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# **Heat shock protein gp96 and NAD(P)H oxidase 4 play key roles in Toll-like receptor 4-activated apoptosis during renal ischemia-reperfusion injury**

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Running title: **Role of gp96 and NOX4 in TLR4-mediated apoptosis**

## Summary

Ischemia/reperfusion injury (IRI) causes inflammation and cell injury as a result of activating innate immune signaling. Toll-like receptor 4 (TLR4) plays a key role in mediating kidney damages during IRI, but the downstream signaling pathway(s) stimulating apoptosis remains debated. Here we show that TLR4 mediates MyD88-dependent activation of TNF receptor-associated factor 2 (TRAF2), apoptosis signal-regulatory kinase 1 (ASK1), and JNK and p38 MAP kinases in ischemic-reperfused kidneys and post-hypoxic renal tubule epithelial cells (RTECs). Hypoxia stimulated the expression of the endoplasmic-resident gp96, which co-immunoprecipitated TLR4, while silencing gp96 mRNA expression impaired hypoxia-induced apoptosis in TLR4-expressing RTECs. NAD(P)H oxidase 4 (NOX4) was shown to interact with TLR4 and to be required in lipopolysaccharide-induced production of reactive oxygen species (ROS). IRI stimulated the expression of a 28-kDa NOX4 spliced isoform abundantly expressed in wild-type RTECs, which co-immunoprecipitated with TLR4, but not with gp96 in TLR4-deficient RTECs. Silencing NOX4 mRNA expression impaired hypoxia-induced activation of ASK1 and both JNK and p38, leading to the inhibition of ROS production and apoptosis in post-hypoxic TLR4-expressing RTECs. These findings demonstrate that, concomitantly to the activation of p38, the gp96/TLR4 interaction is required for activation of ASK1/JNK signaling in post-hypoxic mouse RTECs, and that the 28-kDa NOX4 plays a key role in TLR4-mediated apoptosis during renal IRI.

**Key words:** kidney; gp96; Toll-like receptor 4; NAD(P)H oxidase 4; apoptosis

**Abbreviations:** ASK1, apoptosis signal-regulating kinase 1; DAMP, damage-associated molecular pattern; DPI, diphenyleneiodonium chloride; HMGB1, high-mobility group box 1; Hsp, heat shock proteins; JNK, Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MyD88, myeloid differentiation factor-88; NOX, NAD(P)H oxidase; PP5, protein phosphatase 5; ROS, reactive oxygen species; RTEC, renal tubule epithelial cell; siRNA, small interfering RNA; TRAF, TNF receptor-associated factor; UPR, unfolded protein response

## Introduction

Renal ischemia/reperfusion injury (IRI) is one of the most common causes of acute renal failure, which is associated with increased morbidity and mortality.<sup>1</sup> It is also an unavoidable event during transplantation, and may compromise subsequent renal graft function.<sup>2</sup> IRI induces inflammatory response and variable tissue damage<sup>1</sup> resulting from the capacity of endogenous ligands, known as damage-associated molecular patterns (DAMPs) that are secreted or released from hypoxic or necrotic cells, to activate immune signaling pathways via common pattern recognition receptors. Among DAMPs, the high-mobility group box 1 (HMGB1), heat shock proteins (Hsps), and the matrix components biglycan and heparan sulfate were shown to activate both TLR2 and TLR4.<sup>3</sup> The role of TLR4 in the inflammation caused by IRI has been established in hepatic IRI, cardiac IRI, and hemorrhagic shock.<sup>4-6</sup> TLR4 and TLR2 also mediate inflammatory responses and kidney damage during IRI: mice deficient for TLR2, TLR4, or for the adaptor protein MyD88 withstand IRI better than wild-type mice.<sup>7-9</sup> Renal tubule epithelial cells (RTECs) constitutively express TLR2 and TLR4, the expression of both of which are increased in ischemic RTECs,<sup>10,11</sup> produce cytokines and undergo apoptosis after ischemia.<sup>7,9</sup> However, less is known about the TLR4-mediated downstream pro-apoptotic pathway(s) activated during IRI. The endoplasmic-reticulum (ER) resident gp96, homologue to cytosolic Hsp90, is a master chaperone for most TLRs.<sup>12</sup> We have shown that gp96 interacts with the protein phosphatase 5 (PP5) to control selective TLR2-mediated activation of the mitogen-activated protein kinase (MAPK) ERK<sub>1/2</sub>, but not of JNK.<sup>13</sup> The question therefore arises as to whether gp96, shown to interact with TLR4 in intestinal epithelial cells,<sup>14</sup> is required for TLR4-activated apoptosis during renal IRI.

The production of reactive oxygen species (ROS) by hypoxic cells plays an important role in the induction of apoptosis. ROS-induced cell injury has been attributed in part to the activation of MAPK.<sup>15</sup> The serine/threonine MAP kinase apoptosis signal-regulating kinase 1 (ASK1) plays a pivotal role in cytokine- and stress-induced apoptosis.<sup>16,17</sup> In response to ROS

stimulation, the negative redox sensor thioredoxin dissociates from the endogenous inactive ASK1 complex, and then forms an active high molecular mass complex after the recruitment of TNF receptor-associated factors 2 (TRAF2) and 6 (TRAF6), and the downstream activation of Jun N-terminal kinase (JNK).<sup>18</sup> Lipopolysaccharide (LPS) was shown to activate a TRAF6/ASK1/p38 pathway through ROS production, indicating that this pathway plays a key role in TLR4-mediated innate immunity.<sup>19</sup> However, TLR4-mediated downstream signaling controlling apoptosis during IRI still remains not well known.

