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NADPH Oxidase 1 Activity and ROS Generation Are Regulated by Grb2/Cbl-Mediated Proteasomal Degradation of NoxO1 in Colon Cancer Cells

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Abstract

The generation of reactive oxygen species (ROS) is required for proper cell signaling, but must be tightly regulated to minimize deleterious oxidizing effects. Activation of the NADPH oxidases (Nox) triggers ROS production and, thus, regulatory mechanisms exist to properly control Nox activity. In this study, we report a novel mechanism in which Nox1 activity is regulated through the proteasomal degradation of Nox organizer 1 (NoxO1). We found that through the interaction between NoxO1 and growth receptor–bound protein 2 (Grb2), the Casitas B–lineage lymphoma (Cbl) E3 ligase was recruited, leading to decreased NoxO1 stability and a subsequent reduction in ROS generation upon epidermal growth factor (EGF) stimulation. Additionally, we show that EGF-mediated phosphorylation of NoxO1 induced its release from Grb2 and facilitated its association with Nox activator 1 (NoxA1) to stimulate ROS production. Consistently, overexpression of Grb2 resulted in decreased Nox1 activity, whereas knockdown of Grb2 led to increased Nox1 activity in response to EGF. CRISPR/Cas9-mediated NoxO1 knockout in human colon cancer cells abrogated anchorage-independent growth on soft agar and tumor-forming ability in athymic nude mice. Moreover, the expression and stability of NoxO1 were significantly increased in human colon cancer tissues compared with normal colon. Taken together, these results support a model whereby Nox1 activity and ROS generation are regulated by Grb2/Cbl-mediated proteolysis of NoxO1 in response to EGF, providing new insight into the processes by which excessive ROS production may promote oncogenic signaling to drive colorectal tumorigenesis. *Cancer Res; 76(4); 855–65. ©2016 AACR.*

Introduction

It is firmly established that coupling of NADPH oxidase (Nox) activity with receptor signaling stimulates the transient generation of reactive oxygen species (ROS), which function as a second messenger in cell signaling (1–5). However, uncontrolled ROS generation through the pathophysiologic state of mitochondria or through hyperactivation of Nox results in cytotoxic damage to intracellular molecules, including lipid, DNA, and proteins (3–5). Therefore, ROS generation by Nox isozymes should be tightly regulated.

It has long been proposed that phosphorylation of regulatory proteins in Nox2 complex identified from phagocytic cells such as neutrophils and macrophages plays an important role in modulating Nox2 activity (6). Nox2 as a catalytic protein requires integral protein p22phox and three cytosolic proteins: p47phox, p67phox, and Rac. In the resting stage, p47phox is maintained as an inactive form resulting from the intramolecular interaction of two SH3 domains with the autoinhibitory region (AIR). Upon stimulation of epidermal growth factor (EGF), phosphorylation of p47phox by protein kinase C (PKC) results in exposure of the tandem SH3 domain in the central region of p47phox for interaction with the proline-rich region (PRR) domain of $p22^{phox}$ and one PRR in the COOH-terminal region of p47phox for the binding to the SH3 domain of $p67^{phox}$, leading to the activation of the Nox2 complex. Since the identification of Nox1 from the colon epithelium (7, 8), Nox activator 1 (NoxA1) as a homolog of Nox2 regulatory proteins $p67^{phox}$ and Nox organizer 1 (NoxO1) as a homolog of $p47^{phox}$ have been reported. Adaptor protein NoxO1 contains two SH3 domains in the central region for interaction with the PRR domain of p22phox and one PRR in the COOH-terminal region for binding to the SH3 domain of NoxA1, respectively. It has generally been assumed that the Nox1 complex is constitutively active because a domain homologous to the AIR domain of $p47^{phox}$ is missing in NoxO1 (9, 10). However, it has been reported that Nox1 activity is transiently increased in response to various agonists, including EGF (11–16), suggesting that an additional mechanism is in operation for the regulation of Nox1 activity.

Here, we show for the first time that proteasome-based degradation of NoxO1 regulates the integrity and activity of the

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Nox1–NoxA1–NoxO1 complex. Specifically, NoxO1 interacts with Grb2, which in turn recruits Cbl, leading to ubiquitination and degradation of NoxO1. We also demonstrate that EGF induces NoxO1 release from Grb2–Cbl and that the consequently increased NoxO1 protein level leads to an additional active Nox1 complex, ultimately resulting in augmentation of ROS generation.

Materials and Methods

Cell culture

HEK293T (human embryonic kidney cells) and HCT116 (human colorectal carcinoma cells) were obtained from the ATCC. HEK293T and HEK293 stably expressing Nox1 (designated HEK293-Nox1; ref. 12) were maintained at 37°C under an atmosphere of 5% CO_2 in culture dishes containing Dulbecco's Modified Eagle's Medium (JBI) supplemented with 10% fetal bovine serum (FBS; JBI) and 1% antibiotic–antimycotic solution (Invitrogen Life Technologies). HCT116 cells were cultured under the same condition with the exception of using RPMI-1640 medium supplemented with 10% FBS and 1% antibiotic–antimycotic solution.

Assay of intracellular H_2O_2 production

Intracellular H_2O_2 production was assayed after stimulation of cells with EGF (100 ng/mL) for 3 minutes. Confluent cells were washed with Hank's Balanced Salt Solution and incubated for 15 minutes with 5 μmol/L of Peroxy Orange-1 (PO-1) in the dark at 37°C. PO-1 is oxidized by H_2O_2 to the brightest fluorescence. After being washed with PBS, the cells were then examined with a laser scanning confocal microscope (LSM510; Zeiss) equipped with an argon laser tuned to an excitation wavelength of 540 nm and a Zeiss Axiovert objective lens. Images were digitized and stored at a resolution of 512 by 512 pixels. Five groups of cells were randomly selected from each sample, and the mean relative fluorescence intensity for each group of cells was measured with a Zeiss vision system and then averaged for all groups. All experiments were repeated at least three times.

NoxO1 knockout HCT116 cell lines

Human HCT116 colon cancer cells were maintained in McCoy's 5A Medium supplemented with 10% FBS. CRISPR/Cas9-mediated genome modification was used to generate NoxO1 knockout cells according to the manufacturer's protocol (ToolGen Genome Engineering; refs. 17, 18).

Soft agar colony formation assay

To examine anchorage-independent growth, a cell suspension (2×10^4 cells) was suspended in 0.4% agar in growth medium and seeded in triplicate on 60-mm dishes precoated with 0.8% agar in growth medium and incubated at 37°C, 5% CO_2. After 14 days, colonies were photographed and counted in four randomly chosen fields and expressed as means of triplicates, representative of two independent experiments (19).

Animal experiments

Tumors were formed by subcutaneous inoculation of HCT116 colon cancer cells (1×10^6 cells) into the right flank of athymic Balb/c female nude mice (5 weeks of age; Orient). Tumor size was measured with a caliper (calculated volume = shortest diameter^2 × longest diameter/2) at 2-day intervals. This study was reviewed and approved by the Institutional Animal Care and Use Committee of Center for Laboratory Animal Sciences, Medical Research Coordinating Center, HYU industry–University Cooperation Foundation.

