

Loss of glutathione peroxidase 7 promotes TNF- α -induced NF- κ B activation in Barrett's carcinogenesis

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Esophageal adenocarcinoma (EAC) is a classic example of inflammation-associated cancer, which develops through GERD (gastroesophageal reflux disease)-Barrett's esophagus (BE)-dysplasia-adenocarcinoma sequence. The incidence of EAC has been rising rapidly in the USA and Western countries during the last few decades. The functions of glutathione peroxidase 7 (GPX7), an antioxidant enzyme frequently silenced during Barrett's tumorigenesis, remain largely uncharacterized. In this study, we investigated the potential role of GPX7 in regulating nuclear factor-kappaB (NF- κ B) activity in esophageal cells. Western blot analysis, immunofluorescence and luciferase reporter assay data indicated that reconstitution of GPX7 expression in CP-A (non-dysplastic BE cells) and FLO-1 (EAC cells) abrogated tumor necrosis factor- α (TNF- α)-induced NF- κ B transcriptional activity ($P < 0.01$) and nuclear translocation of NF- κ B-p65 ($P = 0.01$). In addition, we detected a marked reduction in phosphorylation levels of components of NF- κ B signaling pathway, p-p65 (S536), p-I κ B- α (S32) and p-IKK α / β (S176/180), as well as significant suppression in induction of NF- κ B target genes [TNF- α , interleukin (IL)-6, IL-8, IL-1 β , CXCL-1 and CXCL-2] following treatment with TNF- α in GPX7-expressing FLO-1 cells as compared with control cells. We validated these effects by knockdown of GPX7 expression in HET1A (normal esophageal squamous cells). We found that GPX7-mediated suppression of NF- κ B is independent of reactive oxygen species level and GPX7 antioxidant function. Further mechanistic investigations demonstrated that GPX7 promotes protein degradation of TNF-receptor 1 (TNFR1) and TNF receptor-associated factor 2 (TRAF2), suggesting that GPX7 modulates critical upstream regulators of NF- κ B. We concluded that the loss of GPX7 expression is a critical step in promoting the TNF- α -induced activation of proinflammatory NF- κ B signaling, a major player in GERD-associated Barrett's carcinogenesis.

Introduction

Chronic gastroesophageal reflux disease (GERD), where esophageal epithelium of the lower esophagus is abnormally exposed to a mixture of acid and bile salts, leads to the development of Barrett's esophagus (BE) that consists of metaplastic glandular intestinal cells, the main risk factor for the development of esophageal adenocarcinoma (EAC) (1,2). Chronic GERD-associated mucosal injury and inflammation not

Abbreviations: BE, Barrett's esophagus; EAC, esophageal adenocarcinoma; GERD, gastroesophageal reflux disease; GPX7, glutathione peroxidase 7; IKK, I κ B kinase; IL, interleukin; MOI, multiplicity of infection; mRNA, messenger RNA; NF- κ B, nuclear factor-kappaB; RIP1, receptor-interacting protein 1; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; shRNA, short hairpin RNA; TNF- α , tumor necrosis factor- α ; TNFR1, TNF-receptor 1; TRAF2, TNF receptor-associated factor 2.

only mediate the development of BE but also the progression to EAC (3–5). In the USA, the incidence rate for EAC has increased 4–10% per year among men since 1976, more rapidly than for any other type of cancer (6). The risk of developing EAC is 30 times higher for chronic GERD patients and up to 125 times higher for GERD patients with BE than for the general population (3,7). In this regard, EAC is considered an example of inflammation-associated cancer (8).

The link between chronic inflammation and human carcinogenesis has been established in several cancer types (9,10). A large body of evidence suggests that nuclear factor-kappaB (NF- κ B) is one of the few key regulatory signaling molecules; the aberrant activation of which is invariably associated with inflammation and cancer in mouse models and human disease (11–14). Activation of NF- κ B plays an important role in integrating multiple stress stimuli and regulating immune responses (14,15). A variety of stimuli can trigger NF- κ B activation such as infections, inflammatory cytokines, ultraviolet irradiation and oxidative stress. Bile acids, particularly deoxycholic acid, have been shown to activate NF- κ B pathway (16,17). Inactive NF- κ B is located in the cytoplasm. Once it becomes phosphorylated and activated, NF- κ B is translocated to the nucleus where it binds to promoters of specific target genes and can upregulate the transcription of cytokines [tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , IL-6, and IL-8] and chemokines (CXCL-1 and CXCL-2) (14,18). There is a complex signaling cascade leading to NF- κ B activation which is mediated by I κ B kinase (IKK) complex (19). TNF- α , a proinflammatory cytokine, is a known key NF- κ B activator and also a target gene of activated NF- κ B (20). TNF-receptor 1 (TNFR1) is the primary receptor triggering TNF- α -mediated induction of NF- κ B transcription factors (19,21). TNF- α activates IKK signaling cascade through the TNFR1 and TRADD/receptor-interacting protein 1 (RIP1)/TNF receptor-associated factor 2 (TRAF2) complex, leading to activation of NF- κ B transcription factors (19,22). Although there is evidence that immune cells are the main source of TNF- α , autocrine production by cancer cells can also contribute to its secretion, particularly when NF- κ B is activated within the cancer cells (13,14). It has been reported that the expression levels of TNF- α and NF- κ B are significantly increased in BE and EAC (5,20).

The glutathione peroxidases (GPXs) are a major antioxidant enzyme family composed of eight members, GPX1-GPX8 (23). Generally, GPXs catalyze the reduction of hydrogen peroxide, organic hydroperoxide and lipid peroxides by reduced glutathione (23,24). GPX7 has been shown to have very limited GPX enzyme activity due to its lack of glutathione binding selenocysteine (25,26). GPX7 can reduce oxidative stress generated from polyunsaturated fatty acid metabolism (27). It can also neutralize hydrogen peroxide *in vitro* in absence of glutathione and protect normal esophageal epithelia from acidic bile salts-induced oxidative stress, oxidative DNA damage and double strand breaks (25). A recent study suggested that GPX7 may act as an oxidative stress sensor and functions through regulating GRP78 chaperone activity to reduce oxidative stress (28). We have recently shown that GPX7 possesses tumor suppressor functions in EAC (29). This finding is further supported by the recent discovery that GPX7 deficiency in mice leads to systemic oxidative stress, increased tumor incidence and shortened life span (28). We have reported that loss of expression and dysfunction of GPX7 are frequent in EAC and its precancerous lesions (25,29,30). In the present study, we investigated the role of GPX7 in regulating NF- κ B and demonstrated that GPX7 can suppress TNF- α -induced activation of NF- κ B pathway, suggesting its potential role in modulating the inflammation associated with Barrett's carcinogenesis.