Members of the NAD(P)H oxidase (NOX) family producing ROS, which are homologues of the gp<sup>91phox</sup> catalytic subunit of phagocytic NAD(P)H oxidase (also designated NOX2), have been shown to be involved in cellular functions related to innate immunity, signal transduction, proliferation, and/or apoptosis.<sup>20,21</sup> NOX1, all the components of the phagocytic NOX2, and NOX4 are expressed in the kidney, NOX4 being the most abundant isoform expressed in this tissue, mainly in RTECs.<sup>22,23</sup> NOX4 was shown to be required for LPS-induced H<sub>2</sub>O<sub>2</sub> generation in TLR4-expressing HEK 293T cells, and to interact directly with TLR4 in regulating NF- $\kappa$ B activation.<sup>24</sup> NOX4 induction has been reported in a variety of cell types subjected to ER stress, hypoxia or ischemia, suggesting that this non-phagocytic NOX might play a role in inducing cell damage during IRI.<sup>20</sup> In this study, we investigated the roles of gp96 and NOX4 in controlling TLR4-mediated apoptotic signaling in a murine experimental model of renal IRI, and in primary cultures of wild-type, *Tlr4*<sup>-/-</sup>, and *MyD88*<sup>-/-</sup> RTECs subjected to hypoxia. gp96 which interacts with TLR4 in RTECs, appears to be essential for the induction of the TLR4-mediated ASK1/JNK apoptosis pathway. We also identified a NOX4 spliced isoform predominantly expressed in RTECs, which interacts with TLR4 and acts as a key regulator of the TLR4-mediated apoptosis in post-hypoxic RTECs.

## Results

**TLR4 mediates the activation of the TRAF2/ASK1/JNK pathway.** Renal IRI caused by bilateral clamping of the renal pedicles from wild-type mice induces marked renal dysfunction and tubule cell damages, which peaked during the first two days following the reperfusion of the kidneys.<sup>9,13</sup> The fact that renal dysfunction and tubule cells apoptosis were significantly less marked in *Tlr4*<sup>-/-</sup> mice than in wild-type kidneys,<sup>9,13</sup> led us to analyze the activation of MAPK pathways in post-ischemic, reperfused wild-type and *Tlr4*<sup>-/-</sup> kidneys. IRI increased the expression level of TRAF2, and phosphorylated (p-) ASK1 and p-JNK, and to a lesser extent p-p38, in day-1 and day-2 post-ischemic wild-type kidneys, but not in post-ischemic *Tlr4*<sup>-/-</sup> kidneys (Figure 1a). The pro-apoptotic Bcl-2 homologue BAX, but not the anti-apoptotic Bcl-2,<sup>25</sup> also significantly increased in wild-type, but not in *Tlr4*<sup>-/-</sup> post-ischemic kidneys (Figure 1a). These findings indicate that TLR4 mediates predominant activation of p-ASK1 and p-JNK, two key signal events responsible for apoptosis during IRI,<sup>13</sup> in post-ischemic kidneys.

**TLR4 and MyD88 mediate ROS production and apoptosis in post-hypoxic renal tubule cells.** Experiments were carried out on primary cultures of RTECs isolated from the kidneys of wild-type, *Tlr4*<sup>-/-</sup>, and *MyD88*<sup>-/-</sup> mice to analyze the pathways controlling ROS production and apoptosis in RTECs subjected to hypoxia by incubating the cells with mineral oil.<sup>7,13</sup> The expressions levels of TRAF2 over  $\beta$ -actin, p-ASK1, p-p38, and p-JNK, and BAX, but not of Bcl-2, were also greater in post-hypoxic than in untreated wild-type RTECs (Figure 1b). In contrast, the amounts of p-ASK1, p-p38, p-JNK, and BAX remained barely detectable in post-hypoxic *MyD88*<sup>-/-</sup> and no or only moderately increased in post-hypoxic *Tlr4*<sup>-/-</sup> RTECs (Figure 1b). As previously reported,<sup>9</sup> the number of active caspase-3 positive apoptotic cells was significantly lower in the 24 h post-hypoxic *Tlr4*<sup>-/-</sup> and *MyD88*<sup>-/-</sup> RTECs than in wild-type RTECs (Supplementary Figure S1a and b). Imaging assays using 2'-7'-dichlorofluorescein-diacetate (DCFH-DA) to measure ROS produced by intact cells<sup>26</sup> revealed that hypoxia

stimulated ROS production in wild-type RTECs, which was inhibited by adding 2  $\mu$ M diphenyleneiodonium chloride (DPI), a potent flavoprotein inhibitor (Supplementary Figure S1c). In contrast, the DPI-inhibitable production of ROS remained significantly lower in post-hypoxic *Tlr4*<sup>-/-</sup> and *MyD88*<sup>-/-</sup> RTECs than in wild-type counterparts (Supplementary Figure S1c).