Clinicopathologic characteristics of colorectal cancer patients

Colorectal cancer tissue samples were obtained from the archives of the Department of Pathology, Yonsei University (Seoul, Korea) and from the Cancer Specimen Bank of the National Research Resource Bank Program of the Korea Science and Engineering Foundation of the Ministry of Science and Technology. Colon cancer tissues were subjected to immunohistochemical analysis with antibody against NoxO1 (Life Span BioScience Inc.). Authorization for the use of these tissues for research purposes was obtained from the Institutional Review Board of Yonsei University of College of Medicine (IRB number 4-2012-0026). Colorectal cancer tissue samples from 222 patients with primary colorectal cancers of stages I to IV were used in this study. All patients had undergone colorectal resection between 2004 and 2006.

Detailed information on mass spectrometry, site-directed mutagenesis of NoxO1, small interfering RNA for Grb2, ubiquitination assay, and NoxO1 knockout HCT116 cell lines are described in the Supplementary Methods.

Results

Identification of Grb2 as a binding partner for NoxO1

To isolate candidate proteins with a regulatory effect on Nox1 complex, GST-NoxO1 pulldown assays were carried out using HEK293 cell lysates. NoxO1 binding proteins were subjected to tandem mass spectrometry (MS/MS) analysis for peptide sequencing, leading to identification of Grb2 as a binding partner for NoxO1 (Fig. 1A; Supplementary Fig. S1). Subsequently, we verified interaction of NoxO1 with Grb2 using coimmunoprecipitation analysis. HEK293T cells were transfected with HA-tagged NoxO1 together with or without FLAG-tagged Grb2. Immunoprecipitation with antibody against HA confirmed that NoxO1 binds to Grb2 (Fig. 1B). To determine the functional domain of Grb2 that interacts with NoxO1, we performed a yeast two-hybridization assay. The assay showed that the SH3 domain of Grb2 interacted with NoxO1 (Supplementary Fig. S2A). Next, we used an immunoprecipitation assay to show that the carboxyl terminal PRR domain of NoxO1 was required for the interaction with Grb2 (Supplementary Fig. S2B). To evaluate the role of Grb2 in intracellular H_2O_2 generation via regulation of Nox1 activity, Flag-tagged Grb2 was transfected into HEK293 cells stably expressing Nox1 (HEK293-Nox1) as well as NoxA1 and NoxO1, and intracellular H_2O_2 generation in response to EGF was measured. In these experiments, we used boronate compound PO-1 dye, which is specific to H_2O_2. Interestingly, increasing the amount of ectopic Flag-tagged Grb2 resulted in decreased intracellular EGF-induced H_2O_2 generation (Fig. 1C and 1E). In contrast, knockdown of Grb2 by RNA interference led to increased intracellular H_2O_2 generation in response to EGF (Fig. 1D and 1F). These results suggested that Grb2 is involved in the negative regulation of Nox1 activity.

Interaction of NoxO1 with the Grb2–Cbl complex results in its degradation

It has been well established that apart from its role as a positive regulator in the RTK–Ras–MAPK pathway, Grb2 functions as a

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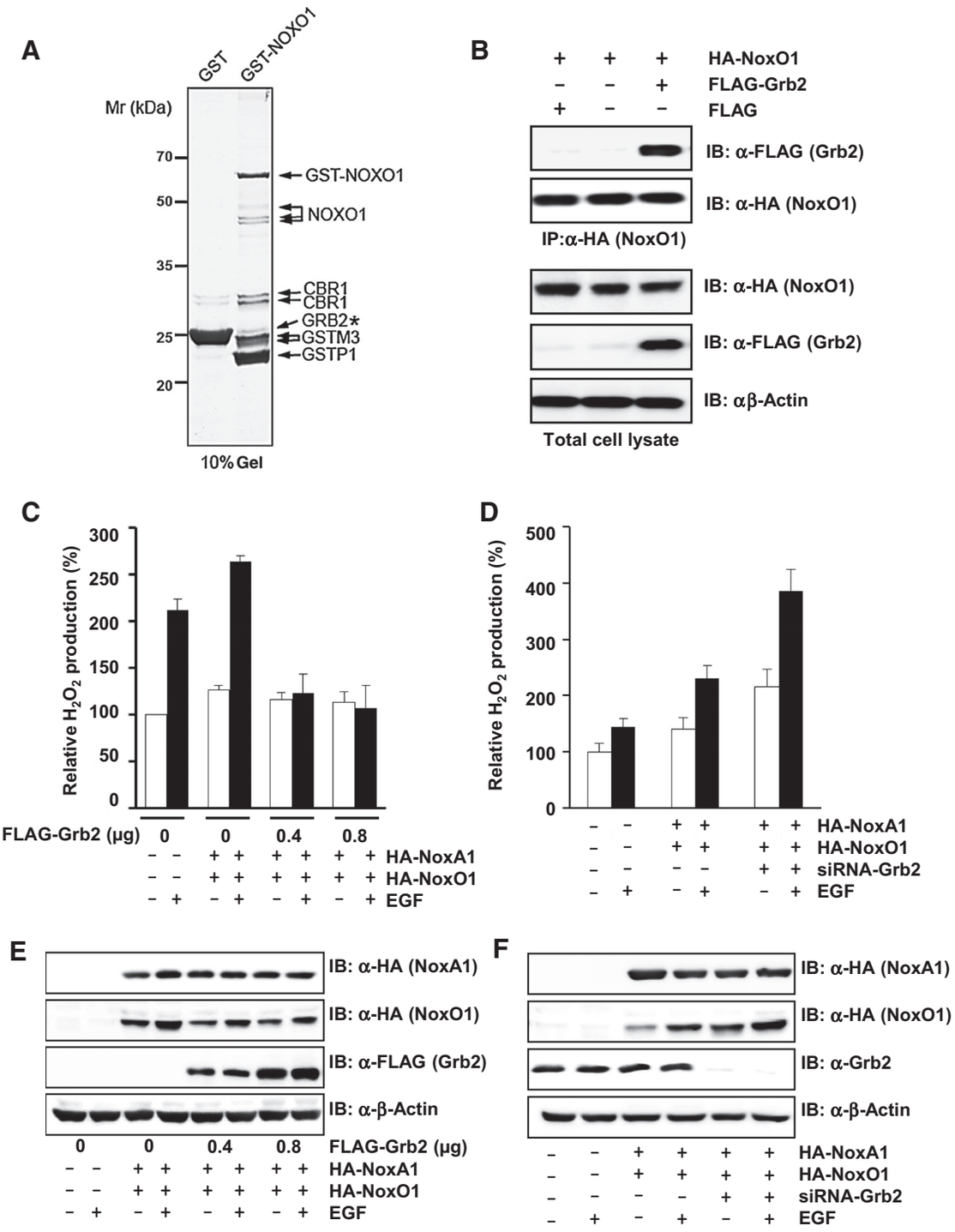

Figure 1.