Materials and methods

Cell lines

The immortalized human esophageal epithelial cell line HET1A and the EAC cell line FLO-1 were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (Invitrogen, Carlsbad, CA). Immortalized BE cell line CP-A (ATCC) were cultured with epithelial cell medium 2 (ScienCell, Carlsbad, CA), supplemented with 5% fetal bovine serum and antibiotics on primary plates and flasks (BD Biosciences, Bedford, MA). All cell lines were grown at 37°C in 5% carbon dioxide.

Antibodies

Anti-GPX7 antibody (rabbit) was obtained from Proteintech Group (Chicago, IL). Anti-p65 antibody (rabbit), anti-phospho-p65 antibody (S536, rabbit), anti-I κ B- α antibody (mouse), anti-phospho-I κ B- α antibody (S32, rabbit), anti-IKK- α antibody (rabbit), anti-phospho-IKK α / β (S176/180, rabbit), anti-TRAF2, (rabbit), anti-TNFR1 (rabbit), anti-Histone 3 (rabbit), anti-actin antibody (rabbit), horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA).

Quantitative real-time RT-PCR analysis of gene expression

Total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA). Single-stranded complementary DNA was subsequently synthesized from RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using a CFX Connect real-time system (Bio-Rad) with the threshold cycle number determined by the use of Bio-Rad's CFX manager 3.0 software. The sequences of primers are provided in [Supplementary Table 1](#), available at [Carcinogenesis Online](#). Relative messenger RNA (mRNA) expression fold was determined as described previously (30,31).

GPX7 expression plasmids and GPX7 knockdown lentiviral shRNA

Construction of GPX7 expression plasmids and GPX7 knockdown lentiviral short hairpin RNA system were described previously (25). Cells were infected with GPX7-expressing adenovirus particles [Ad-GPX7, 5 multiplicity of infection (MOI)] and control adenovirus (Ad-CTRL, 5 MOI).

NF- κ B luciferase reporter stable cell lines and NF- κ B reporter luciferase assay

The pGL4.32[luc2P/NF- κ B-RE/Hygro] Vector (Promega, Madison, WI) contains five copies of an NF- κ B response element (NF- κ B-RE) that drives transcription of the luciferase reporter gene luc2P. FLO-1 and CP-A cells were transfected with the pGL4.32[luc2P/NF- κ B-RE/Hygro] Vector and selected under hygromycin at 100 μ g/ml (for CP-A) or 200 μ g/ml (for FLO-1) to generate stable expression cell lines. These cells were infected with GPX7-expressing adenovirus particles (Ad-GPX7, 5 MOI) and control adenovirus (Ad-CTRL, 5 MOI). Twenty-four hours after infection, cells were seeded into 24-well plates at the density of 1×10^5 cells per well. The next day, cells were treated with TNF- α (100 ng/ml) for 6 h, and then luciferase assay (Promega) was used to measure the NF- κ B reporter activity. The relative NF- κ B reporter activity was normalized to the value of the untreated cells. FLO-1 cells stably expressing PcDNA-GPX7 were cotransfected with NF- κ B luciferase reporter plasmid and β -galactosidase reporter plasmid using DNAfectin™ 2100 Transfection Reagent (Applied Biological Materials, Richmond, BC). Forty-eight hours after cotransfection, cells were treated with TNF- α (100 ng/ml) for 6 h, and then dual luciferase assay (Promega) was used to measure NF- κ B reporter activity. The relative NF- κ B reporter activity was first normalized to the value of β -galactosidase then compared with the value of the untreated cells. Bay 11-7082 (40 μ M, a known NF- κ B inhibitor by blocking TNF- α -induced phosphorylation of I κ B- α) and Tiron [10 mM, a reactive oxygen species (ROS) scavenger] were administered 1 h before adding TNF- α .

Immunofluorescence assay

FLO-1 cells stably expressing GPX7 or empty vector (PcDNA) were plated in 8-well slide chambers. The next day, cells were treated with TNF- α (100 ng/ml) for 30 min and then subjected to immunofluorescence assay for detection of nuclear NF- κ B-p65 protein, as described previously (13). Briefly, cells were fixed with fresh 4% paraformaldehyde solution for 15 min at room temperature, followed by incubation in 10% normal goat serum blocking solution (Invitrogen) for 20 min at room temperature. Cells were incubated with primary antibody against NF- κ B-p65 (1:400; GenScript, Piscataway, NJ) overnight at 4°C in a humidified chamber followed by incubation in fluorescein isothiocyanate-tagged secondary antibody (1:1000; Jackson ImmunoResearch, West Grove, PA) for 45 min at room temperature. The cells were then mounted with Vectashield with DAPI (Vector Laboratories, Burlingame, CA) and

visualized using an Olympus BX51 fluorescence microscope (Olympus Co., Center Valley, PA). At least 400 cells were counted from each experiment. Quantification was performed using ImageJ software (NIH, <http://www.uhnr-research.ca/facilities/wcif/imagej/>).

Western blot analysis

Protein lysates were prepared using radioimmunoprecipitation assay buffer, adding proteinase and phosphatase inhibitors before use. Western blot analysis was performed using standard protocol. To evaluate nuclear translocation of NF- κ B, FLO-1 cells stably expressing GPX7 or control PcDNA were treated with TNF- α (100 ng/ml) for 30 min, then nuclear and cytoplasmic protein fractions were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL) following the manufacturer's instructions. Equal amounts of nuclear and cytoplasmic proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membranes were incubated with NF- κ B-p65 or p-NF- κ B-p65 (Ser536) antibodies. To examine protein stability of TNFR1 and TRAF2, FLO-1 cells were infected with adenoviruses expressing GPX7 (5 MOI) or control (5 MOI). Forty-eight hours after infection, cells were treated with cycloheximide (50 μ M) for 0.5, 1, 2, 4 and 7 h, cell lysates were then prepared and western blot analysis was performed as described above. The cytoplasmic and nuclear protein fractions were normalized to β -actin and Histone 3, respectively. Quantification of the intensity of the immunoblot bands was performed using ImageJ software.

Detection of intracellular ROS

FLO-1 cells infected with GPX7-expressing (Ad-GPX7) or control (Ad-CTRL) adenovirus were seeded into 96-well plates. The next day, cells were treated with TNF- α (100 ng/ml) for 30 min or 2 h. After treatment, cells were washed in phosphate-buffered saline and incubated with CM-H₂DCFDA (10 μ M) in phenol-free medium in the dark at 37°C for 20 min. Cells were then washed in phosphate-buffered saline and the fluorescence intensity of the samples was measured using a FLUO Star OPTIMA microplate reader (BMG Labtech, Cary, NC). The relative fluorescence unit was normalized to the wells containing phosphate-buffered saline (16). Cells treated with hydrogen peroxide (100 μ M) for 30 min served as positive control. Each treatment was performed in triplicate.

