Highlights:

- Nox4-derived ROS mediate the growth and migration of glioblastoma cells.
- Nox4 transmits growth-and migration-regulating signals via PTP1B.
- Tyrosine-phosphorylated coronin-1C is identified as a novel substrate of PTP1B.

Nox4 redox regulation of PTP1B contributes to the proliferation and migration of glioblastoma cells by modulating tyrosine-phosphorylation of coronin-1C

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Abbreviations: ROS, reactive oxygen species; Nox4, NADPH oxidase 4; PTP1B, protein tyrosine phosphatase 1B; IAF, 5'-iodoacetamide fluorescein; EGF, epidermal growth factor; PTEN, phosphatase and tensin homologue

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Abstract

Glioblastoma multiforme is a common primary brain tumor in adults and one of the most devastating human cancers. Reactive oxygen species (ROS) generated by NADPH oxidase (Nox) 4 have recently been a focus of attention in the study of glioblastomas, but the molecular mechanisms underlying the actions of Nox4 remain elusive. In this study, we demonstrated that silencing of Nox4 expression by Nox4-targeted siRNA suppressed cell growth and motility of glioblastoma U87 cells, indicating the involvement of Nox4. Furthermore, Nox4-derived ROS oxidized and inactivated protein tyrosine phosphatase (PTP):1B: PTP1B in its active form downregulates cell proliferation and migration. By affinity purification with the substrate-trapping mutant of PTP1B, tyrosine-phosphorylated coronin-1C was identified as a substrate of PTP1B. Its tyrosine phosphorylation level was suppressed by Nox4 inhibition, suggesting that tyrosine-phosphorylation of coronin-1C is regulated by the Nox4-PTP1B pathway. Finally, ablation of coronin-1C attenuated the proliferative and migratory activity of the cells. Collectively, these findings reveal that Nox4-mediated redox regulation of PTP1B serves as a modulator, in part through coronin-1C, of the growth and migration of glioblastoma cells, and provide new insight into the mechanistic aspect of glioblastoma malignancy.

Keywords: NADPH oxidase (Nox)4; glioblastoma; PTP1B; coronin-1C; cell proliferation; cell migration.

Introduction

Although oxidative stress is related to cytotoxicity in general, there is now a growing body of evidence that reactive oxygen species (ROS) generated by the NADPH oxidase (Nox) family are involved in signal transduction in normal physiological processes such as host defense, vascularity, apoptosis, and cell growth [1]. Their catalytic subunits constitute seven isoforms of transmembrane proteins Nox1~5 and Duox1 and 2, each of which has a different regulatory mode and tissue distribution and displays similar but distinct structural and functional characteristics [2]. Nox isoforms catalyze the reduction of molecular oxygen by NADPH to generate superoxide and its metabolite, hydrogen peroxide. Among them, Nox4 appears to be a constitutively active enzyme that is regulated at the transcriptional level [2]. Nox4 proteins are localized not only in perinuclear regions and the endoplasmic reticulum [ER] but also in the plasma membrane and focal adhesions, and associated with mitochondria [3-7]. Although initially considered kidney-specific [8], Nox4 is also expressed in other tissues, including the blood vessel, heart, liver, and neurons [1,2]. It exerts mediating roles in various cellular functions, such as angiotensin II-induced vascularization [9] and insulin-triggered glucose uptake [10]. In contrast, aberrant control of Nox4 could contribute to human diseases, including cancer. Upregulated expression of Nox4 has been implicated in neoplastic transformation and tumor growth. For example, increased Nox4-derived ROS are required for the survival of pancreatic cancer cells [11,12] and contribute to the cell proliferation of melanoma cells [13] as well as von Hippel-Lindau-deficient renal cell carcinoma [14].

Malignant glioblastoma is the most aggressive tumor in the central nervous system; it is characterized by its rapid growth and high invasivity and has a very poor prognosis [15]. It is necessary to better understand the molecular pathogenesis of gliomas in order to provide a framework for improved treatment. Studies on the biology of gliomas have revealed multiple alterations in both gene expression and intracellular signalings. These include activation of PI3K/AKT anti-apoptotic and growth pathways due to genetic loss of phosphatase and tensin homologue (PTEN) [16-18] and dysregulation of Ras and PI3K/AKT pathways caused by overexpression or gain-of-function mutations in receptor tyrosine kinases such as the EGF receptor and PDGF receptor [19,20]. While these studies have greatly extended our understanding of the molecular basis of gliomagenesis, the function of ROS in the development of gliomas is less understood. With respect to the involvement of Nox isoform-derived ROS in gliomas, Nox4 overexpression has been specifically detected in glioblastoma cell lines [21] and patient glioma samples [22], and Nox4 was suggested to play mediating roles in the proliferation and survival activity of glioma cells [22] and in tumor growth via hypoxia inducing factor (HIF)-1 activation under hypoxia [23]. However, the detailed signaling mechanism for Nox4 action in glioblastoma cells is still undefined, mostly owing to a lack of information about downstream effectors for Nox4. In this study, we report characterization of the regulation and function of the Nox4 redox signaling pathway via tyrosine phosphatase PTP1B and describe that PTP1B utilizes coronin-1C, whose expression and activity were previously implicated in the malignancy of glioblastomas, as a physiological substrate [24].

Materials and Methods

Cell culture, reagents and plasmid

U87 cells were obtained from American Type Cell Collection (Rockville, MD, USA). SF188 and HUG31 cells were provided by Dr. T. Takeshita. All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. 5'-iodoacetamide fluorescein (IAF) and anti-fluorescein antibodies were purchased from Molecular Probes (Eugene, OR, USA). Luminol and horseradish peroxidase were obtained from Sigma-Aldrich (St. Louis, MO, USA). The pMT2-PTP1B and pMT2-PTP1B C215A plasmids were described [25]. The human wild type pCN-HA-PTP1B and mutant pCN-HA-PTP1B-D181A plasmids [26] were kindly provided by Dr. Z-Y Zhang.

RT-PCR and siRNA constructs

RT-PCR was performed as described previously [27]. The Nox primers used were described previously [28]. pSilencer human Nox4 siRNA and scrambled siRNA were described previously [27]. Nox4 siRNA duplexes (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were also used. siRNA molecules were designed as follows: 5′-UGACCAUAGUCGGAUUAAATT-3′ and 5′-UUUAAUCCGACUAUGGUCATT-3′ for PTP1B RNAi. Coronin-1C siRNA and control siRNA duplexes were purchased from Santa Cruz Biotechnology.