Interaction of NoxO1 with Grb2. A, GST-NoxO1 was incubated with HEK 293 cell lysates and protein complex was subjected to SDS-PAGE. B, HA-tagged NoxO1 was transiently expressed in HEK293T cells with FLAG-tagged Grb2. Cell lysates were immunoprecipitated with antibody to HA and subjected into immunoblotting with antibody to FLAG or Grb2. C, HEK293-Nox1 cells were transfected with increasing amounts of FLAG-tagged Grb2 in the presence of HA-tagged NoxA1 and NoxO1. EGF-induced ROS generation was monitored by confocal microscopic analysis of DCF fluorescence. D, HEK293-Nox1 cells were transfected with small interfering RNA for Grb2 or control siRNA in the presence of HA-tagged NoxA1 and NoxO1. ROS generation in response to EGF was monitored by confocal microscopic analysis of DCF fluorescence. Data in C and D represent three repeated experiments and are shown as mean ± SD ($n = 3$). E, HEK293-Nox1 cells were transfected with increasing amounts of FLAG-tagged Grb2 in the presence of HA-tagged NoxA1 and NoxO1. F, HEK293-Nox1 cells were transfected with small interfering RNA for Grb2 or control siRNA in the presence of HA-tagged NoxA1 and NoxO1. Expression of NoxA1, NoxO1, and Grb2 was analyzed by immunoblotting with antibody to HA or FLAG (E and F).

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negative modulator of receptor-mediated signaling by recruiting Cbl E3 ligase and promoting receptor ubiquitination (20–22). We hypothesized that the Grb2–Cbl complex interacts with NoxO1 and thereby regulates Nox1 activity. To verify the tertiary complex formation, HEK293T cells were transfected with FLAG-Grb2 and HA-NoxO1 and were examined in the coimmunoprecipitation experiment with antibodies against FLAG or Cbl. Immunoblotting clearly showed that Grb2 simultaneously interacted with NoxO1 as well as endogenous Cbl (Fig. 2A). To evaluate the function of Cbl in modulating protein levels of Nox1, NoxA1, and NoxO1, a Cbl-expressing plasmid was transfected into HEK293 cells expressing Nox1, NoxA1, or NoxO1. Overexpression of Cbl had no effect on protein levels of Nox1 and NoxA1 (Supplementary Fig. S3). However, NoxO1 protein was significantly destabilized by Cbl in a dose-dependent manner, and this degradation was completely blocked by pretreatment with the proteasome inhibitor MG132 (Fig. 2B). Next, we explored whether NoxO1 ubiquitination was dependent on Cbl activity. Transfection of wild-type Cbl into HEK293T cells resulted in significantly increased polyubiquitination of NoxO1, whereas NoxO1 ubiquitination was inhibited in the presence of C3AHN E3 ligase–defective RING mutant of Cbl (Fig. 2C; ref. 23). The result indicated that NoxO1 protein is ubiquitinated by Cbl as its specific E3 ligase. Because $p47^{phox}$ in the Nox2 complex is the homolog of NoxO1 in the Nox1 complex, we also analyzed the ubiquitination of $p47^{phox}$ by Cbl. In contrast with NoxO1, overexpression of Cbl failed to ubquitinate $p47^{phox}$, indicating that Cbl-mediated ubiquitination is not a regulatory mechanism of Nox2 complex (Supplementary Fig. S4A).

To examine the effect of Cbl-dependent NoxO1 degradation on EGF-induced H_2O_2 generation, we used HCT116 colon carcinoma cells, which endogenously express NoxA1 and NoxO1 as well as Nox1. Stimulation of HCT116 cells with EGF resulted in increased NoxO1 protein levels coinciding with the rapid generation of intracellular H_2O_2 (Fig. 2D and 2E). Pretreatment of HCT116 cells with MG132 demonstrated an increased basal level of NoxO1 protein compared with control cells (Fig. 2D). The kinetics of NoxO1 expression matched that of intracellular H_2O_2 generation in response to EGF in the presence of MG132 (Fig. 2D and 2E). However, the mRNA level of NoxO1 did not change in HCT116 cells in response to EGF (Supplementary Fig. S4B). These results strongly indicated that NoxO1 protein in the resting stage is maintained at a low level through Cbl-dependent degradation and that upon stimulation with EGF, NoxO1 degradation is inhibited, leading to a sustained high level of NoxO1 expression and intracellular H_2O_2 generation.

Phosphorylation of NoxO1 induces Grb2 release and formation of active Nox1 complex

We next questioned whether the interaction of NoxO1 with Grb2 was regulated by EGF. Using HEK293-Nox1 cells expressing HA-NoxO1 and FLAG-Grb2, we observed that the NoxO1–Grb2 interaction decreased in response to EGF stimulation and recovered 20 minutes after the initial exposure to EGF (Fig. 3A). The result suggested that interaction of NoxO1 with Grb2 is transiently abrogated by exposure to EGF. To investigate the interaction between endogenous NoxO1 and Grb2 in HCT116 cells, we performed a coimmunoprecipitation experiment with antibody against Grb2 and then immunoblotted with antibody to NoxO1. Stimulation of HCT116 cells with EGF resulted in reduced interaction of Grb2 with NoxO1 in the coimmunoprecipitated complex and a concomitant increased endogenous NoxO1 stability in total cell lysate (Fig. 3B).

It has been recently reported that phosphorylation of $serine^{154}$ on NoxO1 by PKC plays an important role in Nox1 complex formation (24). To validate PKC-dependent NoxO1 stability, staurosporine as a PKC inhibitor and PMA as a PKC activator were applied in analyses of association of NoxO1 with Grb2 and NoxO1 stability. Pretreatment of staurosporine resulted in association of NoxO1 with Grb2 in response to EGF, whereas EGF or PMA alone stimulated the dissociation of the complex (Supplementary Fig. S5A). Moreover, pretreatment of staurosporine showed suppressed ROS generation and stability of NoxO1 in response to EGF (Supplementary Fig. S5B). In contrast, incubation of PMA led to increased stability of NoxO1 (Supplementary Fig. S5C). These results indicate that activation of PKC is involved in NoxO1 stability and ROS generation in response to EGF. Consistently, while dissociation of wild-type NoxO1 from Grb2 was facilitated in response to EGF, NoxO1 S154A mutant failed to show a similar response (Fig. 3C).

It is well known that the association of another cytosolic regulator NoxA1 to NoxO1 is required for the formation of an active Nox1 complex (1, 4, 7). We duly showed that interaction of NoxO1 with Grb2 was inhibited in the presence of an increasing amount of NoxA1 (Fig. 3D). To test the binding preference of NoxO1 to either Grb2 or NoxA1, GFP-NoxO1, HA-NoxA1, and Flag-Grb2 were transfected into HEK293-Nox1 cells. Interaction of NoxO1 with Grb2 was decreased in response to EGF stimulation, whereas binding of NoxO1 to NoxA1 was reciprocally increased (Fig. 3E). This shift in the binding preference of NoxO1 by EGF stimulation indicated that NoxA1 plays a crucial role in protecting NoxO1 from Grb2, leading to the formation of an active Nox1 complex for ROS generation.