Statistical analysis

Data are expressed as the mean \pm SD for parametric data. An unpaired Student's *t*-test was performed for two independent variables. All statistical analyses were done using GraphPad Prism 4 software (GraphPad Software, La Jolla, CA). For all analyses, $P \leq 0.05$ is considered statistically significant.

Results

GPX7 suppresses TNF- α -induced NF- κ B reporter activity and NF- κ B-p65 nuclear translocation

Because TNF- α is a major component of the inflammatory process involved in GERD and Barrett's tumorigenesis, we examined if GPX7 could regulate the activation of NF- κ B by TNF- α . NF- κ B reporter activity was determined by luciferase reporter assay in FLO-1 EAC cells with stable expression of GPX7 (Figure 1A) or transient expression of GPX7 (Figure 1B). In both conditions, the TNF- α treatment significantly induced activation of NF- κ B reporter activity in control cells, whereas this induction was significantly suppressed in GPX7-expressing cells ($P < 0.01$). Similar results were obtained using a non-dysplastic Barrett's cell line, CP-A ($P < 0.001$) (Figure 1C). The NF- κ B pharmacologic inhibitor, Bay 11-7082, was used as a control. To confirm these results, immunofluorescence assay and western blot analysis for nuclear and cytoplasmic protein fractions were performed after TNF- α treatment. The results indicated that GPX7 inhibited the nuclear translocation of NF- κ B-p65 as demonstrated by immunofluorescence (Figure 1D and E) and western blot analysis (Figure 1F). The expression of GPX7 inhibited the TNF- α -mediated phosphorylation of NF- κ B-p65 at serine 536 (Figure 1F). Taken together, these results indicate that GPX7 can suppress the TNF- α -induced NF- κ B phosphorylation and nuclear localization.

GPX7 inhibits the TNF- α -induced NF- κ B signaling and expression of its target genes

Because of the aforementioned results, we next examined if GPX7 inhibits NF- κ B-p65 phosphorylation through regulating the phosphorylation of the upstream signaling components, I κ B and IKK.

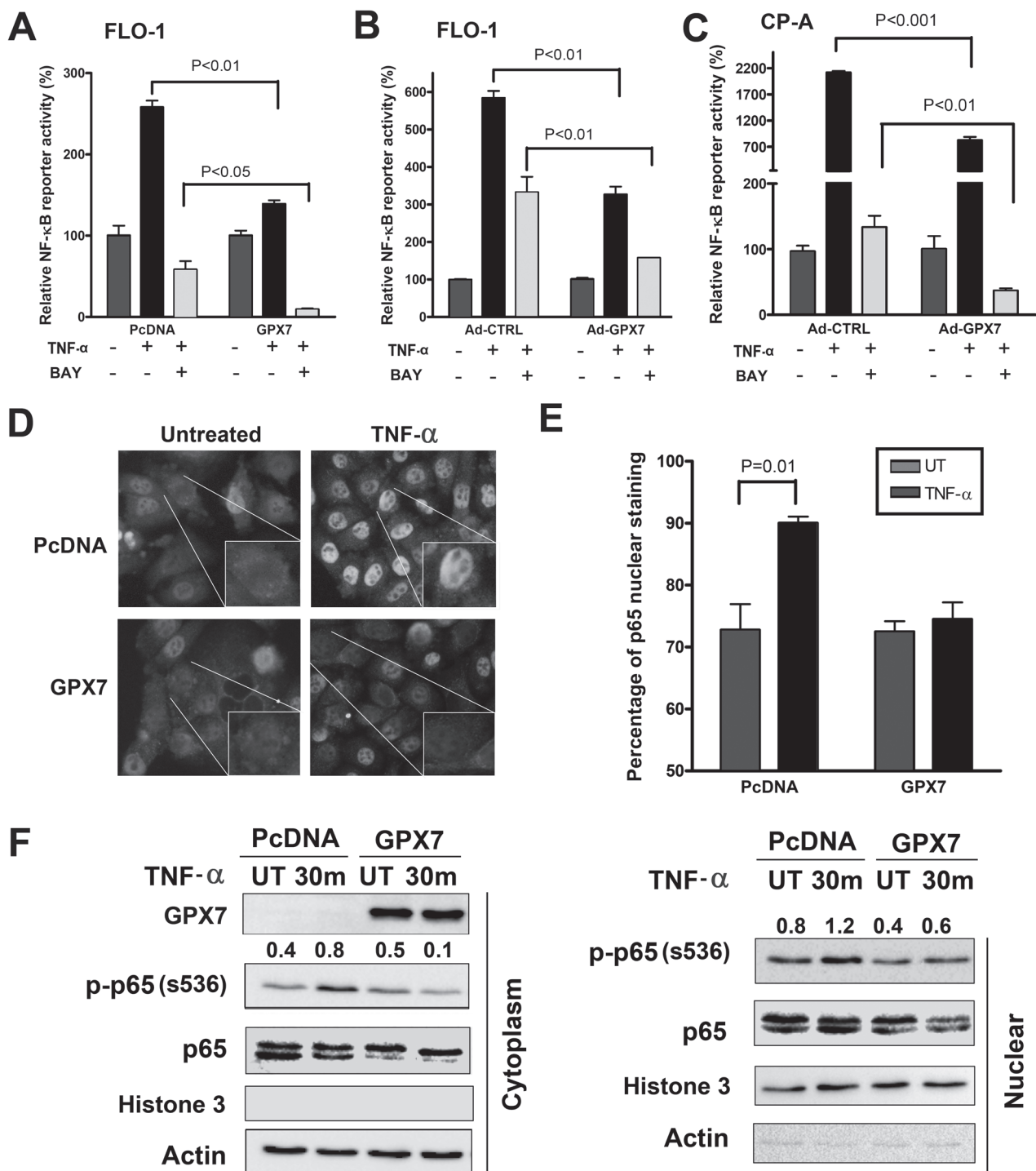


Fig. 1. GPX7 suppresses TNF- α -induced NF- κ B reporter activity and NF- κ B-p65 translocation to the nucleus. NF- κ B reporter luciferase assay was performed in FLO-1 and CP-A cells. (A) FLO-1 cells stably transfected with GPX7 or PcDNA 3.1 plasmids. (B) FLO-1 cells infected with GPX7 (Ad-GPX7) or control (Ad-CTRL) adenoviruses. (C) CP-A cells infected with GPX7 (Ad-GPX7) or control (Ad-CTRL) adenoviruses. Cells were treated with TNF- α (100 ng/ml) alone or in combination with Bay 11-7082 for 6 h. Bay 11-7082 was added to the medium 1 h before treatment with TNF- α . (D) Immunofluorescence staining of NF- κ B-p65 protein in FLO-1 cells. In control cells (PcDNA), treatment with TNF- α (100 ng/ml) resulted in a significant increase in p65 nuclear staining. However, in GPX7-expressing cells (GPX7), the TNF- α -induced increase of p65 nuclear staining was abrogated. Insets with high magnification ($\times 200$) are shown in the lower right corner of each panel. (E) Quantitative data of p65 nuclear staining are shown. (F) Western blot analysis of p65 protein in cytosolic (left) and nuclear (right) protein fractions in FLO-1 cells after exposure to TNF- α (100 ng/ml). The quantification was performed using ImageJ and normalized to β -actin or histone 3. UT, untreated.