Transfection

Transfection with plasmid DNAs and siRNAs was performed by using Lipofectamine-2000 and Oligofectamine (Invitrogen, Carlsbad, CA, USA) or SCB

system (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were harvested 36-48h after transfection.

Immunoprecipitation and immunoblotting Cells were lysed in buffer A (25mM Tris-HCl, pH 7.5, 50mM NaCl, 0.5% Nonidet P-40, 1mM PMSF) or RIPA buffer with phosphatase inhibitors cocktail, and lysates were subjected to immunoprecipitation and immunoblotting as described [29]. The following antibodies were used: rabbit-anti Nox4 provided by Dr. A. Shah, mouse-anti HA, mouse-anti p21 and β-actin from Sigma-Aldrich, mouse-anti Rho from Cell Signaling (Lake Placid, NY, USA), rabbit-anti PTP1B and mouse-anti phosphotyrosine (clone 4G10) from Millipore (Temecula, CA, USA), and mouse-anti coronin-1C from Santa Cruz Biotechnology.

5'-IAF labeling

Cells were lysed in the lysis buffer (50mM MES-NaOH pH6.5, 0.5% TritonX-100, 1mM PMSF, 1μg/ml leupeptin, 1μg/ml aprotinin), and the lysates were incubated with 12μM 5'-IAF for 1h at 4°C under anaerobic condition [30,31]. Unbound 5'-IAF was removed by dialysis against PBS. The labeled proteins were subjected to immunoprecipitation with anti-fluorescein antibodies, followed by SDS-PAGE.

Purification of HA-tagged protein complexes

U87 cells were transfected with control vectors, HA-wtPTP1B and HA-PTP1B-D181A , and the cell lysates were prepared in buffer A with phosphatase inhibitor cocktail. Then, cell lysates were incubated in the spin column containing anti-HA bead suspension (Medical & Biological Laboratories, Nagoya, Japan) for 1 h at 4°C. The column was washed, and bound proteins were eluted with HA-peptides. The eluted

proteins were analyzed by immunoblotting.

GST-PTP1B pull down assay and MS analysis

PTP1B cDNA was subcloned into pGEX-6p-1 at BamHI and XhoI sites. GST and GST-PTP1B fusion proteins were expressed in JM109 cells and immobilized to glutathione-S-transferase beads according to the company's protocol (Amersham Pharmacia Biotechnology, Piscataway, NJ, USA). U87 cell lysates were prepared in buffer A with phosphatase inhibitor cocktail and incubated with GST- or GST-PTP1B-coupled beads at 4° for 3h. Proteins retained to the resins were analyzed by CBB staining and immunoblotting. Affinity-purified proteins were digested by trypsin and the peptides were fractionated by using a nanoscale RPC column (ZORBAX C18, 3mm, 100A, 75mm, i.d.; Agilent Technologies, Santa Clara, CA, USA). Peptide fragments were subjected to LC-MS/MS analysis by using a QSTAR quadrupole-TOF mass spectrometer (Applied Biosystems/MDS SCIEX, Concord, Ontario, Canada).

Growth assay

The numbers of cells that were not stained by trypan blue was counted.

Cell migration assay

Migration was assayed by using Boyden chambers (Nalge Nunc, Rochester, NY). Cells (1×10^4) were plated to the upper well of the chambers in serum-free DMEM, while the lower well was filled with DMEM containing 5% FBS as a cue. After 20h of incubation, migrating cells were counted.

Measurement of ROS generation

ROS generation was measured by using luminol according to the published method [32].

Cells (2.5×10^4) were suspended in Hank's buffer supplemented with calcium/magnesium (HBCM). Cells were mixed with 200 μM luminol and 0.8unit horseradish peroxidase in 500 μl HBCM and incubated at room temperature for 20min. Luminescence was quantified by a luminometer Lumart LB9507 (Berthold, Bad Wildbad, Germany).

Measurement of the Rho activity

GST-Rho binding domain-coupled glutathione S-transferase resins were prepared as described previously [31]. Cells were lysed in buffer A supplemented with 5mM $MgCl_2$ and phosphatase inhibitors cocktail, and the lysates were incubated with the resins for 3h at 4℃. The active Rho・GTP retained to the resins were analyzed by immunoblotting with anti-Rho antibodies.

Statistics

Differences or correlations between two groups were assessed by Student's t test. Differences with values $p<0.05$ were considered to be statistically significant.

Results

Nox4 mediates cell proliferation and migration of glioma cells

We first examined the expression of Nox isozymes in a subset of human glioma cell lines. Consistent with the previous reports [21], RT-PCR analysis showed that Nox4 mRNA was prominently expressed in the U87, SF188, and HUG31 cell lines, whereas little or no expression was detected with other Nox isozymes (Fig.1A). To investigate the bioactivity of Nox4 in glioma cells, we therefore introduced either pSilencer-Nox4 siRNAs or Nox4 siRNA duplex mixtures into the cells. RT-PCR and immunoblotting

analyses verified that different origin of Nox4 siRNAs effectively suppressed the expression of endogenous Nox4 in U87 cells, ensuring the specificity of Nox4 knockdown (Fig.1B). Furthermore, spontaneous ROS production in the cells was significantly inhibited by the transduction of Nox4 siRNAs suggesting that Nox4 is involved in ROS generation in glioma cells (Fig.1C). When the biological effect of Nox4 silencing was examined, Nox4 siRNAs exhibited an inhibitory effect on cell proliferation compared with scrambled siRNAs (Fig.2A), which is consistent with the previous report [22]. In order to give more specificity to this observation, the expression level of the cell cycle inhibitor p21 was examined. Nox4 knockdown resulted in a significant increase in the level of p21 expression, supporting the mitogenic effect of Nox4 (Fig.2B). In addition, we found that the strong migratory activity characteristic of glioblastoma cells was also repressed by Nox4 knockdown, as determined by Boyden chamber migration assay (Fig.2C). We then examined whether Nox4 affects the activity of small G-protein Rho, a key regulator of cytoskeletal contractility. GST-Rho-binding domain pull down assay demonstrated that the level of active Rho・GTP complexes was increased following transfection of Nox4 siRNAs (Fig.2D). This is consistent with the idea that Nox4-derived ROS promote cell motility by down-regulating the Rho activity, as in the case of Nox1-mediated migration in Ras transformed cells and colon cancer cells [33]. Taken together, these data suggest that Nox4 participates in the control of cell proliferation and migration in glioma cells. While Nox4 regulation of growth in glioblastoma cells was previously suggested [22], no study has reported the involvement of Nox4 in their motility.