Next, we investigated the effect of Grb2 knockdown on membrane translocation of NoxO1 and NoxA1 as an indication of Nox1 complex formation in response to EGF. Silencing of Grb2 expression by transfection of siRNA specific to Grb2 (siGrb2) in HCT116 cells failed to induce enhanced EGF-dependent membrane translocation of NoxO1 and NoxA1 compared with control siRNA-transfected cells (siCon; Supplementary Fig. S6A). We also tested EGF-dependent membrane translocation of NoxO1 S154A mutant in HEK293 cells. NoxO1 S154A mutant led to decreased membrane translocation in response to EGF compared with wild-type NoxO1. Expression of NoxO1 S154A mutant failed to induce membrane translocation of NoxA1 in response to EGF (Supplementary Fig. S6B). These results indicated that phosphorylation of $serine^{154}$ of NoxO1 is essential for the interaction of NoxO1 with Grb2 and membrane translocation of NoxO1.

Deficiency of NoxO1 attenuates tumor growth of HCT116 cells

Several recent reports indicate that intracellular ROS generation by the Nox1 complex appears to play an important role in cellular transformation (25, 26). To evaluate the function of NoxO1 in Nox1-mediated ROS generation and cellular transformation, we attempted NoxO1 knockout from HCT116 cells using CRISPR/Cas9-mediated genome modification (17, 18). We established multiple NoxO1 knockout cell lines, from which we selected three for further experiments: NoxO1-KO1 (40-bp deletion/1-bp insertion), NoxO1-KO4 (1-bp deletion/166-bp insertion), and NoxO1-KO5 (56-bp deletion/93-bp

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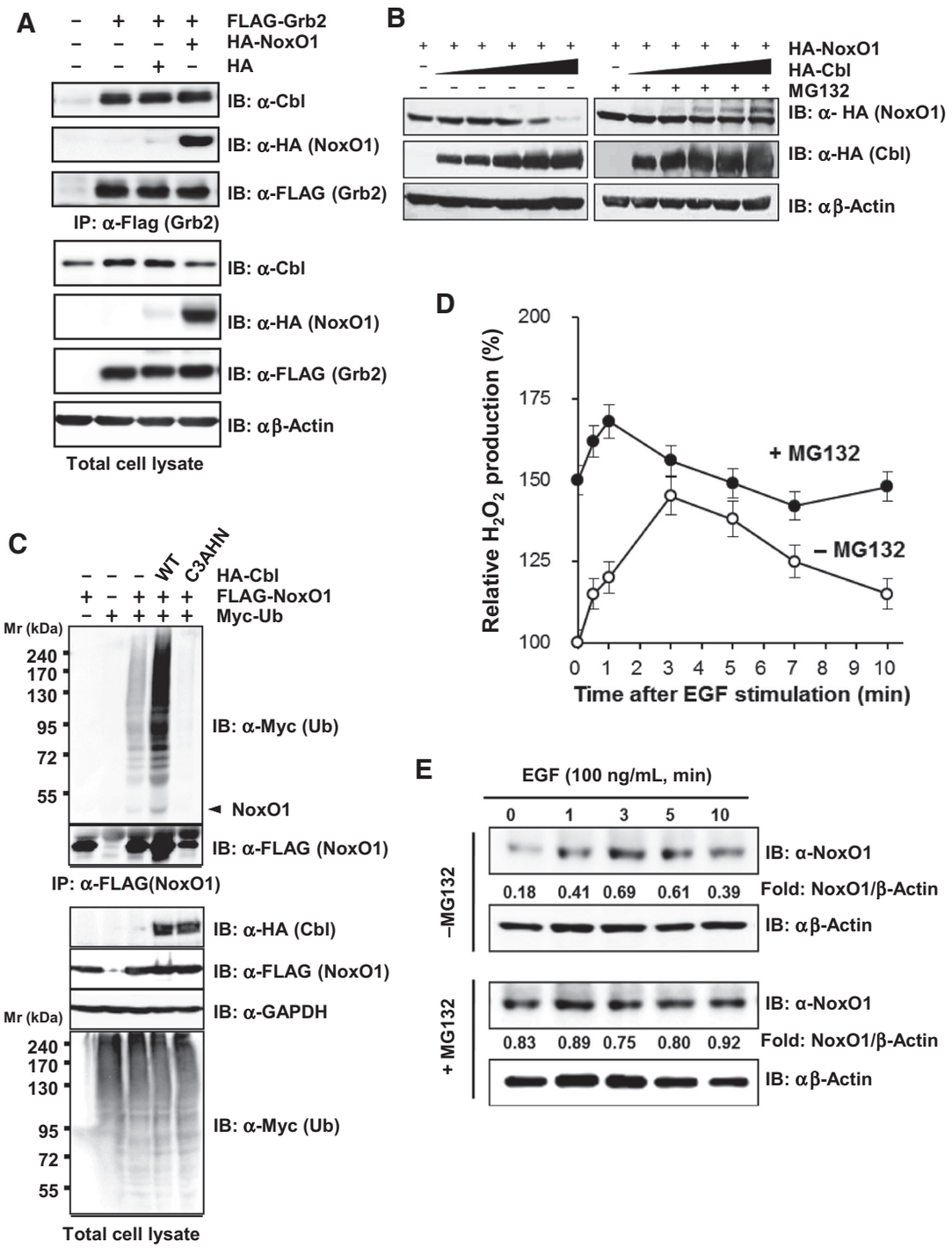

Figure 2.

Ubiquitin-dependent proteosomal degradation of NoxO1. A, Grb2 interaction with Cbl as well as NoxO1. HA-tagged NoxO1 was transiently expressed in HEK293T cells with FLAG-tagged Grb2. Cell lysates were immunoprecipitated with antibody to FLAG and subjected to immunoblotting with antibody to HA or Cbl. B, HEK293-Nox1 cells were transfected with increasing amounts of expression vector for Cbl in the presence of NoxO1. Cells were treated with 0.5 μmol/L MG132 for 6 hours. Total cell lysates were subjected to immunoblot analysis with antibody to HA. C, HA-tagged wild-type Cbl (WT) or mutant Cbl (C3AHN) was transiently expressed with combination of FLAG-tagged NoxO1 and Myc-tagged ubiquitin (Ub) in HEK293T cells as indicated. Cells were treated with 2 μmol/L MG132 for 12 hours. Total cell lysates were subjected to immunoprecipitation with antibody to FLAG, and the resulting precipitates were subjected to immunoblot analysis with antibodies against FLAG, HA, or Myc. D, effect of MG132 on ROS generation in response to EGF stimulation in HCT116 cells. EGF-induced ROS generation was monitored by confocal microscopic analysis of DCF fluorescence. E, the expression level of endogenous NoxO1 in EGF-stimulated HCT116 cells was analyzed by immunoblotting with antibody to NoxO1. HCT116 cells were pretreated with 0.5 μmol/L MG132 for 6 hours and cell lysates were subjected to immunoblotting with antibody to NoxO1. Data (A–E) represent three repeated experiments and are shown as mean ± SD ($n = 3$).