**Interaction of TLR4 with gp96 is required for apoptosis to be induced in post-hypoxic**

**renal tubule cells.** Acute ischemia induces misfolding of secretory proteins in the ER, and increases the expression of ER chaperones triggered by ER stress signaling pathways. gp96, one of the most abundant Hsps residing in the ER which is induced by the accumulation of misfolded proteins caused by cellular stress, plays an essential role in the maturation and proper folding of membranous and intracellular TLRs.<sup>12,27</sup> The amount of gp96 significantly increased in both wild-type and *Tlr4*<sup>-/-</sup> RTECs subjected to hypoxia (Figure 2a). Co-immunoprecipitation (co-IP) studies revealed that TLR4 interacts with gp96 in lysates from non-hypoxic wild-type RTECs (Figure 2b) or established mouse renal proximal tubule PKSV-PR cells<sup>28</sup> (not shown). Co-IP experiments also showed that hypoxia did not disrupt the interaction between gp96 and TLR4 (Figure 2c). We next analyzed the consequences of gp96 extinction on hypoxia-induced pro-apoptotic signaling. Knockdown *gp96* mRNA expression by silencing RNA (siRNA) (Figure 2d) had no significant effect on p-ASK1 or p-JNK in non-hypoxic wild-type RTECs (not shown), but in contrast, it did prevent the increase in TRAF2, p-ASK1 and p-JNK in post-hypoxic wild-type RTECs (Figure 2e). ROS production and induction of apoptosis were also no longer observed in *gp96* siRNA-transfected RTECs subjected to hypoxia when compared to non-transfected post-hypoxic RTECs or cells transfected with the control siRNA (Figure 2f and g). Transfecting cells with increasing concentrations of *gp96* siRNA also showed that about 70 % inhibition of gp96 protein content was associated with an almost complete inhibition (>90%) of the hypoxia-induced ROS

production and about 80% inhibition of apoptosis in wild-type RTECs (Supplementary Figure S2a and b). Silencing gp96 was shown to reactivate the expression of ERK1/2, but not of ASK1 and JNK, in post-hypoxic *Tlr2*<sup>-/-</sup> RTECs.<sup>13</sup> In contrast to the effect of PP5 silencing mRNA expression on the reinduction of apoptosis in post-hypoxic *Tlr2*<sup>-/-</sup> RTECs, which occurs through reactivation of the p-ASK1/p-JNK pro-apoptotic pathway,<sup>13</sup> silencing gp96 did not induce reactivation of ROS production, nor induction of apoptosis in *gp96* siRNA-transfected post-hypoxic *Tlr4*<sup>-/-</sup> RTECs or *Tlr2*<sup>-/-</sup> RTECs (Figure 2h and i). Because the expression levels of TRAF2 had increased in post-ischemic wild-type kidneys, its participation in the activation of apoptosis was analyzed. Co-IP experiments revealed that TRAF2, which is not associated with ASK1 in non-hypoxic RTECs, co-immunoprecipitated with ASK1 in post-hypoxic wild-type RTECs (Figure 3a). Furthermore, silencing *Traf2* mRNA expression almost fully impaired the activation of NOX4, p-ASK1 and p-JNK, ROS production, and apoptosis in post-hypoxic wild-type RTECs (Figure 3b-d).

**IRI induces NOX4 over-expression in renal tubule cells.** Because NOX enzymes are involved in the regulation of a wide range of physiological functions, including cell death,<sup>20</sup> experiments were carried out to investigate the consequences of IRI on the renal expression of NOX1 and NOX4, which are both predominantly expressed in the kidney.<sup>20,22,23</sup> Western blot analyses revealed the same ~65-kDa NOX1 protein band of the expected size<sup>29</sup> in homogenates of mouse kidney and colon (Figure 4a). Western blot analysis using an antibody raised against the COOH terminus region of mouse NOX4 revealed a major ~65-kDa band similar to NOX1, and a minor 28-kDa band in the colon homogenate (Figure 4b). In sharp contrast, the 28-kDa NOX4 was the major protein band detected in the kidney homogenate, while the 65-kDa NOX4 remained barely detectable (Figure 4b), suggesting that the 28-kDa NOX4 is the main NOX4 isoform expressed in the mouse kidney. Four human *NOX4* splice variants have been identified, including a NOX4 D variant lacking exons 3-11 with a predicted molecular weight of 28-kDa.<sup>30</sup>



We identified the full length *Nox4* (encoding for the 65-kDa NOX4 protein) and a similar *Nox4* variant in the mouse kidney (Supplementary Figure S3a), and in the murine proximal tubule PKSV-PR cells using different sets of primers within mouse NOX4 exons 1 and 14 (Supplementary Figure S3b). The fact that TLR4 was shown to interact with human NOX4 in TLR4-transfected HEK 293T cells<sup>24</sup> led us to test whether endogenous mouse TLR4 interacted with the 28-kDa NOX4, and whether the 28-kDa NOX4 interacted with gp96. TLR4 co-immunoprecipitated with the 28-kDa NOX4 and gp96 in non-hypoxic and post-hypoxic wild-type RTEC lysates (Figure 4c and d). The amount of gp96 and NOX4 bound to TLR4 were greater in post-hypoxic lysates than in non-hypoxic lysates, while the amount of TLR4 bound to immunoprecipitated gp96 remained unchanged or only moderately increased in post-hypoxic RTECs (Figure 2c and 4e, left panel). These findings thus suggest that hypoxia stimulates the binding of gp96 and NOX4 to TLR4. gp96 co-immunoprecipitated with NOX4 in non- and post-hypoxic wild-type RTECs (Figure 4e, left panel), but, in sharp contrast, gp96 no longer co-immunoprecipitated with NOX4 in *Tlr4*<sup>-/-</sup> RTECs (Figure 4e, right panel), indicating that the 28-kDa NOX4 isoform does not directly interact with gp96. Co-IP also confirmed that TLR4 co-immunoprecipitated with both 28-kDa NOX4 and gp96 in non-hypoxic and post-hypoxic wild-type RTECs (Figure 4f), suggesting that they form a trimeric complex (Figure 4g).