PTP1B is involved in glioma cell growth and biochemically links to Nox4

We next intended to dissect the pathway downstream of Nox4 redox signaling involved in the bioactivities of glioma cells. We focused on PTP1B, because it has been suggested to participate in EGF signaling [34] and negatively regulate cell growth by acting as a target of Nox4-produced H_2O_2 upon EGF receptor tyrosine kinase activation [3]. To this end, the endogenous PTP1B expression level was manipulated by RNAi. RNAi-mediated suppression of PTP1B significantly increased the proliferation of U87 cells (Fig.2E), implicating that PTP1B, as a negative regulator, participates in the signaling involved in glioblastoma cell proliferation.

To examine whether there is a biochemical link between PTP1B and Nox4 in glioma cells, the oxidation state of PTP1B was analyzed by 5'-iodoacetamide fluorescein (IAF) labeling. Because it is a redox-sensitive nucleophile, the cysteine-215 residue located in the phosphate-binding motif of PTP1B [35] is attacked by 5'-IAF through competition with intracellular H_2O_2. U87 cells were transfected with Nox4 siRNAs, the cell lysates were incubated with 5'-IAF, and the extent of 5'-IAF labeling of PTP1B was evaluated by immunoprecipitation with anti-fluorescein antibodies. Nox4 siRNA transfection increased the level of the labeled PTP1B compared with scrambled siRNA transfection (Fig.3A), implying that Nox4 modulates PTP1B via oxidation. Since the PTP1B protein contains 10 cysteine residues including Cys215, which to varying degree may be labeled by IAF, we examined modification of this specific catalytic cysteine by using the C215A mutant of PTP1B. wtPTP1B or PTP1B-C215A was overexpressed in HEK293 cells together with exogenous Nox4 and subjected to 5'-IAF labeling. The

data indicated that the labeling of PTP1B-C215A was remarkably down-regulated compared with wtPTP1B, suggesting that Cys215 is preferentially attacked by IAF (Fig.3B). Taken together, the data support the notion that Nox4 biochemically links to PTP1B, and that the Nox4-PTP1B axis plays an important role in the signal transduction controlling the bioactivities of glioma cells.

Identification of the substrates of Nox4-regulated PTP1B

It remains to be determined which phosphoproteins are dephosphorylated by PTP1B in response to Nox4 redox signaling. To identify substrates of PTP1B, we used a substrate-trapping PTP1B-D181A mutant that irreversibly binds to the substrate [36]. U87 cells were transfected with HA-tagged wt PTP1B or PTP1B-D181A, cell lysates were immunoprecipitated with anti-HA antibodies, and the immunoprecipitates were probed by immunoblotting using anti-pTyr antibodies. In cells overexpressing the D181A mutant, tyrosine-phosphorylated p52 was prominently coprecipitated with the mutant PTP1B, whereas little or no p52 was precipitated with the wild type enzymes in cells overexpressing wild type PTP1B (Fig.4A). To further assess the above data, we took an alternative approach using HA-immobilized resins, because immunoprecipitation with antibodies is not always efficient for the recovery of interacting proteins. Lysates prepared from HA-wt PTP1B- or HA-PTP1B-D181A-transfected U87 cells were incubated with HA-conjugated resins, and the bound proteins were subjected to immunoblotting with anti-pTyr antibodies. In addition to the prominent p52, the pTyr-containing proteins p180, p130, p120, p70 and p58 were detected at low levels in cells overexpressing the trapping mutant

compared with the control vector or wt PTP1B transfectants (Fig.4B). The data suggest that the PTP1B mutant binds to these tyrosine-phosphorylated proteins more readily than to its wild type counterpart and protects them from dephosphorylation by endogenous PTP1B. Previous studies [3,36] have shown that the PTP1B-D181A mutant efficiently traps the EGF receptor, which has a molecular mass of ≈180KDa, in response to EGF stimulation. Furthermore, the EGF receptor is frequently amplified in those glioblastomas that include U87 cells [37], and our study indicates that the electrophoretic migration pattern of p180 coincides with that of the highly tyrosine-phosphorylated EGF receptor in EGF receptor-abundant epidermoidcarcinoma A431 cells (Fig.4B). Thus, the p180 band likely represents the EGF receptor.

Identification of p52 as coronin-1C

Since p52 is a prominent substrate of PTP1B in U87 cells, it was partially purified for further characterization. To this end, PTP1B-D181A fused to GST was immobilized to glutathione-Sepharose beads, and p52 proteins were affinity-purified by incubating the resins with lysates of U87 cells (Fig. 5A). Trypsin digests of p52 were fractionated by HPLC, and their primary structures were obtained by LC-MS/MS analysis. The amino acid sequences of three peptide peaks, peptides no.#1, #2, and #3, were found to be identical with residues 132-143, 408-419, and 462-471 of coronin-1C (Fig.5B). Immunoblotting analysis demonstrated that purified p52 is recognized by the antibody directed to the NH_2-terminus of coronin-1C (Fig.5C). From these observations, we conclude that p52 is identical to coronin-1C. Furthermore, coronin-1C was found to be tyrosine-phosphorylated (Fig.5C). This was detected not only in U87 cells but also

in HUG31 cells, which suggests that it is not a particular characteristic of U87 cells but rather common in glioblastoma cells (Fig.5D). To our knowledge, this is the first evidence that coronin-1C is a tyrosine-phosphorylated protein and a substrate of PTP1B. Coronin-1C (coronin3), which consists of 474 amino acids, is enriched at the leading edge of lamellipodia and also present in the cytosol [38,39]. The protein belongs to the coronin family, members of which are implicated in a variety of cellular processes, including signal transduction, transcriptional regulation, and remodeling of the cytoskeleton [40].