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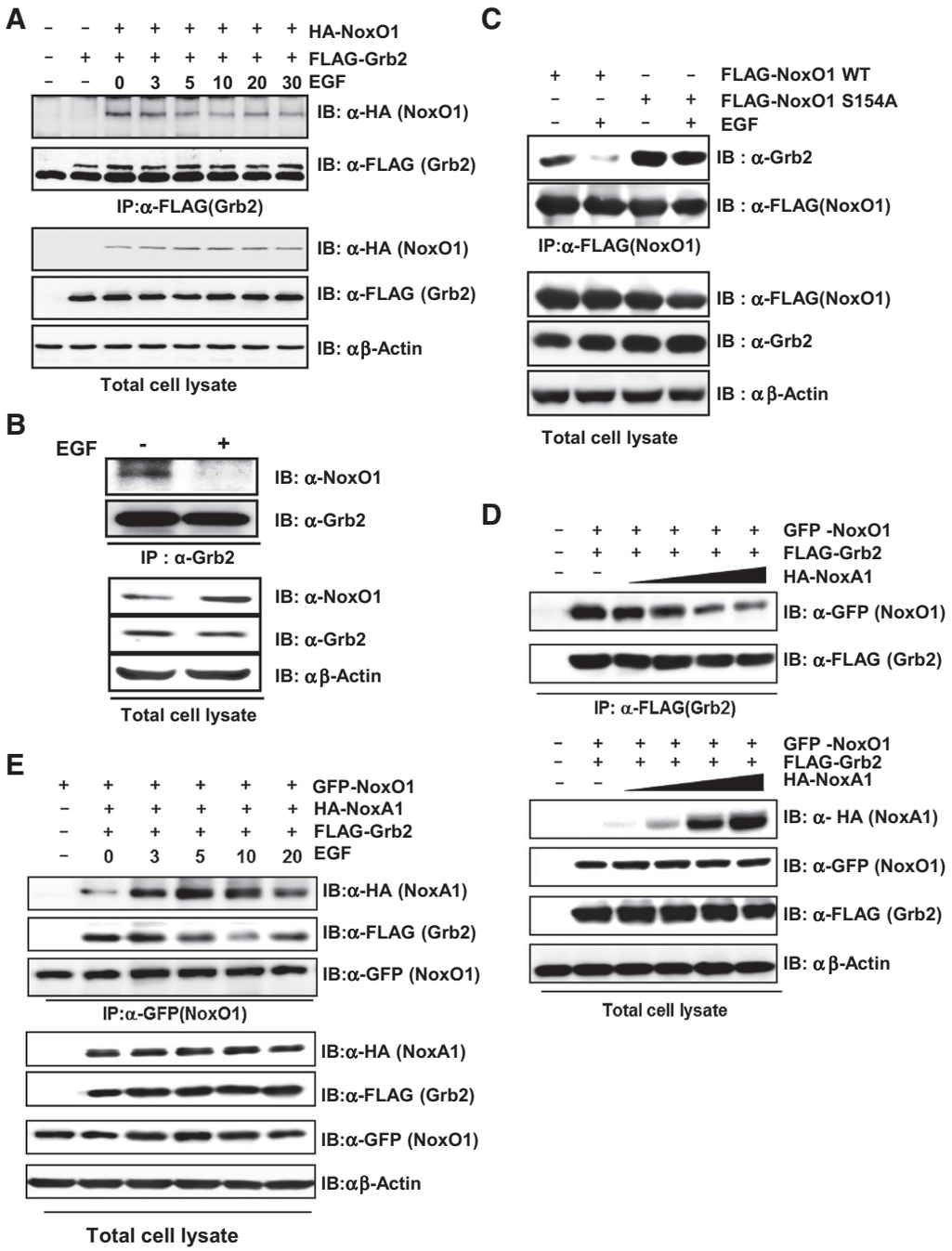

Figure 3.
EGF induces NoxO–NoxA1 interaction. A, effect of EGF stimulation on the interaction between NoxO1 and Grb2. HA-tagged NoxO1 was transiently expressed in HEK293T cells with FLAG-tagged Grb2 as indicated. Total cell lysates were immunoprecipitated with antibody to HA and subjected to immunoblotting with antibody to HA or FLAG. B, endogenous complex of NoxO1 with Grb2 in HCT116 cells. HCT116 cells were treated with EGF for 5 minutes and cell lysates were then subjected to immunprecipitation with antibody to Grb2. Immunoprecipitated complex was subjected to immunoblotting with antibodies to NoxO1, Grb2, or actin. C, FLAG-tagged wild-type NoxO1 (WT) or mutant NoxO1 (S154A) was transiently expressed in HEK293-Nox1 cells as indicated. After stimulation with EGF, total cell lysates were then immunoprecipitated with anti-FLAG antibody, followed by immunoblotting with anti Grb2, anti-FLAG antibody. D, effect of NoxA1 on the interaction of NoxO1 with Grb2. HEK293-Nox1 cells were transfected with increasing amounts of HA-tagged NoxA1 in the presence of FLAG-tagged Grb2 and GFP-tagged NoxO1. Total cell lysates were subjected to immunoprecipitation with antibody to FLAG, and the resulting precipitates were subjected to immunoblot analysis with antibodies against GFP, FLAG, and HA. E, effect of EGF on the interaction between NoxO1 and Grb2 or NoxA1. HEK293-Nox1 cells were transfected with HA-tagged NoxA1, FLAG-tagged Grb2, and GFP-tagged NoxO1. Total cell lysates were subjected to immunoprecipitation with antibody to GFP antibody, and the resulting precipitates were subjected to immunoblot analysis with antibodies against HA, FLAG, or GFP.