Western blot analysis data indicated that TNF- α induced significant upregulation of the p-p65 (S536) protein and its upstream p-I κ B- α (S32) and p-IKK- α / β (S176/180) proteins in the control FLO-1 cells (Figure 2A and B). However, the induction of these proteins by

TNF- α was markedly abrogated in FLO-1 cells transiently or stably expressing GPX7 (Figure 2A and B). To determine the impact of GPX7 on NF- κ B target genes, we performed quantitative real-time RT-PCR. TNF- α treatment induced a significant increase in mRNA

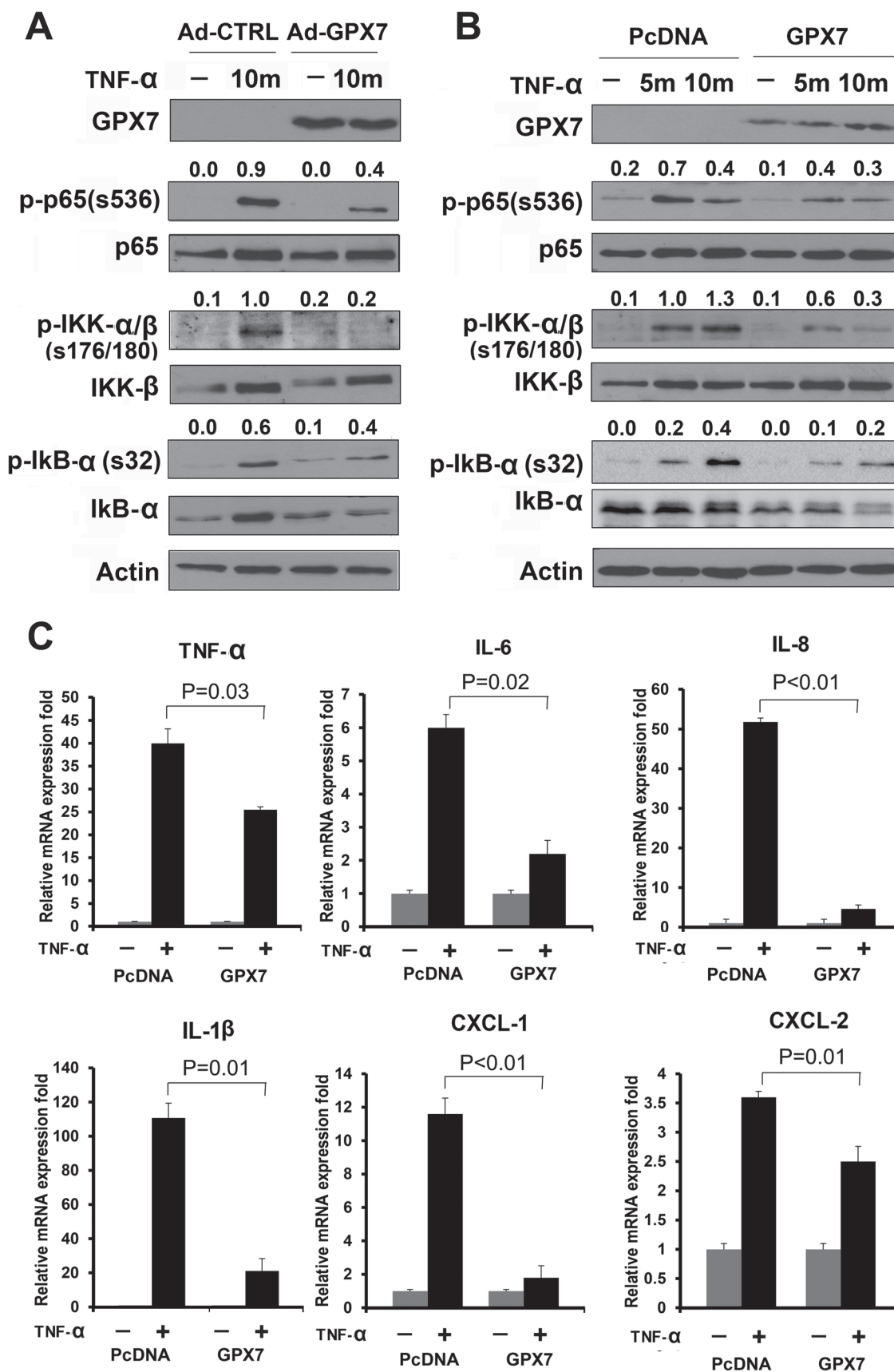


Fig. 2. GPX7 suppresses TNF-α-induced NF-κB canonical pathway activation and the induction of NF-κB target genes. (A and B) Western blot analysis of NF-κB pathway components, p-p65 (S536), p-IκB-α (S32) and p-IKKα/β (S176/180) proteins, upon TNF-α (100 ng/ml) treatment in FLO-1 cells transiently expressing GPX7 (Ad-GPX7) or control (Ad-CTRL) (A), or stably expressing GPX7 (GPX7) or control (PcDNA) (B). (C) Quantitative real-time RT-PCR analysis of gene expression of NF-κB target genes; TNF-α, IL-6, IL-8, IL-1β, CXCL-1 and CXCL-2 are shown.

expression of NF- κ B target genes (Figure 2C) including TNF- α itself, proinflammatory cytokines such as IL-6, IL-8 and IL-1 β ; and chemokines, CXCL-1 and CXCL-2. However, in GPX7-expressing cells, the induction of these NF- κ B target genes by TNF- α was significantly suppressed (Figure 2C), particularly for IL-8 which showed the highest reduction.