We next analyzed the expression of NOX4 compared to that of NOX1 in post-ischemic wild-type kidneys. One and two days after IRI, the amount of the 28-kDa NOX4 isoform had increased whereas that of NOX1 had decreased (Figure 4h). The up-regulation of the 28-kDa NOX4 appears to be closely related to TLR4-dependent renal injury, since the increase in NOX4 was not detected in post-ischemic *Tlr4*<sup>-/-</sup> kidneys (Figure 4h). Immunohistochemical analyses revealed cellular punctuate immunostaining of NOX1 evenly distributed in all tubule cell sections, and NOX4 immunostaining preferentially restricted to some tubule cell sections (Figure 4i, upper panels). 48 h after ischemia, NOX1 was barely detectable in tubule cell sections, whereas intense NOX4 labeling was detected in all tubule cell sections from the

medulla (Figure 4i, lower panels). I/R induces potent inflammatory response, and the influx of neutrophils and macrophages into injured kidneys.<sup>7,9</sup> NOX2, which is expressed in neutrophils,<sup>20,21</sup> but not in RTECs, greatly increased following I/R injury in wild-type kidneys, but not in post-ischemic *Tlr4*<sup>-/-</sup> kidneys (Supplementary Figure S4a). However, NOX2 did not seem to play any significant role in ischemia-induced apoptosis, since the number of TUNEL-positive cells did not significantly differ in post-ischemic *Nox2*<sup>-/-</sup> and wild-type kidneys (Supplementary Figure S4b). These findings indicate that the renal 28-kDa NOX4 spliced isoform is up-regulated after IRI, suggesting that this NOX4 isoform is involved in the induction of tubule cell apoptosis in injured kidneys after IRI.

**Interaction between TLR4 and 28-kDa NOX4 is required for ROS production and for**

**apoptosis in post-hypoxic renal tubule cells.**

The interaction between TLR4 and NOX4 were shown to be essential for the LPS-induced production of ROS to occur.<sup>24</sup> The question arises as to whether the 28-kDa NOX4 isoform identified in the mouse kidney might play a role in TLR4-mediated ROS production and apoptosis during IRI. NOX4 is considered to be a constitutive reactive oxygen species (ROS)-generating NAD(P)H oxidase.<sup>20,31</sup> However, the ability of the 28-kDa NOX4 variant to generate ROS remains elusive. Experiments were carried out on post-hypoxic wild-type RTECs transfected or not with various *Nox4* siRNAs or a control siRNA to further assess the role of the 28-kDa NOX4 isoform in TLR4-mediated apoptosis. Silencing both the full length 65-kDa and 28-kDa *Nox4* mRNAs using two siRNAs that target the sequences of exons 1 and 3 (designated as *Nox4*<sub>65-28</sub> siRNA) inhibited by more than 80% the increase in both 28- and 65-kDa NOX4 mRNA and protein expressions (Figure 5a and b, left panels), ROS production and apoptosis caused by hypoxia (Figure 5c). Similarly, silencing the 28-kDa *Nox4* isoform using a siRNA directed against a target sequence overlapping exons 2 and 12 (designated as *Nox4*<sub>28</sub> siRNA) (Figure 5a, right panel) which selectively blunted the expression of the 28-kDa NOX4 protein by about 60 % (Figure 5a and b, right panel), led to a

almost complete inhibition of ROS production and about 80% inhibition of apoptosis in post-hypoxic RTECs (Figure 5c). Consistent with these findings, transfecting cells with various concentrations of the *NOX4*<sub>28</sub> siRNA led to a concentration-dependent inhibition in both ROS production and apoptosis caused by hypoxia (see Supplementary Figure S2). These data further suggest that the 28-kDa NOX4 isoform is required in the induction of TLR4-mediated apoptosis in post-hypoxic RTECs.

**NOX4 controls JNK-mediated renal tubule cell apoptosis induced by hypoxia.** We next investigated the role of the 28-kDa NOX4 in inducing the TRAF2/ASK1/JNK pro-apoptotic pathway in post-hypoxic, wild-type RTECs. As in post-ischemic kidneys, the amount of the 28-kDa NOX4, but not that of NOX1, increased significantly in the 24 h post-hypoxic, wild-type RTECs, but not in post-hypoxic *Tlr4*<sup>-/-</sup> or *MyD88*<sup>-/-</sup> RTECs (Figure 6a). The *Nox4*<sub>28</sub> siRNA did not impair the expression levels of TRAF2 in post-hypoxic, wild-type RTECs, but did reduce the hypoxia-induced p-ASK1 and p-JNK activation in post-hypoxic RTECs (Figure 6b). In contrast, the stimulation of NOX4, p-ASK1, p-JNK, ROS production, and apoptosis were not altered in post-hypoxic *Nox1*<sup>-/-</sup> RTECs (Supplementary Figure S5). Wild-type RTECs were then incubated with the JNK inhibitor SP600125 to test whether JNK could reciprocally regulate NOX4 and inhibit apoptosis. Pre-incubating cells with SP600125 (2 μM), which did not affect the activation of the other MAPK kinases (Supplementary Figure S6a), reduced the activation of the c-Jun, the substrate of JNK, and NOX4 (Figure 6c). Moreover, SP600125 significantly inhibited ROS production and the percentage of apoptotic cells, respectively, caused by hypoxia (Figure 6d and e). Altogether, these findings indicate that NOX4 controls the activation of the JNK/ASK1 pro-apoptotic pathway in post-hypoxic RTECs.