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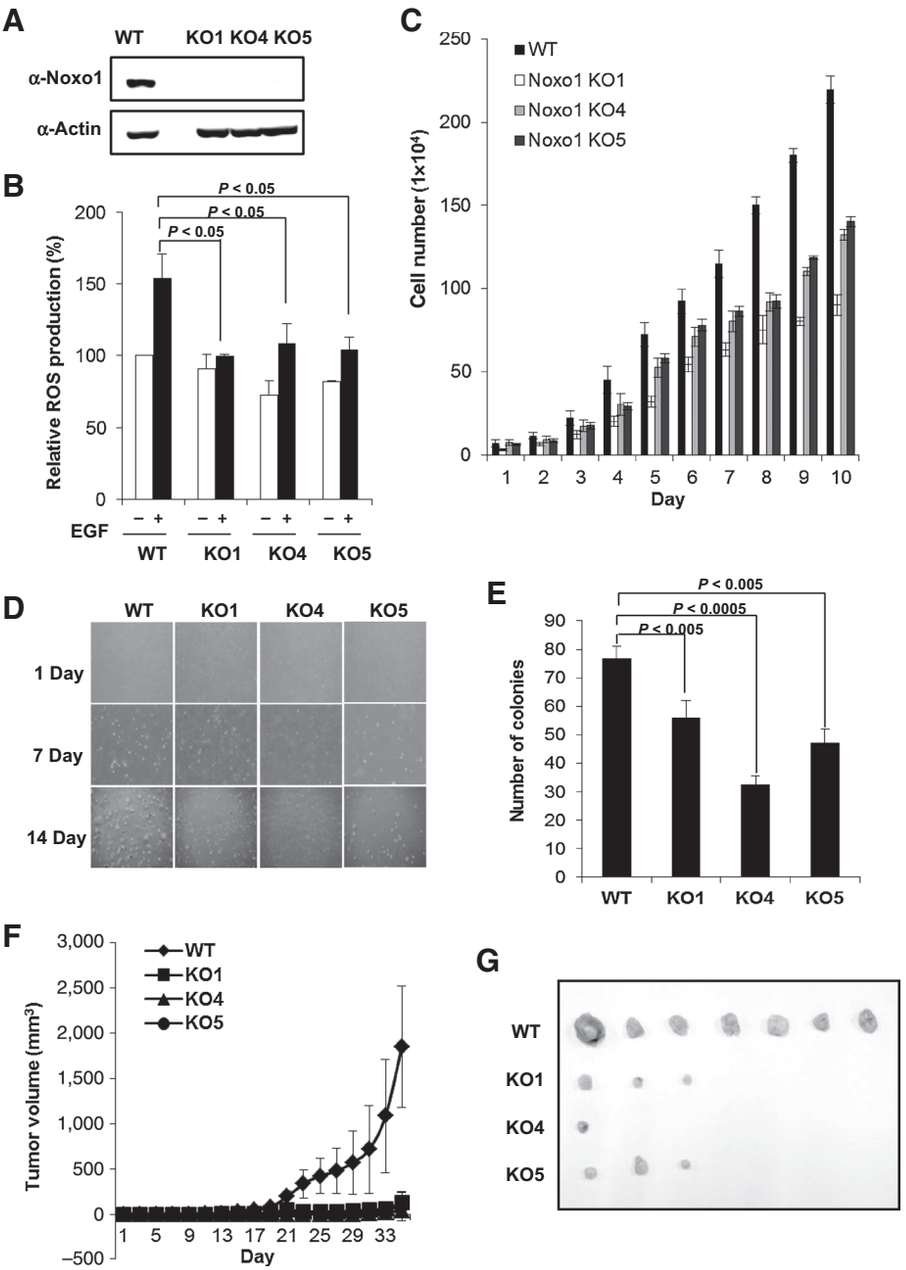

Figure 4.
NoxO1 is a critical regulator for tumor growth in HCT116. A, immunoblotting of NoxO1 and actin in HCT116 cells and three NoxO1 knockout cell lines (NoxO1-KO1, NoxO1-KO4, and NoxO1-KO5). B, ROS generation was monitored by confocal microscopic analysis of DCF in EGF stimulated cells. Data represent three repeated experiments and are shown as mean ± SD ($n = 3$). C, effect of NoxO1 expression on HCT116 cell growth. Data represent three repeated experiments and are shown as mean ± SD ($n = 3$). D, soft agar assay with wild-type cells or three NoxO1 knockout HCT116 cell lines. Data represent three repeated experiments and are shown as mean ± SD ($n = 3$). E, soft agar assay with wild-type cells or three NoxO1 knockout HCT116 cell lines. Data represent three repeated experiments and are shown as mean ± SD ($n = 3$). F, tumor growth curves of xenografts derived from HCT116 cells or three NoxO1 knockout HCT116 cell lines. Graph shows that tumor size of athymic nude mice derived from HCT116 cells ($N = 7$) or three NoxO1 knockout HCT116 cell lines (NoxO1-KO1, $N = 7$; NoxO1-KO4, $N = 7$; and NoxO1-KO5, $N = 5$). G, tumor growth of xenografts derived from HCT116 cells or three NoxO1 knockout HCT116 cell lines after 35 days of injection.

deletion). Deletion of NoxO1 was confirmed by an immunoblot assay with antibody against NoxO1. Expression of NoxO1 in the three cell lines was completely ablated (Fig. 4A). Importantly, stimulation of the three cell lines with EGF failed to generate ROS, compared with control cells (Fig. 4B). To examine the effect of NoxO1 on tumorigenic capacity of HCT116 cells and the three cell lines (NoxO1-KO1, NoxO1-KO4, and NoxO1-KO5), we first analyzed cell proliferation as measured by trypan blue exclusion assay (Fig. 4C). The growth rate of wild-type HCT116 cells was higher than three NoxO1 KO HCT116-mutant cells (Fig. 4C).

We wondered if the lack of NoxO1 in the three HCT116 derivative cell lines would free up Grb2 and lead to additional stimulation of the EGF signaling pathway. We examined Erk phosphorylation as a measure of EGFR–Ras pathway activation. Indeed, stimulation of three NoxO1 knockout HCT116 cells with EGF resulted in significantly increased Erk phosphorylation compared with wild-type HCT116 cells (Supplementary Fig. S7A). Pretreatment of MG132 as a proteasome inhibitor did not affect Erk phosphorylation in HCT116 cells (Supplementary Fig. S7B). However, three NoxO1 knockout HCT116 cells showed lower ROS generation and growth rate than wild-type HCT116 cells, consistent with the role of ROS in cell proliferation (Fig. 4B and 4C). This represents a paradox of a kind as RAS–Erk signaling is apparently activated but proliferation is retarded. We examined the possibility that cell apoptosis in three NoxO1 knockout

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HCT116 cells is enhanced. Populations of apoptotic cells in three NoxO1 knockout HCT116 cells were higher in proportion than in wild-type cells (Supplementary Fig. S7C). Several studies reported that scavenging of ROS by addition of antioxidant chemical compounds or proteins leads to cell death (27–32). Taken together, our data indicate that a concerted action of two signaling cascades, Nox-dependent ROS generation and ROS-independent signaling event such as Erk phosphorylation, plays an important role in cell growth.

We next performed an anchorage-independent colony forming assay. Notably, knockout of NoxO1 (NoxO1-KO) drastically suppressed the anchorage-independent colony forming ability of HCT116 colon cancer cells (Fig. 4D and 4E). To confirm this result *in vivo*, we injected HCT116 cells as a control and three NoxO1-KO derivative cell lines (NoxO1-KO1, NoxO1-KO4, and NoxO1-KO5) subcutaneously into the right flank of athymic nude mice. In agreement with the *in vitro* data, tumor formation was significantly retarded in xenograft mice injected with NoxO1-depleted HCT116 colon cancer cells compared with the control group (Figs. 4F and G). Taken together, these results suggest that NoxO1 is a critical regulator for tumor formation in HCT116 cancer cells.

