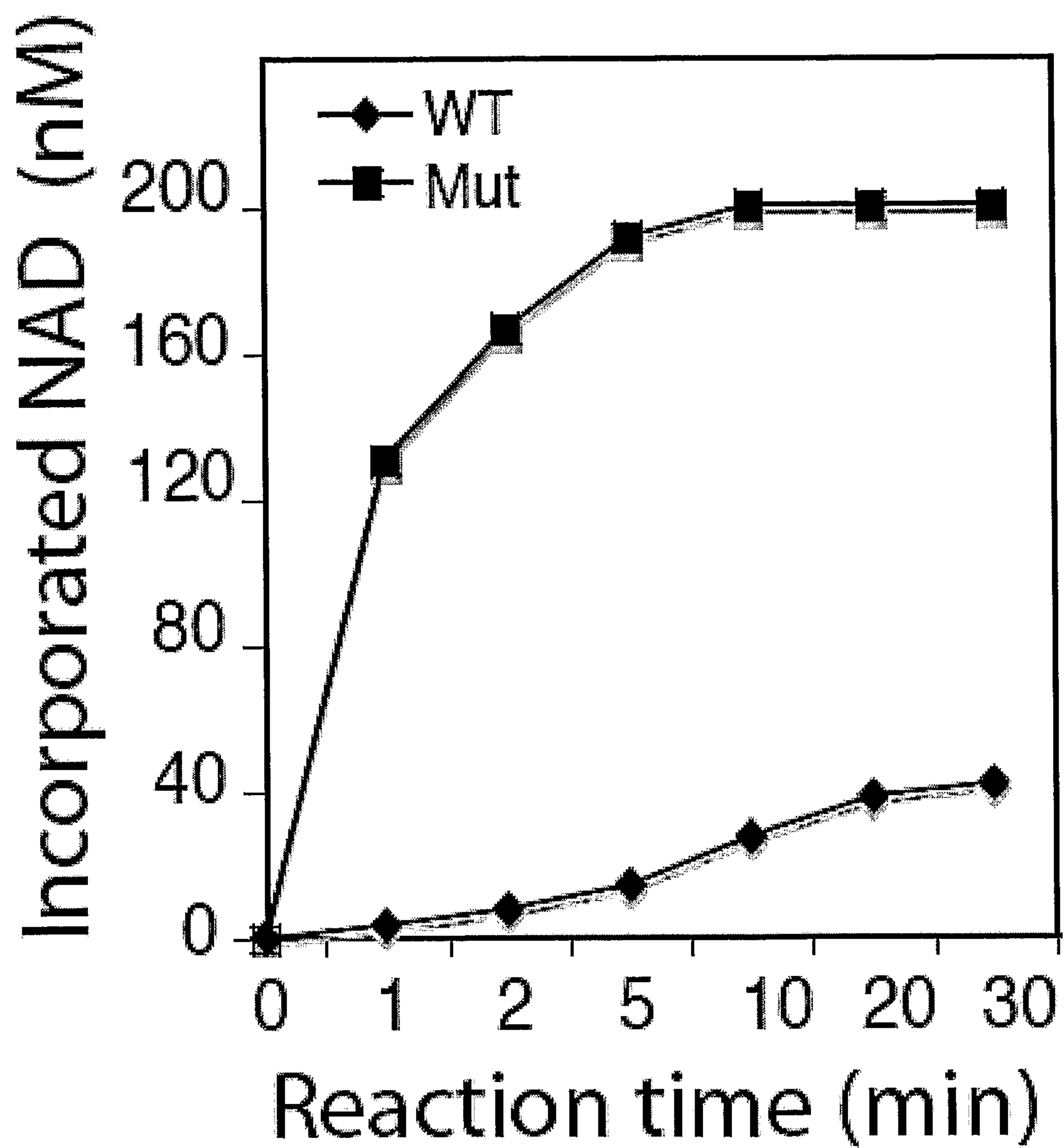


Figure 21