**Silencing NOX4 impairs the activation of p38 in post-hypoxic renal tubule cells.** Because hypoxia induced the activation of p-p38, together with that of p-ASK1 and p-JNK, in post-

hypoxic RTECs, the question arises as to whether NOX4 also controls the activation of p38. Silencing *Nox4*<sub>28</sub> siRNA expression, in addition to its inhibitory action on p-ASK1 and p-JNK, fully inhibited the activation of p-p38 in post-hypoxic wild-type RTECs (Figure 7a). We then tested the consequence of p38 activity inhibition on ROS production and apoptosis. RTECs were incubated with 10  $\mu$ M of the selective p38 inhibitor SB203580, which prevented the activation of the p38 substrate MAPKAPK2 (Figure 7b) without affecting the activation of the other MAPK kinases (Supplementary Figure S6b). SB203580 significantly reduced the hypoxia-induced ROS production (Figure 7c), but had only a borderline significant inhibitory action on apoptosis in post-hypoxic RTECs (Figure 7d). Because the selective inhibition of p38 did not impair the activation of p-JNK, we tested whether the inhibition of both p38 and JNK activity could fully impair cell apoptosis induced by hypoxia. Pre-incubation of wild-type RTECs with 2  $\mu$ M SP600125 plus 10  $\mu$ M SB203580 was more efficient than SP600125 alone or SB203580 alone to inhibit apoptosis in post-hypoxic wild-type RTECs (Figure 6e and Figure 7d). Collectively, these findings indicated that induction of apoptosis in post-hypoxic RTECs results from the combined activation of ASK1 and both JNK and p38 MAPKs.

## Discussion

Ischemia/reperfusion injury induces the activation of the innate immune response that leads to a rapid inflammatory response and apoptosis. Previous studies indicate that both TLR4 and TLR2 play central roles in the pathogenesis of renal IRI,<sup>7-9</sup> and that TLR4/MyD88 signaling contributes to the induction of RTEC apoptosis.<sup>9</sup> Here, we show that TLR4 complexed with gp96, mediates the pro-apoptotic TRAF2/ASK1/JNK pathway in post-hypoxic RTECs. We also found that the non-phagocytic 28-kDa NOX4 isoform, which interacts with TLR4, plays a

central role in controlling TLR4-mediated ROS production and apoptosis of post-hypoxic RTECs.

The accumulation of unfolded proteins in the ER, as a result of ER stress, induces a compensatory unfolded protein response (UPR) to permit cell survival, but if this response is not controlled properly it can also trigger cell death.<sup>32</sup> The accumulation of misfolded proteins, like that of other Hsps, stimulates the production of gp96,<sup>33</sup> although not essential for cell viability,<sup>27</sup> controls the proper folding and export of most of the TLRs, including TLR4, in a pre-B cell line and macrophages.<sup>13,27</sup> We show that TLR4 co-immunoprecipitated with both gp96 and NOX4, and that hypoxia triggered the binding of TRAF2 to ASK1. Furthermore, silencing *gp96* or *Traf2* mRNA expression both impaired the up-regulation of p-ASK1 and p-JNK, ROS production and the induction of RTEC apoptosis caused by hypoxia. Overall, these findings strongly suggest that the TLR4-gp96 complex is required for the proper transduction of the TLR4-mediated TRAF2/ASK1/JNK pathway in post-hypoxic renal cells.

There is growing evidence that ROS are important intracellular intermediates involved in regulating cytokine expression, cell proliferation, and apoptosis. ROS can be produced by the mitochondrial respiratory chain, xanthine oxidase, xanthine-oxygenase, lipoxygenase, and NOX enzymes in stressed cells.<sup>34</sup> Functional TLR4 signaling has been shown to be required for ROS production, and subsequent release of endogenous HMGB1 in hepatocytes subjected to hypoxia.<sup>4</sup> Here, we show that the 28-kDa NOX4 isoform, similar to the human NOX4 D variant previously identified in human lung A549 cells and lung tissue,<sup>30</sup> represents the main NOX4 isoform expressed in mouse RTECs. The fact that ROS production is significantly reduced in transfected post-hypoxic wild-type RTECs with the selected *Nox4*<sub>28</sub> siRNA, which targets the 28-kDa NOX4 isoform, indirectly suggests that this NOX4 isoform is involved in the ROS production induced by hypoxia. Human NOX4 has been shown to be a constitutive ROS-generating enzyme that requires the membrane-associated subunit p22<sup>phox</sup>,<sup>35</sup> but, unlike NOX1, NOX2 or NOX3, does not require the presence of organizers NOXAs and NOXOs

subunits.<sup>20</sup> NOX4 is thought to be an inducible NOX isoform regulated at the mRNA level.<sup>31</sup> NOX4 expression has been shown to be increased by transforming growth factor- $\beta$ 1 in human pulmonary smooth muscle cells and by angiotensin II in mesangial cells (reviewed in ref 20). Here we show that the mouse 28-kDa NOX4 isoform is upregulated in post-ischemic kidneys and post-hypoxic RTECs. However, the exact mechanism by which this NOX4 variant, which lacks most transmembrane domains, interferes in ROS production remains to be determined. Furthermore, it may not in fact directly control the production of ROS induced by hypoxia. LPS-induced ROS generation and NF- $\kappa$ B activation have been shown to be mediated by direct interaction between the COOH-terminus region of NOX4 and the cytoplasmic tail of TLR4.<sup>24</sup> Because the endogenous 28-kDa NOX4 isoform also co-immunoprecipitated with TLR4 in RTECs, the lack of ROS production and apoptosis observed after silencing the 28-kDa NOX4 may reflect the efficient protection of RTECs against hypoxia due to the absence of TLR4-mediated activation, and the subsequent activation of ROS produced by pathways other than that involving ROS-generating NOX4. NOX4 which is complexed with TLR4, but not with gp96, appears to be an important effector of the ASK1/JNK pathway that is activated in response to hypoxia-induced ER stress. The fact that JNK exerts reciprocal control over NOX4, ROS production and apoptosis in post-hypoxic RTECs, further confirms the role of the TLR4-mediated TRAF2/ASK1/JNK pathway in controlling apoptosis.