Nox4 redox regulation of coronin-1C tyrosine phosphorylation

To investigate the functional relationships between coronin-1C and Nox4 signaling, we first examined whether tyrosine-phosphorylation of coronin-1C is regulated by Nox4. U87 cells were transfected with Nox4 siRNAs, and tyrosine phosphorylation levels of endogenous coronin-1C bound to GST-PTP1B-D181A-affinity resins were evaluated. Overexpression of Nox4 siRNAs supressed coronin-1C tyrosine phosphorylation compared with that of scrambled siRNAs (Fig.5E). It is likely that depletion of Nox4-derived ROS restores the active state of PTP1B, thereby dephosphorylating tyrosine-phosphorylated coronin-1C. Thus, the data suggest that Nox4-generated ROS control the phosphorylation status of coronin-1C through oxidation of PTP1B. Because Nox4 siRNA-inhibited growth/migratory activities (Fig. 2A and 2C) is correlated with Nox4 siRNA-inhibited tryrosine phosphorylation of coronin-1C (Fig.5E), we believe that phosphorylation status of coronin-1C is critical for proliferation and migration of glioma cells.

Coronin-1C mediates proliferation and migration of glioma cells

If coronin-1C is an integral component of the Nox4-PTP1B signaling pathway, one might expect that, like Nox4 and PTP1B, coronin-1C also participates in regulation of the proliferation and migration of glioma cells. To test this possibility, the effect of coronin-1C siRNAs on these biological activities was examined with U87 cells. Ablation of coronin-1C suppressed the growth of U87 cells (Fig.6A). Likewise, cell migration was attenuated by coronin-1C knockdown (Fig.6B). Immunoblotting confirmed the inhibition of the expression of coronin-1C by coronin-1C siRNAs (Fig.6C). The data indicate that coronin-1C modulates the proliferation and motility of glioma cells, which is in agreement with the previous observation [24].

Discussion

Glioblastoma is the most aggressive tumor in the central nervous system and is characterized by rapid cell proliferation and high-level invasiveness. Nox4 expression was previously reported to be upregulated in glioblastoma cells and considered to play a role in cellular functions related to the malignant phenotype [22, 23], but its regulatory mechanism is largely unknown. Here, we show that Nox4-derived ROS signaling mediates cell proliferation and migration in glioblastoma cells via PTP1B as a ROS sensor, suggesting a critical role of Nox4 in the maintenance of glioblastoma malignancy. Furthermore, our studies reveal that coronin-1C is tyrosine-phosphorylated and acts as a substrate for PTP1B, thereby mediating signal transmission by the Nox4-PTP1B axis involved in the regulation of cell proliferation and migration. To our knowledge, this is the first evidence that Nox4-dependent redox-regulation of PTP1B is biochemically linked to tyrosine-phosphorylated

coronin-1C (see the model in Fig.7).

In Nox isoform-mediated cellular signal transduction, Nox1-derived ROS are believed to utilize protein tyrosine phosphatases as signal transducers. These include PTP1B, PTEN, Src-homology region 2 domain-containing phosphatase1, and low-molecular-weight protein tyrosine phosphatase [41]. The catalytic domain of these phosphatases contains nucleophilic, redox-sensitive cysteine-SH residues that undergo H_2O_2-caused oxidation, which results in impaired catalytic activity [35, 42]. As for Nox4, it has been described that PTP1B acts to terminate EGF receptor tyrosine kinase-dependent signaling, and that Nox4-dependent oxidative inactivation of PTP1B in the ER promotes autophosphorylation of the EGF receptor, maintaining EGF signaling [3]. Selective targeting of PTP1B has also been detected with ROS generated during EGF receptor-mediated fibroblast cell proliferation [34]. Consistent with these observations, our data indicated that Nox4-derived ROS regulate cell proliferation and motility of glioblastoma cells via PTP1B, which reinforces the importance of the Nox4-PTP1B pathway in cellular redox signaling. Despite numerous characterizations of the biochemical links between Nox isozymes and PTPs, the nature of the substrates for these PTPs is poorly understood. To help clarify this issue, substrate-trapping mutants have been developed and have proven to be very useful for isolating PTP1B substrates, as illustrated by identification of the EGF receptor tyrosine kinase in A431 cells [35, 36]. We have applied this strategy to identification of physiological PTP1B substrates in glioblastoma cells and demonstrated that tyrosine-phosphorylated coronin-1C is an as-yet-unidentified substrate of PTP1B.

Coronin-1C is an F-actin-binding protein that crosslinks and bundles actin filaments [38-40, 43]. Since the actin cytoskeleton dynamics is critically involved in cell division and migration of both normal and tumor cells, coronin-1C is expected to influence these biological events. Indeed, it has recently been shown that coronin-1C expression correlates with the grade of malignancy of diffuse gliomas, and that knockdown of coronin-1C results in a reduced level of cell proliferation, cell motility and invasion of glioblastoma cells *in vitro* [24], which was also confirmed in the present study. This suggests the close association of coronin-1C with the malignancy of glioblastomas. However, much of the biochemical mechanism of coronin-1C activity has remained to be determined. In this context, the current finding of coronin-1C as a pTyr-containing, PTP1B substrate has significant implications. The available data suggest that tyrosine phosphorylation of coronin-1C could promote cell proliferation and motility, although direct evidence for this is needed.

Tyrosine phosphorylation levels of coronin-1C can be controlled through the balance between phosphorylation by tyrosine kinases and dephosphorylation by PTP1B. Since coronin-1C is abundant at the leading edge of lamellipodia, receptor and nonreceptor tyrosine kinases such as the EGF receptor, FAK, and Src, which are enriched in the vicinity of the membrane compartments, may modulate coronin-1C. Thus, in a future study, it would be of great interest to define a tyrosine kinase (s) responsible for phosphorylation of coronin-1C.

Amplification and activation mutation of receptor kinases, including the EGF receptor, are frequently observed, leading to the activation of downstream Ras [19, 20].

Our results suggested that, in addition to coronin-1C, PTP1B likely recognizes the EGF receptor as a substrate (Fig.4B). Given these observations and the involvement of Nox4 in EGF receptor signaling [3], the Nox4-PTP1B axis may modulate the EGF receptor as well as coronin-1C-dependent pathways in glioblastoma cells (see the model in Fig.7).

In summary, our study highlights a novel link between Nox4-dependent redox regulation of PTP1B and tyrosine-phosphorylated coronin-1C. Furthermore, Nox4 seems to exert biological effects in glioblastoma cells by regulating tyrosine-phosphorylation of coronin-1C whose expression and function are closely associated with the malignant phenotype of glioblastomas. Thus, the discovery suggests a critical mediating role of Nox4 redox signaling in gliomagenesis. Nox4 may serve as a potential molecular target in the development of therapeutic agents for malignant gliomas.