High NoxO1 stability provides a basis of ROS-contributed development of colon cancer

Since Nox1 was first identified from colon epithelium, several reports suggested that Nox1-mediated ROS generation is involved in chronic inflammation of the epithelium and development of colorectal cancer (25, 33, 34). We thus questioned whether NoxO1 stability was increased in colorectal cancer cells. Cell lysates from colorectal cancer and adenoma tissues of 12 independent patients were subjected to immunoblot analysis with antibody to NoxO1. The NoxO1 protein level was significantly increased in colorectal cancer tissues compared with normal tissues (Fig. 5A and B). We next investigated NoxO1–Grb2–Cbl tertiary complex formation in the normal colon tissues and tumor tissues. NoxO1 stability was an increase in whole-cell lysate of colon cancer tissues (Fig. 5C). While the protein complex isolated with the antibody to Grb2 from normal tissues contained NoxO1 and Cbl, the complex from colon cancer tissues were negative for these proteins (Fig. 5C). The result indicated that the NoxO1–Grb2–Cbl complex is disrupted in growing cancer tissues. To investigate interaction of the endogenous NoxA1/NoxO1/p22^{phox}/Nox1 complex in cancer tissues, we performed a coimmunoprecipitation experiment with antibody against NoxA1 and then immunoblotting with antibodies to NoxO1, p22^{phox}, or Nox1. Complex of NoxA1 with NoxO1/p22^{phox}/Nox1 was increased in amount in cell lysate in cancer tissues compared to normal tissues (Supplementary Fig. S7D). We next investigated Erk phosphorylation in the normal colon tissues and tumor tissues. The level of Erk phosphorylation was increased in the cell lysate of colon cancer tissues compared with normal control tissues

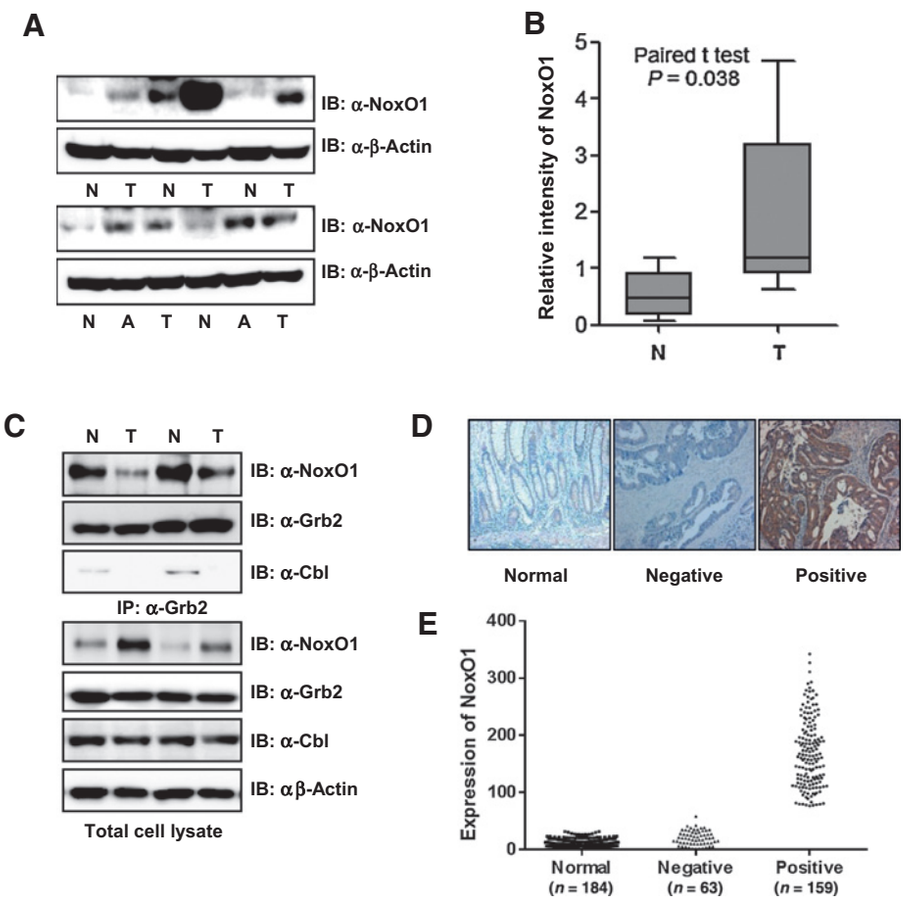

Figure 5.
High stability of NoxO1 in colon cancer tissues. A, immunoblotting of NoxO1 and actin in human colon cancer tissues (T), and matched nontumorous colonic mucosal tissues (N; top), or human colon cancer tissues (T), and matched adenocarcinoma mucosal tissues and matched nontumorous colonic mucosal tissues (N; bottom). B, expression of NoxO1 in NoxO1-positive colon cancer tissues (T, $n = 12$) compared with nontumorous colonic mucosal tissues (N, $n = 12$) by immunoblotting of NoxO1 and actin in T, and matched N. Statistical analysis was conducted with a paired *t* test ($P < 0.05$). C, cell lysates from normal and colon cancer tissues were immunoprecipitated with antibody to Grb2 and subjected to immunoblotting with antibody to NoxO1, Grb2, or Cbl. D, immunohistochemical analysis of NoxO1 protein expression in colon cancer tissues. E, the amount of NoxO1 expression was analyzed by using the ImageJ program.

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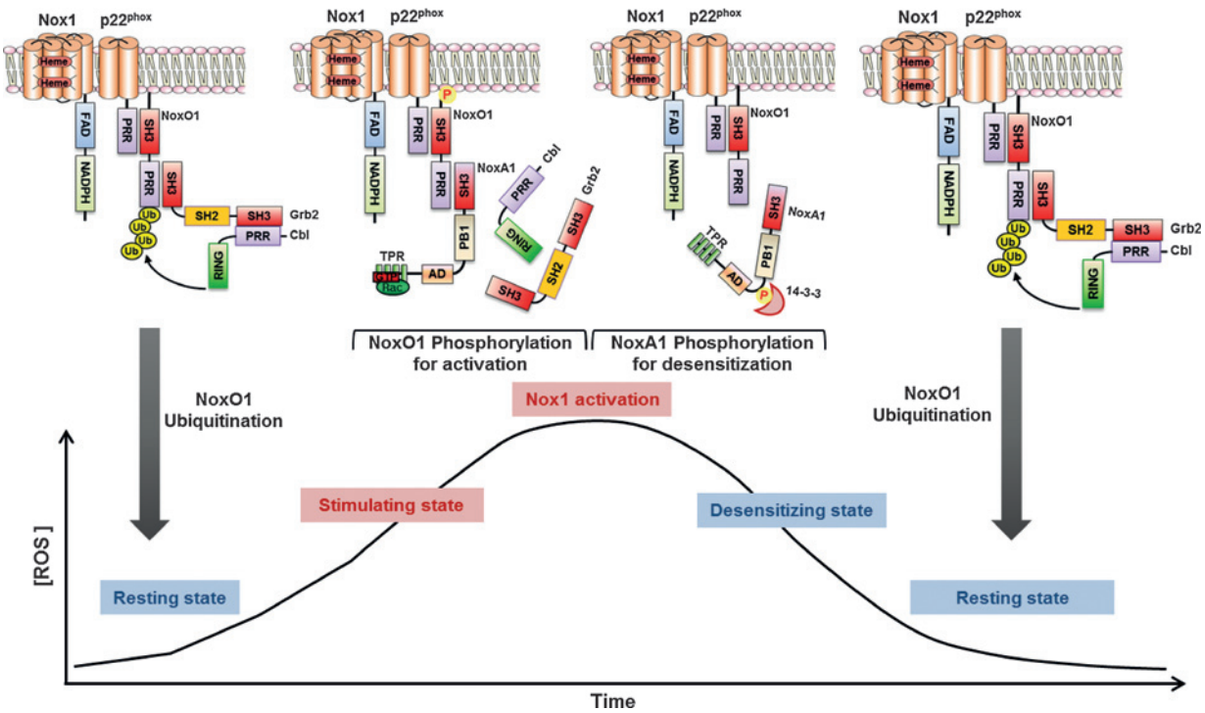