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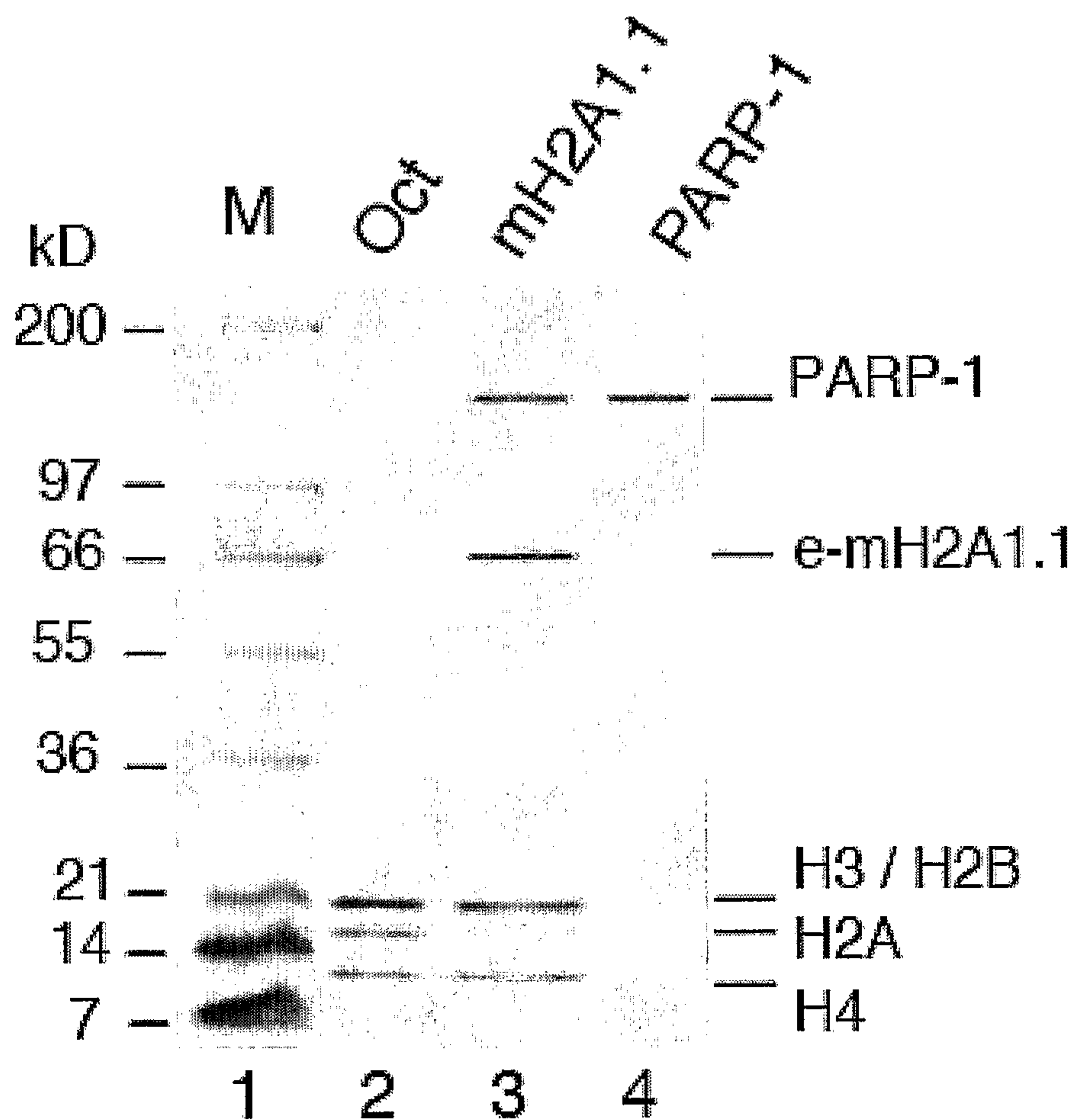