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References

[1] Lambeth, J. D. Nox enzymes, ROS, and chronic disease: an example of antagonistic pleiotropy. *Free Radic Biol Med* **43:**332-347; 2007.

[2] Lambeth, J. D.; Kawahara, T.; Diebold, B. Regulation of Nox and Duox

enzymatic activity and expression. *Free Radic Biol Med* **43:**319-331; 2007.

[3] Chen, K.; Kirber, M. T.; Xiao, H.; Yang, Y.; Keaney, J. F., Jr. Regulation of ROS signal transduction by NADPH oxidase 4 localization. *J Cell Biol* **181:**1129-1139; 2008.

[4] Kuroda, J.; Ago, T.; Matsushima, S.; Zhai, P.; Schneider, M. D.; Sadoshima, J. NADPH oxidase 4 (Nox4) is a major source of oxidative stress in the failing heart. *Proc Natl Acad Sci U S A* **107:**15565-15570; 2010.

[5] Hilenski, L. L.; Clempus, R. E.; Quinn, M. T.; Lambeth, J. D.; Griendling, K. K. Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* **24:**677-683; 2004.

[6] Block, K.; Gorin, Y.; Abboud, H. E. Subcellular localization of Nox4 and regulation in diabetes. *Proc Natl Acad Sci U S A* **106:**14385-14390; 2009.

[7] Kuroda, J.; Nakagawa, K.; Yamasaki, T.; Nakamura, K.; Takeya, R.; Kuribayashi, F.; Imajoh-Ohmi, S.; Igarashi, K.; Shibata, Y.; Sueishi, K.; Sumimoto, H. The superoxide-producing NAD(P)H oxidase Nox4 in the nucleus of human vascular endothelial cells. *Genes Cells* **10:**1139-1151; 2005.

[8] Geiszt, M.; Kopp, J. B.; Varnai, P.; Leto, T. L. Identification of renox, an NAD(P)H oxidase in kidney. *Proc Natl Acad Sci U S A* **97:**8010-8014; 2000.

[9] Lassegue, B.; Sorescu, D.; Szocs, K.; Yin, Q.; Akers, M.; Zhang, Y.; Grant, S. L.; Lambeth, J. D.; Griendling, K. K. Novel gp91(phox) homologues in vascular smooth muscle cells : nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways. *Circ Res* **88:**888-894; 2001.

[10] Mahadev, K.; Motoshima, H.; Wu, X.; Ruddy, J. M.; Arnold, R. S.; Cheng, G.; Lambeth, J. D.; Goldstein, B. J. The NAD(P)H oxidase homolog Nox4 modulates insulin-stimulated generation of H2O2 and plays an integral role in insulin signal transduction. *Mol Cell Biol* **24:**1844-1854; 2004.

[11] Vaquero, E. C.; Edderkaoui, M.; Pandol, S. J.; Gukovsky, I.; Gukovskaya, A. S. Reactive oxygen species produced by NAD(P)H oxidase inhibit apoptosis in pancreatic cancer cells. *J Biol Chem* **279:**34643-34654; 2004.

[12] Mochizuki, T.; Furuta, S.; Mitsushita, J.; Shang, W. H.; Ito, M.; Yokoo, Y.; Yamaura, M.; Ishizone, S.; Nakayama, J.; Konagai, A.; Hirose, K.; Kiyosawa, K.; Kamata, T. Inhibition of NADPH oxidase 4 activates apoptosis via the AKT/apoptosis signal-regulating kinase 1 pathway in pancreatic cancer PANC-1 cells. *Oncogene*

25:3699-3707; 2006.

[13] Yamaura, M.; Mitsushita, J.; Furuta, S.; Kiniwa, Y.; Ashida, A.; Goto, Y.; Shang, W. H.; Kubodera, M.; Kato, M.; Takata, M.; Saida, T.; Kamata, T. NADPH oxidase 4 contributes to transformation phenotype of melanoma cells by regulating G2-M cell cycle progression. *Cancer Res* **69:**2647-2654; 2009.

[14] Maranchie, J. K.; Zhan, Y. Nox4 is critical for hypoxia-inducible factor 2-alpha transcriptional activity in von Hippel-Lindau-deficient renal cell carcinoma. *Cancer Res* **65:**9190-9193; 2005.

[15] Demuth, T.; Berens, M. E. Molecular mechanisms of glioma cell migration and invasion. *J Neurooncol* **70:**217-228; 2004.

[16] Li, J.; Yen, C.; Liaw, D.; Podsypanina, K.; Bose, S.; Wang, S. I.; Puc, J.; Miliaresis, C.; Rodgers, L.; McCombie, R.; Bigner, S. H.; Giovanella, B. C.; Ittmann, M.; Tycko, B.; Hibshoosh, H.; Wigler, M. H.; Parsons, R. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* **275:**1943-1947; 1997.

[17] Holland, E. C.; Celestino, J.; Dai, C.; Schaefer, L.; Sawaya, R. E.; Fuller, G. N. Combined activation of Ras and Akt in neural progenitors induces glioblastoma formation in mice. *Nat Genet* **25:**55-57; 2000.

[18] Sonoda, Y.; Ozawa, T.; Aldape, K. D.; Deen, D. F.; Berger, M. S.; Pieper, R. O. Akt pathway activation converts anaplastic astrocytoma to glioblastoma multiforme in a human astrocyte model of glioma. *Cancer Res* **61:**6674-6678; 2001.

[19] Feldkamp, M. M.; Lala, P.; Lau, N.; Roncari, L.; Guha, A. Expression of activated epidermal growth factor receptors, Ras-guanosine triphosphate, and mitogen-activated protein kinase in human glioblastoma multiforme specimens. *Neurosurgery* **45:**1442-1453; 1999.

[20] Rajasekhar, V. K.; Viale, A.; Socci, N. D.; Wiedmann, M.; Hu, X.; Holland, E. C. Oncogenic Ras and Akt signaling contribute to glioblastoma formation by differential recruitment of existing mRNAs to polysomes. *Mol Cell* **12:**889-901; 2003.

[21] Cheng, G.; Cao, Z.; Xu, X.; van Meir, E. G.; Lambeth, J. D. Homologs of gp91phox: cloning and tissue expression of Nox3, Nox4, and Nox5. *Gene* **269:**131-140; 2001.