Matsuzawa et al.<sup>19</sup> demonstrated that the ROS-dependent activation of the TRAF6/ASK1/p38 pathway is required in TLR4-mediated cell activation. Here we show that p-p38 is activated in post-hypoxic RTECs, and to a lesser extent in post-ischemic intact kidneys. We also show that silencing *NOX4*<sub>28</sub> mRNA expression inhibited both the activation of p-ASK1, p-JNK and p-p38 in post-hypoxic RTECs, suggesting that NOX4 controls the ASK1 downstream effectors p-38 and JNK. In accordance with these findings, selective inhibition of the two JNK and p38 MAPKs leads to marked inhibition in cell apoptosis caused by hypoxia. In vivo administration of the anti-oxidant selenite was reported to impair the rapid

activation of ASK1/MAKK3/p38 signaling cascade and RTECs apoptosis 3 h after the reperfusion of adult male rat kidneys subjected to ischemia for 45 min.<sup>36</sup> Because selenite inhibited the activation of p-ASK1, it cannot be excluded that this antioxidant agent, in addition to its inhibitory action on p38, also inhibited the activation of p-JNK during renal IRI. However, the effect of selenite on JNK activation was not investigated in this study. In the present work, a 30 min clamping of the renal pedicles induced only a discrete activation of p-p38 in the 24 h post-hypoxic kidneys from female mice. However, the activation of p-p38 was detected in the 24 h post-hypoxic wild-type RTECs following addition of mineral oil for 30 min. Therefore, the apparent differences observed in the activation of p-p38 may reflect differences in the time course of MAPK activation during IRI, and in cell susceptibility to hypoxia between the *in vivo* and *in vitro* experimental conditions. They also may reflect the differences in susceptibility between male and female animals to renal IRI, since kidneys of males exhibit much higher susceptibility to I/R than females, due to the presence of testosterone.<sup>37</sup>

NOX1 has also been shown to cause JNK activation, and the subsequent cell death of cultured mouse, alveolar type-II, epithelial cells.<sup>38</sup> In contrast, renal IRI leads to a decrease in NOX1, and experiments using *Nox1*<sup>-/-</sup> RTECs clearly show that the extinction of *Nox1* mRNA does not affect the activation of p-ASK1 and p-JNK in post-hypoxic RTECs, suggesting that NOX1- and NOX4-mediated cell apoptosis are tissue specific.

Although TLR4 downstream signaling plays an important role in the control of apoptosis during IRI, we and others have reported that ischemic kidneys from *Tlr2*<sup>-/-</sup> mice are also better preserved than ischemic wild-type kidneys to almost the same extent as those of *Tlr4*<sup>-/-</sup> mice.<sup>8-10</sup> However, the TLR2 and TLR4 downstream signaling pathways engaged during IRI are probably different. For example, the activation of ERK1/2, which depends on the dissociation of the gp96/PP5 complex, occurs in post-hypoxic wild-type and *Tlr4*<sup>-/-</sup> RTECs, but not in post-hypoxic *Tlr2*<sup>-/-</sup> RTECs.<sup>13</sup> To conclude, this study has revealed that gp96 and the 28-

kDa NOX4 isoform, both of which interact with TLR4, are involved in TLR4-mediated apoptosis in post-hypoxic RTECs. The identification of NOX4 as a sensor in the ER stress-induced, TLR4-mediated, pro-apoptotic signaling cascade has important implications for selective anti-superoxide-generating enzyme strategies intended to prevent the apoptosis of ER stress-induced RTECs that may contribute to the deterioration of renal function.

## **Materials and Methods**

**Mice.** Pathogen-free, 10-12 week-old female wild-type C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, MA, USA). *Tlr4*<sup>-/-</sup> and *MyD88*<sup>-/-</sup> null mice (originally obtained from S. Akira, Osaka University, Osaka, Japan), and *Nox1*<sup>-/-</sup> knockout mice<sup>39</sup> kindly provided by K. H. Krause (Geneva University, Switzerland) were also used. All the mice were further backcrossed at least eight times with C57BL/6J mice to ensure that they all had similar genetic backgrounds. All experiments were conducted in accordance with the guidelines of the French Agricultural Office, and in compliance with the legislation governing animal studies.

**Renal ischemia/reperfusion in mice.** Renal I/R injury was induced as previously described, but with some slight modifications.<sup>7,13</sup> Mice were anesthetized by the intraperitoneal injection of 2,2,2-Tribromoethanol (5 mg/mouse) (Sigma-Aldrich, S<sup>t</sup> Louis, MO). The kidneys were exposed by bilateral lumbar incisions, and the renal pedicles were clamped with nontraumatic microvascular clamps for 30 min. The clamps were then removed, and the lumbar incisions were sutured after checking that the blood flow had been restored. Sham-operated mice (referred as Controls) underwent the same procedure, except that the renal vessels were not clamped. Surgery was performed on a homeothermic table to maintain a body temperature between 36.5°C and 38°C. All mice received 200 µl of sterile 0.9% NaCl dripped onto the lumbar incisions to keep the tissue moist. Mice (6-10 mice per group) were sacrificed 1, 2 and



7 days after surgery. Before sacrifice, a blood sample was collected, and the level of serum creatinine was determined using an Olympus AU400 autoanalyzer.