Figure 6.
Proposed mechanism of Nox1 regulation by ubiquitination of NoxO1. In the resting state, NoxO1 is rapidly degraded by Cbl-mediated ubiquitination. Stimulation of EGF results in increased NoxO1 stability, leading to formation of an active Nox1 complex for ROS generation. After the peak of ROS generation, interaction between 14-3-3 and phosphorylated NoxA1 disintegrates the active Nox1 complex for the desensitization of ROS generation. ROS level subsequently returns to the basal state.

(Supplementary Fig. S7E). These results are consistent with the hypothesis that both of Nox-dependent ROS generation and ROS-independent signaling through Erk phosphorylation likely contribute to cell proliferation.

We also evaluated the NoxO1 protein level in 222 colorectal cancer tissues by immunohistochemistry. NoxO1 expression was hardly detected in normal tissue areas in virtually all samples, whereas the expression was readily detected in the cytoplasm of colorectal cancer cells again virtually in all cases (Fig. 5D and E). The expression of NoxO1 in normal ($n = 184$) and colorectal cancer tissues ($n = 222$) was reevaluated with the ImageJ program and categorized as positive and negative. The intensities of positive NoxO1 expression cases ($n = 159$) were significantly higher than those of negative NoxO1 expression cases ($n = 63$; Fig. 5E). Moreover, all pathological stages in positive NoxO1 expression cases expressed NoxO1 at high levels, suggesting that increased NoxO1 stability is correlated with early developmental stages of colorectal cancer (Supplementary Table S1). It has been well established that Ras mutation plays an important role in tumorigenesis in a large proportion of colorectal cancer cases (35). To investigate the relationship between Ras mutations and ubiquitin-dependent proteolysis of NoxO1, we used cell lines expressing wild-type (RKO and HK29) or K-Ras G13D mutant (HCT116 and DLD). Increasing NoxO1 stability by EGF in cancer cell lines expressing K-Ras G13D mutant was qualitatively similar to that seen in cells expressing wild-type K-Ras (Supplementary Fig. S7F). This result suggests that increased NoxO1 expression in cancer cells is independent of Ras signaling.

Discussion

It has been well established that controlled and moderated levels of ROS play an important role in cell signaling, growth, differentiation, apoptosis, and motility, whereas uncontrolled and excessive levels of ROS lead to unexpected cytotoxic damage and cell death contributing to the development of various diseases such as atherosclerosis, autoimmune disorders, neuronal degeneration, and cancer (36–40). The notion that uncontrolled generation of ROS through hyperactivation of Nox isozyme induces pathologic stages indicates that tight regulatory mechanisms for Nox isozymes must be provided in cells or tissues. One well-established regulatory mechanism for various Nox complexes is phosphorylation of regulatory proteins such as p47phox. In the case of Nox1, however, it has been proposed that no such regulation exists. This was mainly based on that NoxO1 protein lacks AIR region, the target of phosphorylation.

We here provide a novel mechanism for regulation of Nox1 activity involving ubiquitination and degradation of NoxO1 (Fig. 6). Specifically, the Cbl-mediated proteolytic pathway results in negative regulation of ROS generation by Nox1 via ubiquitination and degradation of NoxO1. The rapid degradation of NoxO1 is inhibited by serine 154 phosphorylation of NoxO1, leading to dissociation of NoxO1 from Grb2–Cbl and subsequent association with NoxA1 (Fig. 6). In contrast with NoxO1, stability of p47phox was not affected by Cbl overexpression (Fig. S4A). It is likely that key regulation of p47phox is mediated by phosphorylation of the AIR region rather than the ubiquitination pathway. Recently, Noubade and colleagues

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(41) reported that a negative regulator of ROS (NRROS) directly interacts with Nox2 and stimulates its degradation through the proteasome-dependent ER-associated degradation (ERAD) pathway. Degradation of Nox2 was rescued by p22phox, which resulted in stable complex formation of Nox2-p22phox and then sufficient ROS generation for host defense. Moreover, it has been recently reported that HECT domain and Ankyrin repeat containing E3 ubiquitin protein ligase 1 (HACE1) regulate Nox1 activity through the degradation of active Rac1 (42). HACE1-dependent active Rac1 degradation signifies the turning-off of ROS signaling in response to growth factor.

Nox-mediated ROS has been implicated in cell growth and survival (25). Promitotic effect of Nox-mediated ROS could be deduced from the transient inactivation of protein tyrosine phosphatase through the oxidation of redox-sensitive cysteine residue in the active center (43–46). The balance between protein tyrosine phosphatase and kinase was shifted to enhanced kinase activity, leading to activation of transcriptional factors and increased expression of EGFR for autonomous cell growth and survival (45–47). Proinflammatory cytokines such as TNFα and TRAIL in various cancer cells induce the increased Nox1 and NoxO1 expression, suggesting that Nox1 and its accessory protein expression are regulated at the transcriptional level in cancer cells (48, 49). These reports suggest that sequential activation of EGF-mediated cell signaling cascades including ROS generation induces transcriptional activation of NoxO1 cancer cells. Consistently, a large portion of colon cancer patients (up to 70%) showed increased NoxO1 expression. Whether the transcriptional activation of NoxO1 in colorectal cancer results from activation of Nox1 via a positive feedback loop involving an autocrine requires further investigation (Fig. 5). Moreover, a previous study reported that deficiency of Nox1 is involved in sequential inactivation of PI3K/Akt/Wnt/β-catenin/Notch cascades, leading to control of the cell fate choice of proliferative colonic epithelial cells over post-mitotic goblet cells (34). Therefore, increased NoxO1 stability in colorectal cancer should induce Nox1-mediated ROS generation, which would in turn result in proliferation of colonic epithelial cells and contribute to colorectal tumorigenesis and the progression of cancer.

In summary, we describe a novel mechanism for regulation of functional Nox1 complex formation based on Cbl-mediated ubiquitination of NoxO1, which in turn is demonstrated to be negatively modulated by EGF signaling, leading to increased synthesis of ROS (Fig. 6). Moreover, high NoxO1 stability in the Nox1 complex provides a basis for the ROS-contributed development of colon cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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NADPH Oxidase 1 Activity and ROS Generation Are Regulated by Grb2/Cbl-Mediated Proteasomal Degradation of NoxO1 in Colon Cancer Cells

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