Knockdown of GPX7 enhances the TNF- α -induced activation of NF- κ B and expression of its target genes

To validate the aforementioned results, the endogenous expression of GPX7 was knocked down in normal esophageal HET1A cells using a lentiviral short hairpin RNA system (25). Western blot analysis data showed that knocking down of GPX7 expression enhanced the TNF- α -induced NF- κ B activity, as demonstrated by increased p-p65 (S536), p-I κ B- α (S32) and p-IKK- α/β (S176/180) protein levels as compared with control cells (Figure 3A). In line with these findings, the quantitative real-time RT-PCR data indicated a significant increase in the TNF- α -induced mRNA expression of NF- κ B target genes, TNF- α , IL-6, IL-8, IL-1 β , CXCL-1 and CXCL-2, in GPX7 short hairpin RNA cells as compared with control cells (Figure 3B). Taken together, the results from the reconstitution and knockdown of GPX7 expression demonstrated that GPX7 is a negative regulator of TNF- α -induced activation of NF- κ B signaling pathway in esophageal cells.

Suppression of TNF- α -induced activation of NF- κ B by GPX7 is independent of its antioxidant function

Previous studies have shown that activation of NF- κ B could occur through ROS (22). We have shown previously that GPX7 has an antioxidative capacity by neutralizing intracellular ROS (25). Therefore, we next explored if the observed suppression of TNF- α -induced activation of NF- κ B by GPX7 is attributable to its antioxidant function. First, we checked the intracellular ROS level after TNF- α treatments. Treatment of cells with TNF- α (100 ng/ml) up to 30 min did not induce a significant increase in intracellular ROS (Figure 4A), whereas exposure to TNF- α for 10 min induced a significant increase of p-p65 (S536) protein level (Figure 4D). We next utilized a ROS scavenger, Tiron, which has

been reported to be a universal ROS scavenger (32). As expected, treatment of cells with Tiron, in combination with TNF- α , significantly diminished the intracellular ROS level (Figure 4B). Notably, treatment with Tiron failed to block the TNF- α -induced activation of NF- κ B luciferase reporter activity (Figure 4C) and upregulation of p-p65 (S536) protein in FLO-1 cells (Figure 4D) in control FLO-1 cells. In addition, Tiron treatment had no effect on GPX7's suppression of TNF- α -induced activation of NF- κ B reporter activity and upregulation of p-p65 (S536) protein in FLO-1 cells (Figure 4C and D). We confirmed these data in CP-A cells (Supplementary Figure 1A–C, available at *Carcinogenesis* Online). Collectively, these results suggest that ROS plays a minimal role, if any, in the TNF- α -induced activation of NF- κ B and imply that suppression of TNF- α -induced activation of NF- κ B by GPX7 is independent of its antioxidant function.

GPX7 suppresses TNF- α -induced activation of NF- κ B pathway through regulation of TNFR1/TRAF2 complex

During the process of TNF- α -induced NF- κ B activation, TNF- α binds to its receptor TNFR1 and recruits TRAF2 that associates with RIP1 for IKK activation (14). Because we observed significant suppression of p-IKK- α/β (S176/180) proteins in GPX7-expressing cells upon TNF- α treatment (Figure 2A and B), we sought to explore the role of GPX7 in regulating the upstream cell signaling molecules. Interestingly, the protein levels of TNFR1 and TRAF2 were reduced in GPX7-expressing cells as compared with control cells (Figure 5A and B). The protein levels of TNFR1 and TRAF2 were further diminished following treatment with TNF- α in GPX7-expressing cells. The examination of mRNA levels of TNFR1 and TRAF2 did not show significant differences between control cells and GPX7-expressing cells (Figure 5C and D), ruling out transcriptional regulation of TNFR1 and TRAF2 by GPX7. Following these results, we investigated whether GPX7 regulates the protein stability of TNFR1 and TRAF2 by western blot analysis after treatment with cycloheximide (an inhibitor of protein biosynthesis). The results demonstrated that GPX7 expression leads to a significant increase in the rate of TNFR1 and TRAF2 protein degradation (Figure 5E–G). These findings strongly suggest that GPX7 suppresses TNF- α -induced activation of NF- κ B through the protein degradation of

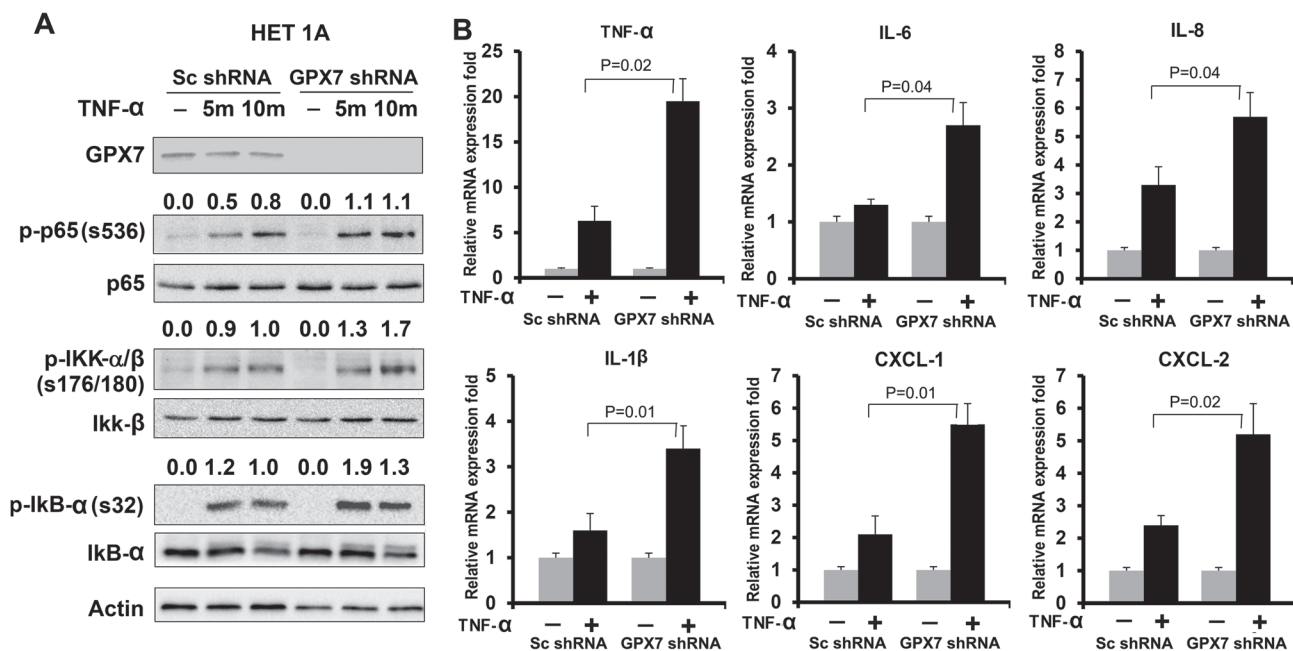


Fig. 3. Knockdown of endogenous GPX7 expression enhances TNF- α -induced NF- κ B activation. (A) Western blot analysis of p-p65 (S536), p-I κ B- α (S32) and p-IKK α/β (S176/180) proteins after TNF- α (100 ng/ml) treatment in HET1A cells with stable knockdown of GPX7 (GPX7 shRNA) or control shRNA (Sc shRNA). (B) Quantitative real-time RT-PCR analysis of gene expression of NF- κ B target genes; TNF- α , IL-6, IL-8, IL-1 β , CXCL-1 and CXCL-2 are shown.