**Cultured Cells.** Experiments were carried out on primary cultured RTECs isolated from the kidneys of naive, wild-type mice, *Tlr4*<sup>-/-</sup> or *MyD88*<sup>-/-</sup> mice using the same procedure as previously described.<sup>13</sup> Experiments were also carried out on established mouse PKSV-PR cells exhibiting features of proximal tubule cells.<sup>28</sup> Hypoxia was induced by immersing RTECs layers in mineral oil as described.<sup>7,13</sup> After replacing with fresh medium, 1 ml sterile mineral oil (Sigma-Aldrich) was added for 30 min (for primary cultured RTECs) or 2 h (for PKSV-PR cells) at 37°C. The mineral oil was then removed by rinsing twice with fresh medium, and the cells were then incubated at 37°C for a further 24 h before being analyzed. Cells were labeled with an active caspase-3 antibody (Promega, Charbonnières, France) to estimate the percentage of apoptotic cells determined on 2 or 3 enlarged fields (x 200) from 3 to 5 separate cultures derived from 2 to 3 different wild-type and/or transgenic mice kidneys for each condition tested. All reagents, media, and buffers used in the assays were checked by using the *Limulus* amoebocyte gelation activity test (BioWhittaker Inc., Walkersville, MD) to ensure that they had not been contaminated by endotoxin.

**Immunohistochemical studies.** Immunohistochemical studies were performed on deparaffinized kidney sections using antibodies raised against mouse NOX1 and NOX4 or activated caspase-3 (Promega, Charbonnières, France) using avidin-biotin blocking, alkaline phosphatase and peroxidase substrate kits (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. To generate antibodies to NOX1 and NOX4, the synthetic peptides <sup>332</sup>KFQRADNKLKTQKIY<sup>346</sup> (NOX1) and <sup>564</sup>NSYGTKFEYNKESFS<sup>578</sup> (NOX4) were used as immunogens, and the antisera obtained from immunized rabbits were affinity-purified using the corresponding synthetic peptides.

**TUNEL.** The number of apoptotic cells analyzed in kidney tissue sections fixed in Dubosc-Brazil solution was determined by the terminal deoxyribonucleotide transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method using the Apop Tag kit (Chemicon Int., Temecula, CA) according to the manufacturer's instructions. The percentage of apoptotic cells was determined on 2 or 3 enlarged fields (x 200) from 2-3 different tissue sections from 3-5 kidneys for each condition tested.<sup>13</sup>

**Small interfering RNA.** Experiments were performed using predesigned HP GenomeWide (Qiagen, Courtaboeuf, France) siRNAs for the murine *gp96* gene (5'-ATGAATGATATCAAACCAATA-3'; sense: GAAUGAUAUCAAAACCAAUAdTdT; antisense: UAUUGGUUUGAUAUCAUUCdAdT), the murine *Traf2* gene (5'-ACGGAGTGTCTGCATGTAAA-3'; sense: GGAGUGUCCUGCAUGUAAATT; antisense: UUUACAUGCAGGACACUCCGT), the murine *Nox4* variant (*Nox4<sub>28</sub>*) encoding for only the 28-kDa NOX4 (5'-AATGTTGGGCTGTCCTACTGA-3'; sense: UGUUGGGCUGUCCUACUGAdTdT; antisense: UCAGUAGGACAGCCCAACAdTdT). For the murine *Nox4* gene encoding both the full length 65-kDa and the 28-kDa isoforms (*Nox4<sub>65-28</sub>*), two siRNAs were used to target two sequences (5'-AACGAAGGGGTTAAACACCTC-3' and 5'-AAAAGCAAGACTCTACACATC-3') (Ildiko Szanto, Geneva, Switzerland). A universal negative control siRNA (target DNA sequence: AATTCTCCGAACGTGTCACGT; sense: UUCUCCGAACGUGUCACGUdTdT; antisense: ACGUGACACGUUCGGAGAAAdTdT) was also used (Qiagen). Single strand sense and antisense RNA nucleotides were annealed (90°C for 1 min, and then 37°C for 1 h) to generate a RNA duplex according to the manufacturer's instructions. Subconfluent cultures of RTECs (day 3-5 after seeding) grown in 48-well plates were incubated with 1 to 6 pmol of each siRNA

tested and 2  $\mu$ l of INTERFERin<sup>TM</sup> prepared according to the manufacturer's instructions (Polyplus-Transfection Inc., San Marcos, CA) for 48 h at 37°C before use.

**ROS production assays.** The production of ROS produced by intact RTECs was also measured by imaging microscopy.<sup>26</sup> Untreated and post-hypoxic RTECs plated on Lab-Tek<sup>®</sup> chambered coverglass slides (Nalge Nunc International, Roskilde, Denmark) were loaded with 50  $\mu$ M 2',7'-dichlorofluorescein-diacetate (DCFH-DA) for 30 min. Cells were then rinsed and after excitation at 488 nm, the green fluorescence of DCF was measured by confocal laser scanning microscopy (CLSM-510-META, Carl Zeiss, Le Pecq, France) equipped with cell culture chamber at 37°C and under an atmosphere containing 5% CO<sub>2</sub>. When used, DPI (2  $\mu$ M) was added 10 min before loading the DCFH-DA.