[22] Shono, T.; Yokoyama, N.; Uesaka, T.; Kuroda, J.; Takeya, R.; Yamasaki, T.; Amano, T.; Mizoguchi, M.; Suzuki, S. O.; Niiro, H.; Miyamoto, K.; Akashi, K.; Iwaki,

T.; Sumimoto, H.; Sasaki, T. Enhanced expression of NADPH oxidase Nox4 in human gliomas and its roles in cell proliferation and survival. *Int J Cancer* **123:**787-792; 2008.

[23] Hsieh, C. H.; Shyu, W. C.; Chiang, C. Y.; Kuo, J. W.; Shen, W. C.; Liu, R. S. NADPH oxidase subunit 4-mediated reactive oxygen species contribute to cycling hypoxia-promoted tumor progression in glioblastoma multiforme. *PLoS One* **6:**e23945; 2011.

[24] Thal, D.; Xavier, C. P.; Rosentreter, A.; Linder, S.; Friedrichs, B.; Waha, A.; Pietsch, T.; Stumpf, M.; Noegel, A.; Clemen, C. Expression of coronin-3 (coronin-1C) in diffuse gliomas is related to malignancy. *J Pathol* **214:**415-424; 2008.

[25] Flint, A. J.; Gebbink, M. F.; Franza, B. R., Jr.; Hill, D. E.; Tonks, N. K. Multi-site phosphorylation of the protein tyrosine phosphatase, PTP1B: identification of cell cycle regulated and phorbol ester stimulated sites of phosphorylation. *EMBO J* **12:**1937-1946; 1993.

[26] Xie, L.; Zhang, Y. L.; Zhang, Z. Y. Design and characterization of an improved protein tyrosine phosphatase substrate-trapping mutant. *Biochemistry* **41:**4032-4039; 2002.

[27] Mitsushita, J.; Lambeth, J. D.; Kamata, T. The superoxide-generating oxidase Nox1 is functionally required for Ras oncogene transformation. *Cancer Res* **64:**3580-3585; 2004.

[28] Mochizuki, T.; Furuta, S.; Mitsushita, J.; Shang, W. H.; Ito, M.; Yokoo, Y.; Yamaura, M.; Ishizone, S.; Nakayama, J.; Konagai, A.; Hirose, K.; Kiyosawa, K.; Kamata, T. Inhibition of NADPH oxidase 4 activates apoptosis via the AKT/apoptosis signal-regulating kinase 1 pathway in pancreatic cancer PANC-1 cells. *Oncogene* **25:**3699-3707; 2006.

[29] Komatsu, D.; Kato, M.; Nakayama, J.; Miyagawa, S.; Kamata, T. NADPH oxidase 1 plays a critical mediating role in oncogenic Ras-induced vascular endothelial growth factor expression. *Oncogene* **27:**4724-4732; 2008.

[30] Wu, Y.; Kwon, K. S.; Rhee, S. G. Probing cellular protein targets of H2O2 with fluorescein-conjugated iodoacetamide and antibodies to fluorescein. *FEBS Lett* **440:**111-115; 1998.

[31] Shinohara, M.; Shang, W. H.; Kubodera, M.; Harada, S.; Mitsushita, J.; Kato, M.; Miyazaki, H.; Sumimoto, H.; Kamata, T. Nox1 redox signaling mediates oncogenic Ras-induced disruption of stress fibers and focal adhesions by down-regulating Rho. *J*

Biol Chem **282:**17640-17648; 2007.

[32] Cheng, G.; Ritsick, D.; Lambeth, J. D. Nox3 regulation by NOXO1, p47phox, and p67phox. *J Biol Chem* **279:**34250-34255; 2004.

[33] Shinohara, M.; Adachi, Y.; Mitsushita, J.; Kuwabara, M.; Nagasawa, A.; Harada, S.; Furuta, S.; Zhang, Y.; Seheli, K.; Miyazaki, H.; Kamata, T. Reactive oxygen generated by NADPH oxidase 1 (Nox1) contributes to cell invasion by regulating matrix metalloprotease-9 production and cell migration. *J Biol Chem* **285:**4481-4488; 2010.

[34] Lee, S. R.; Kwon, K. S.; Kim, S. R.; Rhee, S. G. Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. *J Biol Chem* **273:**15366-15372; 1998.

[35] Barford, D.; Flint, A. J.; Tonks, N. K. Crystal structure of human protein tyrosine phosphatase 1B. *Science* **263:**1397-1404; 1994.

[36] Flint, A. J.; Tiganis, T.; Barford, D.; Tonks, N. K. Development of "substrate-trapping" mutants to identify physiological substrates of protein tyrosine phosphatases. *Proc Natl Acad Sci U S A* **94:**1680-1685; 1997.

[37] Hegi, M. E.; Rajakannu, P.; Weller, M. Epidermal growth factor receptor: a re-emerging target in glioblastoma. *Curr Opin Neurol* **25:**774-779; 2012..

[38] Rybakin, V.; Clemen, C. S. Coronin proteins as multifunctional regulators of the cytoskeleton and membrane trafficking. *Bioessays* **27:**625-632; 2005.

[39] Spoerl, Z.; Stumpf, M.; Noegel, A. A.; Hasse, A. Oligomerization, F-actin interaction, and membrane association of the ubiquitous mammalian coronin 3 are mediated by its carboxyl terminus. *J Biol Chem* **277:**48858-48867; 2002.

[40] Roadcap, D. W.; Clemen, C. S.; Bear, J. E. The role of mammalian coronins in development and disease. *Subcell Biochem* **48:**124-135; 2008.

[41] Byon, J. C.; Kusari, A. B.; Kusari, J. Protein-tyrosine phosphatase-1B acts as a negative regulator of insulin signal transduction. *Mol Cell Biochem* **182:**101-108; 1998.

[42] Rhee, S. G.; Chang, T. S.; Bae, Y. S.; Lee, S. R.; Kang, S. W. Cellular regulation by hydrogen peroxide. *J Am Soc Nephrol* **14:**S211-215; 2003.

[43] Hasse, A.; Rosentreter, A.; Spoerl, Z.; Stumpf, M.; Noegel, A. A.; Clemen, C. S. Coronin 3 and its role in murine brain morphogenesis. *Eur J Neurosci* **21:**1155-1168; 2005.