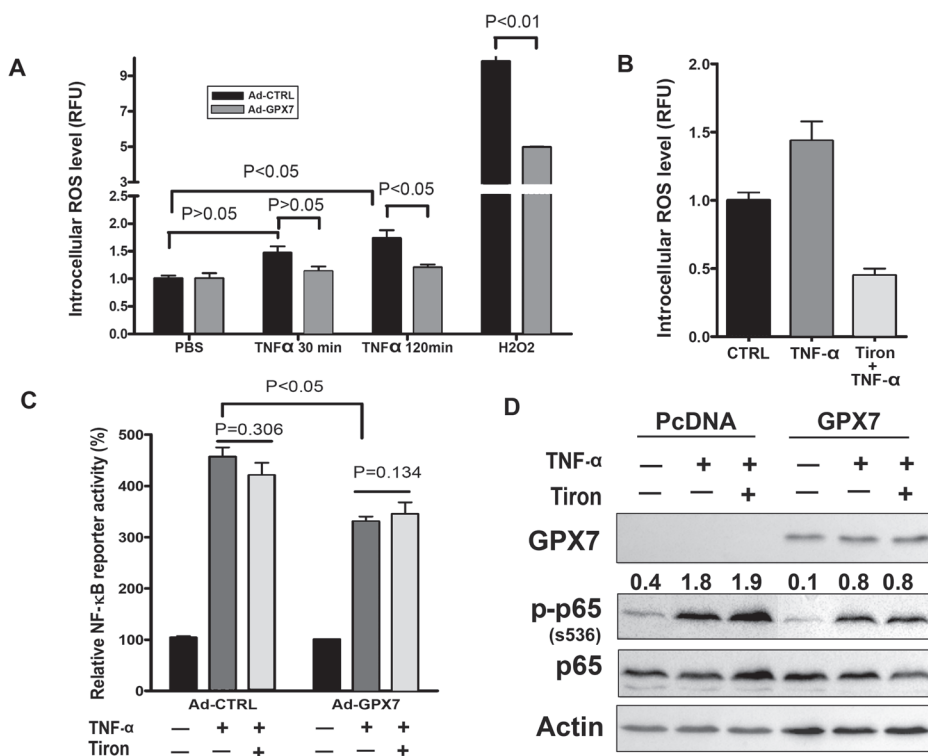


Fig. 4. Suppression of TNF- α -induced NF- κ B activation by GPX7 is independent of its antioxidant function. (A) FLO-1 cells were treated with TNF- α (100 ng/ml) for 30 or 120 min and intracellular ROS level was determined by measuring the fluorescence of a ROS sensitive dye, CM-H₂DCFDA, and presented as relative fluorescence unit (RFU) to phosphate-buffered saline-treated cells. Cells treated with hydrogen peroxide (H₂O₂) (100 μ M) for 30 min were used as positive control. (B) Intracellular ROS level in FLO-1 cells treated with TNF- α (100 ng/ml) for 30 min or Tiron (10 mM) for 1 h followed by TNF- α (100 ng/ml) for 30 min. (C) FLO-1 cells stably expressing pGL4.32[luc2P/NF- κ B-RE/Hygro] were infected with control (Ad-CTRL) or GPX7 (Ad-GPX7) adenoviruses. Forty-eight hours after infection, cells were treated with TNF- α (100 ng/ml) for 6 h or Tiron (10 mM) for 1 h followed by TNF- α (100 ng/ml) for 6 h. NF- κ B reporter luciferase assay was performed to determine the reporter activity. (D) FLO-1 cells stably expressing GPX7 (GPX7) or control (PcDNA) were treated with TNF- α (100 ng/ml) for 10 min or Tiron (10 mM) for 1 h followed by TNF- α (100 ng/ml) for 10 min. Western blot analysis was performed to determine the p65 and p-p65 (S536) protein levels.

the TNFR1/TRAF2 complex, which are essential components for transduction of TNF- α signaling to the downstream effectors of the NF- κ B pathway (Figure 6).

Discussion

Increased NF- κ B activation and TNF- α expression, along with other proinflammatory cytokines, has been reported in Barrett's and EAC tissues (5,20,33). In this study, we have shown that GPX7 can suppress activation of NF- κ B in esophageal epithelial cells upon exposure to TNF- α . In particular, the TNF- α -induced upregulation of NF- κ B target genes, including proinflammatory cytokines and chemokines, was significantly suppressed in cells expressing GPX7. Our data demonstrate, for the first time, that GPX7 suppresses the inflammatory responses associated with Barrett's carcinogenesis. These novel findings provide strong support for the previously suggested potential tumor suppressor properties of GPX7 (29).

Over the past several years, a number of reports emphasized the role of inflammation in carcinogenesis which are supported by the observed reduced risk of many cancers following the long-term use of non-steroidal anti-inflammatory drugs (34). BE develops in response to chronic reflux of acid and bile salts from the stomach and duodenum. The risk of developing EAC is 30 times higher for GERD patients and up to 125 times higher for GERD patients with BE than for the general population (1,4,35). In this regard, the development of BE is strongly linked to chronic GERD-associated inflammation (2,8), which is an added risk for the development of EAC through persisting the proinflammatory oncogenic signaling, angiogenesis and immune suppression. Epidemiological studies demonstrated a decrease in the risk of cancer development in patients with BE with the use of

aspirin and non-steroidal anti-inflammatory drugs (36,37). NF- κ B is a key regulator in the inflammatory process that has been shown to be activated in EAC (5,15,38). TNF- α , released by predominantly inflammatory cells such as macrophages, is one of the major players in regulating NF- κ B activity. In addition, an increase in the TNF- α expression level has been shown in esophageal epithelial cells during progression along the metaplasia-dysplasia-carcinoma sequence (4,20). Our data clearly indicated that GPX7 has an anti-inflammatory tumor suppressor function, as demonstrated by its role as a negative regulator of TNF- α -induced activation of the proinflammatory NF- κ B pathway. Among the cytokines we examined, IL-8 showed the highest induction upon exposure to TNF- α with the largest reduction following reconstitution of GPX7 expression, suggesting that this cytokine may play critical role in the inflammatory process during Barrett's carcinogenesis (39,40). In fact, IL-8 has been shown to promote cancer cell proliferation, survival and migration which are associated with tumor progression and metastasis (41). Because bile salts have been shown to activate NF- κ B pathway in esophageal cells (16,17), our results suggest that GPX7 could have suppressive effects on bile salts-induced activation of NF- κ B and/or proinflammatory cytokines during Barrett's carcinogenesis.