Figure 22

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	mH2A1-Nuc				PARP-1-Nuc			
	NAD							
Time (min) :	0	1	10	20	0	1	10	20

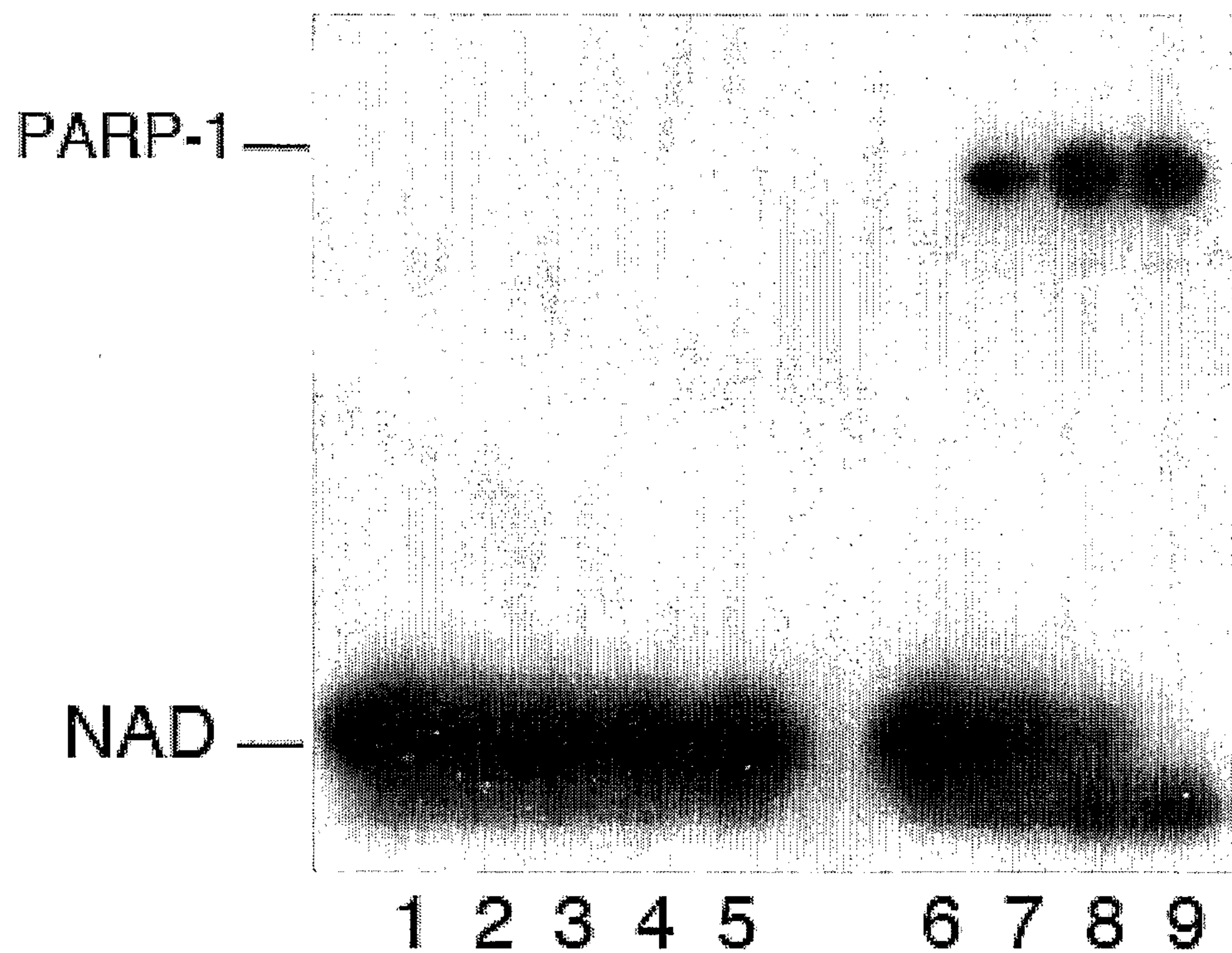


Figure 23

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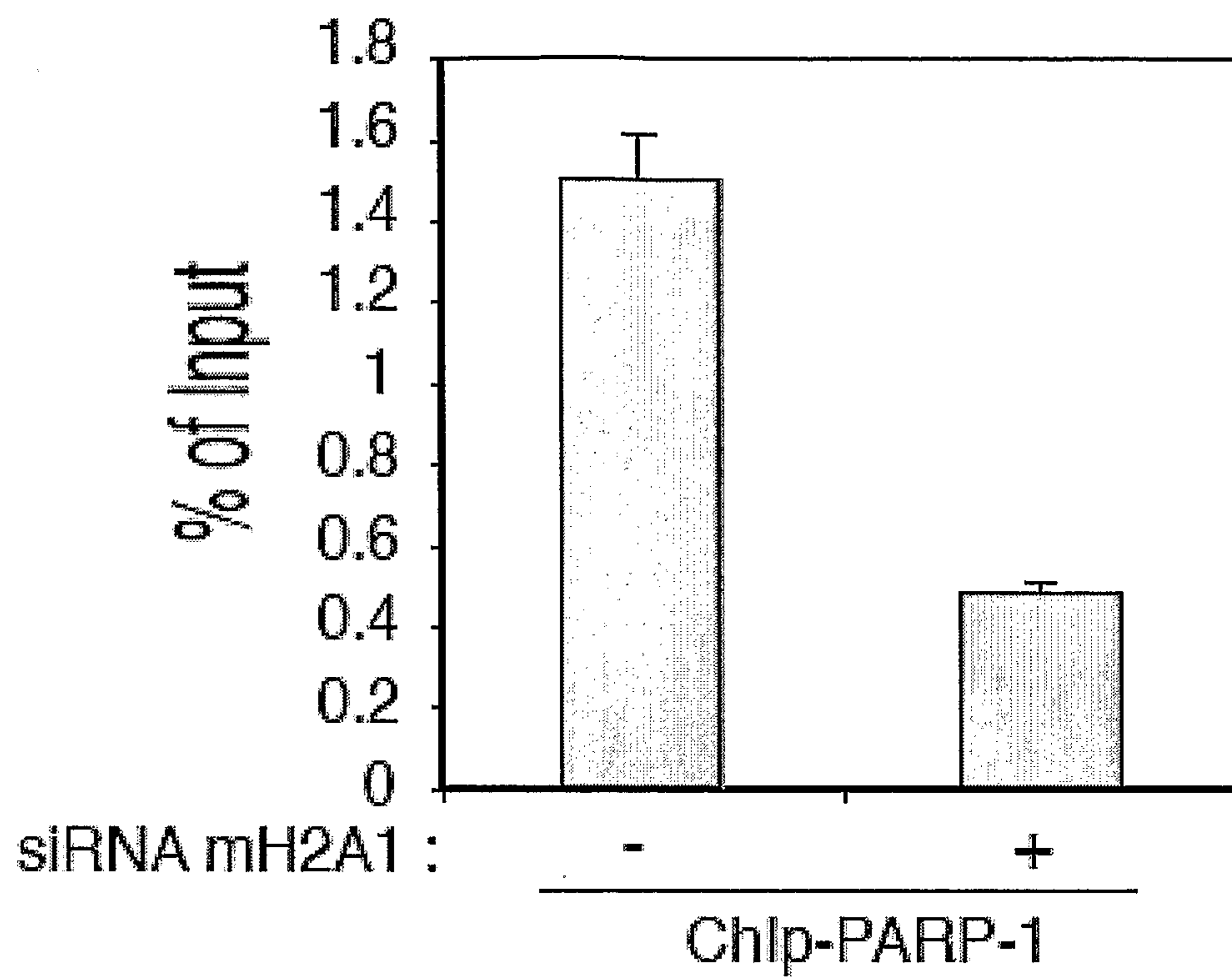


Figure 24