Figure Legends

Figure 1. mRNA expression of Nox family members in glioma cells and suppression of both Nox4 expression and ROS generation by Nox4 siRNAs. A. Total RNAs were extracted from three glioma cell lines, and mRNA expression of Nox family members were analyzed by RT-PCR with EF1α as an internal control. **B**. U87 cells were transfected with pSilencer Nox4 siRNAs or scrambled siRNAs (SC), and 48h later, total RNAs were extracted from transfected cells, and RT-PCR with EF1α as a control was performed to analyze expression of endogenous Nox4 mRNAs. Alternatively, after transfection with Nox4 siRNA duplex mixtures, lysates were prepared and subjected to immunoblotting with rabbit anti-Nox4 antibodies. β-actin is a loading control. **C.** U87 cells were transfected with Nox4 siRNAs or scrambled siRNAs for 48h. ROS levels were measured by luminol assay. The data represent mean ± S. D. (n=3).

Figure 2. Involvement of Nox4 and PTP1B in cell growth and migration of U87 cells. A and **B.** U87 cells were transfected with Nox4 siRNA or scrambled siRNA duplex mixtures and cultured for 3d. The number of cells was counted (**A**) or the expression level of p21 cell cycle inhibitor was evaluated by immunoblotting with anti-p21 antibodies (**B**). **C.** U87 cells were transfected with Nox4 siRNAs or scrambled siRNAs as in A and 48h later, replated into Boyden chambers. After 20h incubation, the number of migrated cells was counted. **D.** U87 cells were transfected with Nox4 siRNA or scrambled siRNA duplex mixtures, and cell lysates were subjected to the Rho activity assay. The level of endogenous Rho was monitored by anti-Rho

antibodies. **E.** U87 cells were transfected with PTP1B siRNAs or scrambled siRNAs and cultured for 3d. The number of cells was counted. The suppression of PTP1B expression with PTP1B siRNAs was examined by immunoblotting anti-PTP1B antibodies. The histograms represent mean ± S. D. (n=3) in **A, C** and **E.** β-actin is a loading control in **B** and **E**.

Figure 3. Nox4 mediates oxidation of PTP1B. **A.** U87 cells were co-transfected with HA-wtPTP1B, Nox4 siRNAs, or scrambled siRNAs. The cells were subjected to 5'-IAF labeling, and labeled PTP1B was detected as described in Materials and Methods. Expression of transfected PTP1B proteins was monitored by immunoblotting with anti-HA antibodies. Histograms represent relative intensity of labeled PTP1B bands (mean ± S. D., n=3). **B.** HEK293 cells were transfected with pMT2-wtPTP1B or pMT2-PTP1B-C215A (CA) together with pcDNA3.0-Nox4 and subjected to 5'-IAF labeling. Expression of transfected wtPTP1B and PTP1B-C215A was monitored by immunoblotting with anti-PTP1B antibodies.

Figure 4. Precipitation of PTP1B substrates by the PTP1B substrate-trapping mutant. **A.** U87 cells were transfected with HA-wtPTP1B, HA-PTP1B-D181A, and control vectors. Lysates were prepared and immunoprecipitated with anti-HA antibodies, and the immunoprecipitates were probed with anti-phosphotyrosine antibodies. Expression of transfected proteins was monitored by immunoblotting with anti-HA antibodies. **B.** U87 cells were transfected with wild type and mutant PTP1Bs as described in **A.** Lysates were prepared and incubated with HA-immobilized resins. The bound proteins were eluted and analyzed by immunoblotting with

anti-phosphotyrosine antibodies. Quality control (QC) for IB indicates EGF-stimulated A431 cell lysates. The heavily phosphorylated protein band with a molecular mass of 180KDa represents EGF receptor. Expression of transfected proteins was monitored as in **A**. In **A** and **B**, an asterisk and arrowheads indicate p52 and other protein bands trapped by PTP1B, respectively.

Figure 5. Purification and identification of tyrosine-phosphorylated p52 as coronin-1C. **A.** GST-PTP1B-D181A was constructed, produced, and purified as described in Materials and Methods. U87 cell lysates were prepared and incubated with GST-PTP1B-D181A-immobilized glutathione agarose beads. The bound proteins were eluted and resolved by SDS-PAGE, followed by Coomassie Brilliant Blue (CBB) staining. An asterisk and an arrowhead indicate p52 and GST-PTP1B-D181A, respectively. **B.** MS analysis of p52 protein. p52 proteins purified as in **A** were digested by trypsin, and the peptides were separated and subjected to MS analysis. Amino acid sequences obtained from peptide peaks #1, #2, and #3 are compared to those of coronin-1C peptides. **C.** p52 proteins eluted from GST-PTP1B-D181A-coupled resins were subjected to immunoblotting with anti-phosphotyrosine antibodies and anti-coronin-1C antibodies. **D.** Cell lysates from U87 and HUG31 cells were immunoprecipitated with anti-pTyr antibodies, and the immunoprecipitates were probed with anti-coronin-1C antibodies. **E.** Inhibition of Nox4 suppresses tyrosine-phosphorylation of coronin-1C. U87 cells were transfected with Nox4 siRNAs or scrambled siRNAs for 48h. Cell lysates were prepared and incubated with GST-PTP1B-D181A-immobilized resins. Coronin-1C bound to resins

was eluted and subjected to immunoblotting with anti-phosphotyrosine and anti-coronin-1C antibodies. Histograms show relative intensity of tyrosine-phosphorylated coronin-1C bands (mean ± S. D., n=3).

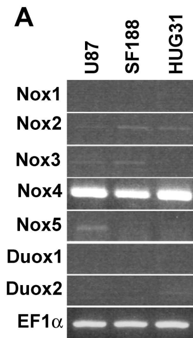
Figure 6. Coronin-1C inhibition blocks cell proliferation and migration.

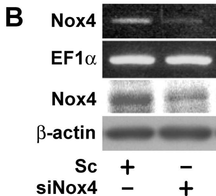
A. U87 cells were transfected with coronin-1C siRNAs or scrambled siRNAs, cultured for 3d, and the number of cells were counted. The data represent mean ± S. D. (n=3). **B.** U87 cells were transfected with coronin-1C siRNAs or scrambled siRNAs as in **A**. 48h later, cells were replated into Boyden chambers, and the number of migrated cells were determined. The data represent mean ± S. D. (n=3). **C.** U87 cells were transfected with coronin-1C siRNAs as in **A**, and cell lysates were subjected to immunoblotting with anti-coronin-1C antibodies. β-actin is a loading control.