Of note, GPX7 has been shown to have antioxidative properties and can decrease intracellular ROS levels in esophageal epithelial cells (25). Therefore, we examined whether suppression of TNF- α -induced activation by GPX7 involves its antioxidative function. Although the intracellular ROS level is known to modulate the activity of NF- κ B (22,42), our data suggest that the observed activation of NF- κ B in response to TNF- α is independent of ROS, and the suppression of TNF- α -induced NF- κ B activation by GPX7 is most likely not related to its antioxidative function.

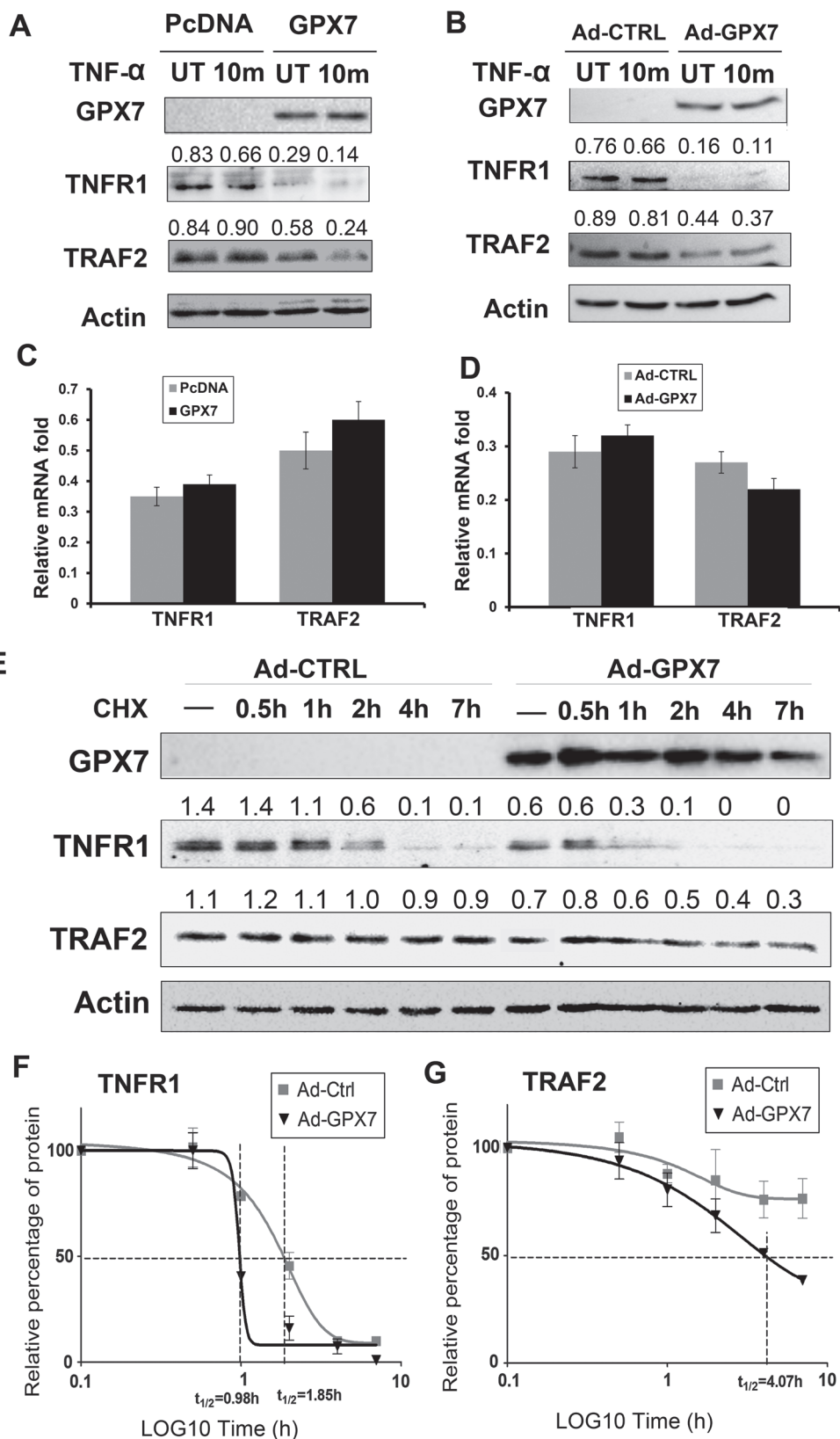


Fig. 5. GPX7 deregulates protein stability of TNFR1 and TRAF2. (A and B) Western blot analysis of TNFR1 and TRAF2 protein levels in FLO-1 cells stably expressing GPX7 (GPX7) or control (PcDNA) (A) or transiently expressing GPX7 (Ad-GPX7) or control (Ad-CTRL) (B). UT, untreated. (C and D) Quantitative real-time RT-PCR analysis of mRNA expression of TNFR1 and TRAF2 in FLO-1 cells stably expressing GPX7 (GPX7) or control (PcDNA) (C) or transiently expressing GPX7 (Ad-GPX7) or control (Ad-CTRL) (D). (E) FLO-1 cells transiently expressing GPX7 (Ad-GPX7) or control (Ad-CTRL) were treated with cycloheximide (CHX, 50 μ M) for the indicated times. TNFR1 and TRAF2 proteins were analyzed by western blot analysis. (F and G) Quantitative data indicating rates of degradation and the half-life ($t_{1/2}$) of TNFR1 (F) and TRAF2 (G) proteins are shown. The percentage of proteins (relative to time 0h) was plotted against the time (log10) with CHX treatment.