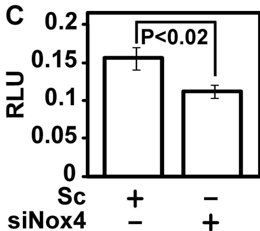
Figure 7. Schematic model for the role of Nox4-PTP1B signaling in glioblastoma cells.

Nox4 expression is upregulated and Nox4-generated ROS are increased in glioblastoma cells. Coronin-1C is tyrosine-phosphorylated by a certain tyrosine kinase (TK). An increase in Nox4-derived ROS may aid the maintenance of maximal tyrosine phosphorylation of coronin-1C by oxidizing and inactivating of PTP1B and thereby, at least in part, contribute to cell proliferation and migration of glioblastoma cells. In addition, the Nox4-PTP1B axis may also modulate EGF receptor signaling pathway.

Figure 1







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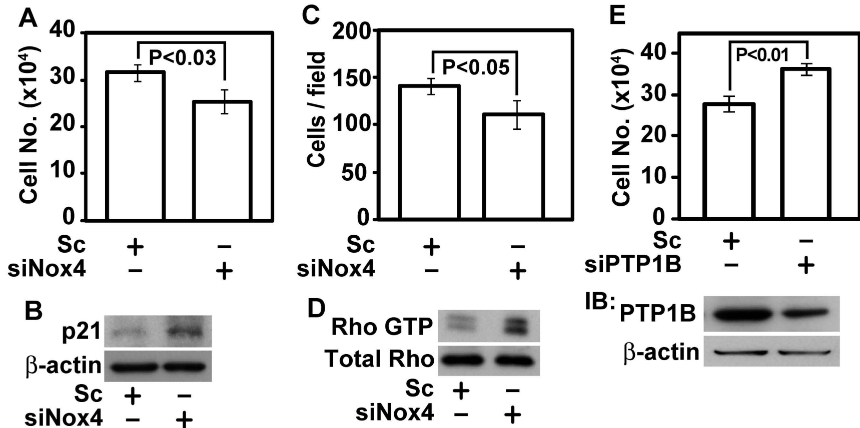

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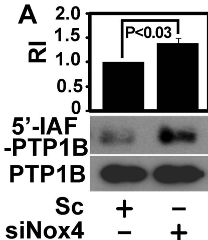

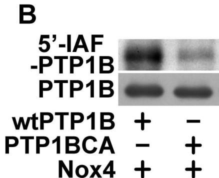

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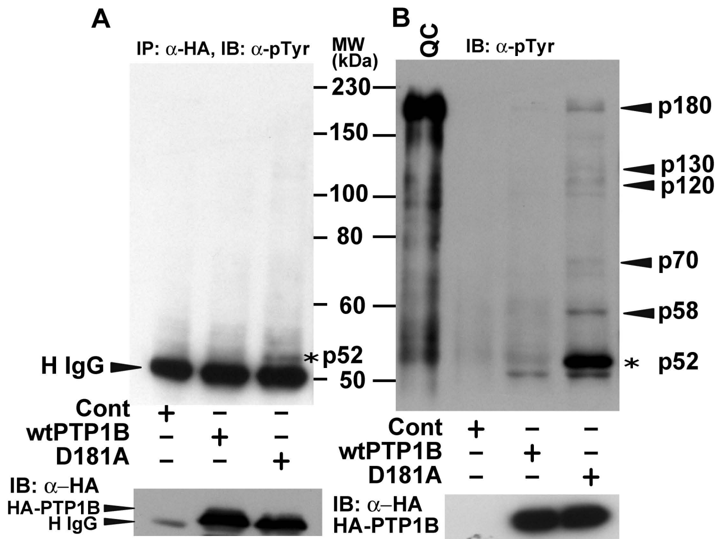

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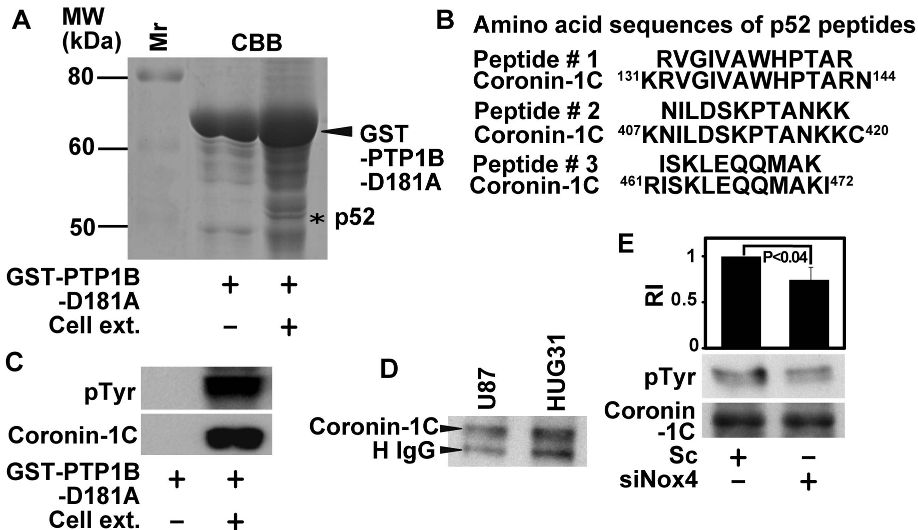

Figure 6

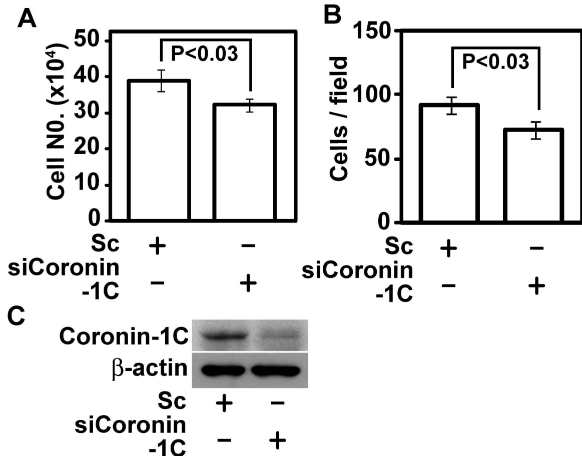

Figure 7