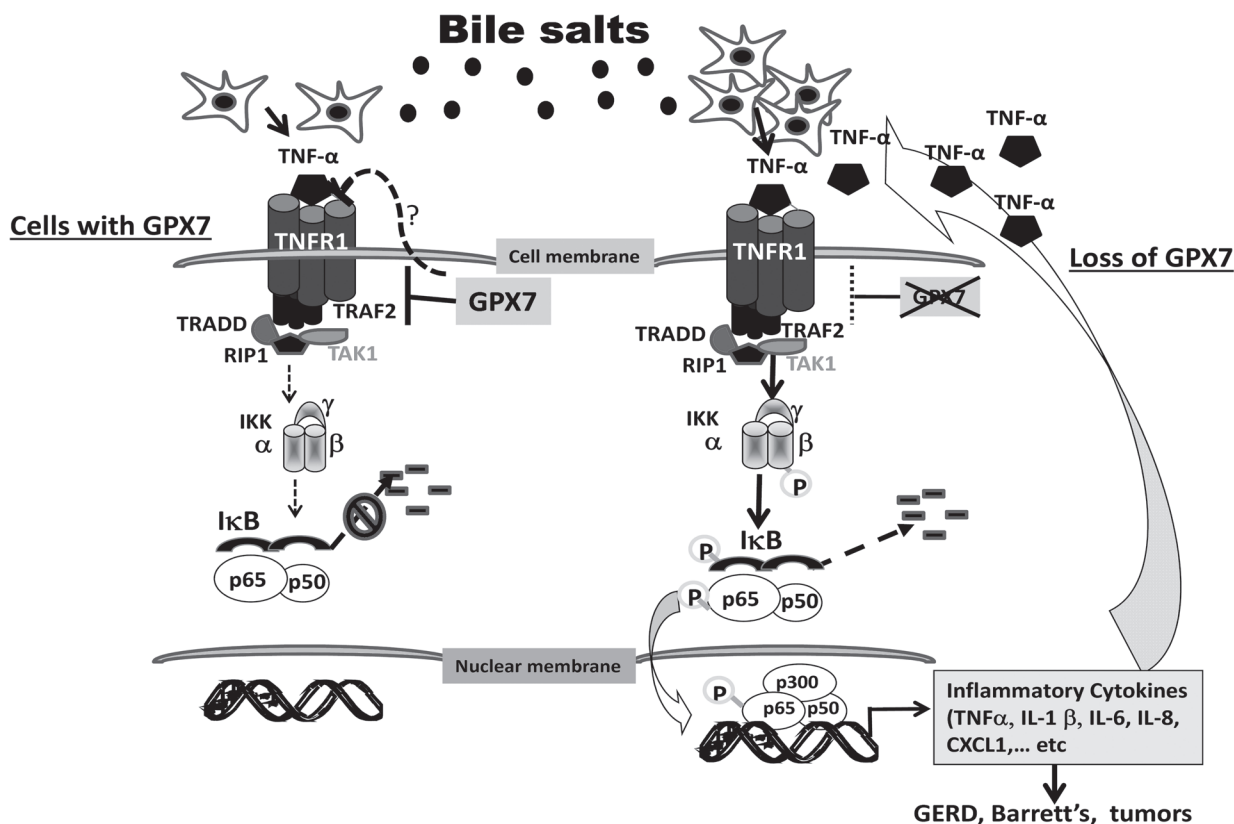


Fig. 6. A diagram depicting the role of GPX7 in regulating inflammation in esophageal epithelial cells. The TNF- α -induced activation of NF- κ B leads to upregulation of several cytokines and chemokines that mediate the inflammatory phenotype. In cells expressing GPX7 (left panel), the activation of NF- κ B is suppressed through degradation of TNFR1 and TRAF2. In the event of GPX7 gene silencing (via DNA hypermethylation), the NF- κ B pathway activation is enhanced, due to loss of the GPX7 inhibitory functions and the positive feedback of TNF- α , leading to persistent proinflammatory signaling that is associated with Barrett's carcinogenesis (right panel).

To identify the molecular mechanism by which GPX7 suppresses TNF- α -induced NF- κ B activation, we investigated the canonical NF- κ B pathway. Indeed, we found that GPX7 regulates the phosphorylation levels of IKK- α/β and I κ B- α . These kinases are essential for rapid NF- κ B activation by proinflammatory signaling cascades, such as those triggered by TNF- α (19). In the absence of cellular stimuli, NF- κ B transcription factors are sequestered in the cytoplasm through binding to the inhibitory I κ B proteins. In response to activating stimuli, I κ B proteins are phosphorylated by IKK- α/β leading to their subsequent degradation. Therefore, the phosphorylation of I κ B proteins is considered a convergence point for signal transduction pathways leading to the release of the I κ B-bound NF- κ B-p65 which translocate along with p50 to the nucleus to drive expression of target genes (19,43). Because our results indicated that GPX7 plays a critical role in suppressing the TNF- α -mediated activation of the IKK- α/β complex, it was important to determine if the regulation of NF- κ B by GPX7 occurs at this level or at a higher upstream signaling level. In response to cellular stimuli like TNF- α , the induction of NF- κ B transcription factors involves binding of TNF- α to its main receptor, TNFR1, leading to binding of TRADD, RIP1 and TRAF2 to form a protein complex that is required for activation of IKK (Figure 6) (14,19,44). We have investigated the possibility that GPX7 may interfere with the upstream TNFR1 signaling and TRADD-RIP1-TRAF2 protein complex. We found that the protein levels of TNFR1 and TRAF2 were markedly downregulated in GPX7-expressing cells as compared with control cells. Examination of mRNA levels of TNFR1 and TRAF2 in the same cells did not show significant difference, ruling out transcriptional regulation of TNFR1 and TRAF2 as a cause of their lower protein levels in GPX7-expressing cells. In contrast, we found that both TNFR1 and TRAF2 proteins degrade at faster rates in GPX7-expressing cells than in control cells. This finding explains

the observed suppression of TNF- α -induced activation of NF- κ B by GPX7 and suggests that GPX7 is likely to be involved in regulating the protein stability of TNFR1 and TRAF2. The degradation of TNFR1 and TRAF2 is known to be regulated by a number of mechanisms (45,46). Further studies are needed to specifically address the exact mechanism(s) by which GPX7 affects the protein stability of TNFR1 and TRAF2.

In summary, our studies suggest that the downregulation of GPX7 expression in Barrett's tumorigenesis could promote TNF- α -induced activation of proinflammatory NF- κ B signaling through loss of the inhibitory effect of GPX7, and positive feedback of TNF- α on NF- κ B pathway (Figure 6). We provide the first evidence that silencing of GPX7 leads to activation of the NF- κ B transcription factors via deregulating the protein levels of TNFR1 and TRAF2, essential components required for transduction of TNF- α signals and activation of NF- κ B. These novel findings establish the critical role of GPX7 loss in coupling inflammation and Barrett's tumorigenesis.

Supplementary material

Supplementary Table 1 and Figure 1 can be found at <http://carcin.oxfordjournals.org/>

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