**Immunoblot and immunoprecipitation analyses.** Frozen kidney samples and cultured RTECs grown in 48-well plates were lysed in 50  $\mu$ l ice-cold lysis buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, and then sonicated for 15 s at 4°C. For each condition tested, cell lysates from 8 separate wells were pooled. Samples (25-50  $\mu$ g/lane) were electrophoresed using 10% SDS-PAGE, and analyzed by immunoblot.<sup>40</sup> Antibodies against TRAF2, p-ASK1, p38, p-p38, p-JNK, p-c-jun, c-jun, MAPKAPK2, p-MAPKAPK2 (Cell Signaling Technology, Danvers, MA), ASK1, JNK (Santa Cruz Biotechnology Inc. Santa Cruz, CA),  $\beta$ -actin (Sigma Aldrich), BAX (Upstate Chemicon, Waltham, MA), Bcl-2 (BD Transduction Laboratories, Lexington, KI), and gp96 (Stressgen, Ann Arbor, MI) were used to detect the corresponding antigens. For the immunoprecipitation studies, confluent cultures of non-hypoxic or day-1 post-hypoxic wild-type or *Tlr4*<sup>-/-</sup> RTECs were lysed, sonicated, centrifuged, and precleared with 75  $\mu$ l protein G Sepharose overnight at 4°C, as described.<sup>13</sup> Precleared supernatants were then sequentially incubated with antibodies against TLR4 (5  $\mu$ g/ml), anti-gp96 (12  $\mu$ g/ml), or ASK1 (10  $\mu$ g/ml) for 2 h at 4°C, and then with 50  $\mu$ l Protein

G Sepharose overnight at 4°C. Bound proteins were rinsed twice in lysis buffers (Roche Diagnostics), and Sepharose-bound proteins were separated on 8%-15% SDS acrylamide gels. After being transferred onto nitrocellulose, the membranes were incubated with antibodies directed against gp96 (1:2000), TRAF2 (1:1000), TLR4 (1:200), and/or NOX4 (1:500), and then with a peroxidase-conjugated secondary antibody. Labeled bands were detected using the ECL+ Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK).

**Reverse transcription PCR.** Total RNA was extracted from confluent cultures of RTECs using the RNeasy mini kit according to the manufacturer's instructions, and reverse-transcribed using Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen, Cergy-Pontoise, France). cDNA and non-reverse transcribed RNA (250 ng) from cultured cells were amplified for 35 cycles in 40 µl total PCR buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4) containing 100 µM dNTP, 1 or 1.5 mM MgCl<sub>2</sub>, 1 U Taq polymerase, 10 pmol *Nox4*<sub>65-28</sub> (NM 015760.4: nt 43-64, nt 1300-1278), 10 pmol *Nox4*<sub>28</sub> (NM 015760: nt 43-64, nt 1137-194) or 10 pmol *GAPDH* (AK 144690: nt 307-327, nt 663-643). The thermal cycling program was 94°C for 30 s, 56°C for 30 s, and then 72°C for 1 min. Amplification products were run on a 2% agarose gel, and then stained with ethidium bromide and autoradiographed.

**Statistical analysis.** Values are given as means ± S.E.M. Data are representative of at least three independent experiments. Statistical differences between groups were analyzed using the Student's *t*-test. A *P* value of less than 0.05 was considered significant.

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## FIGURE LEGENDS

**Figure 1** TLR4-dependent signaling in ischemic, reperfused kidneys and in post-hypoxic renal tubule cells. Immunoblot analysis of TRAF2 and  $\beta$ -actin, phospho (p-) and total ASK1, p38, JNK, BAX and Bcl-2, and  $\beta$ -actin labeled bands in control (C), and day (D)-1, D-2, and D-7 post-ischemic wild-type and *Tlr4*<sup>-/-</sup> kidneys (a), and in non-hypoxic (C) and day-1 post-hypoxic (H) wild-type, *Tlr4*<sup>-/-</sup>, and *MyD88*<sup>-/-</sup> RTECs (b). Bars are the ratio values of densitometric analyses of TRAF2, BAX, and Bcl-2 over  $\beta$ -actin, and p- over total ASK1, p38, and JNK labeled bands. Values are means  $\pm$  S.E.M. from 3 to 4 independent experiments. \*  $P < 0.05$  versus control (C) values.

**Figure 2** gp96 interacts with TLR4 to produce the proper induction of ROS production and apoptosis in post-hypoxic renal tubule cells. (a) Immunoblot analyses of gp96 and the corresponding  $\beta$ -actin in non-hypoxic (C) and post-hypoxic (H) wild-type (WT) and *Tlr4*<sup>-/-</sup> RTECs. (b and c) Lysates from non-hypoxic (C) or 24 h post-hypoxic (H) WT RTECs were subjected to IP using an antibody against TLR4 (b) or gp96 (c). The IP material was then subjected to Western blot analysis, and proteins were detected with anti-gp96 or anti-TLR4 antibodies (b and c). As controls, Western blotting of non-immunoprecipitated cell lysates (None) revealed the presence of equal amounts of TLR4 or gp96 in the cell lysates subjected to immunoprecipitation. (d) Immunoblot analyses of gp96 and the corresponding  $\beta$ -actin in day-1 post-hypoxic (Hypoxia) WT RTECs that had or had not been transfected with a *gp96* siRNA or negative control siRNA. (e) Immunoblot (left panel) and densitometric analyses (right panel) (representative of 3 separated experiments) of TRAF2 and the corresponding  $\beta$ -actin, and phosphorylated (p-) over total ASK1 and JNK in day-1 post-hypoxic (Hypoxia) WT RTECs that had or had not been transfected with the *gp96* siRNA or negative control siRNA. (f, g)

DCF fluorescence recordings of ROS production (**f**), and percentage of apoptotic cells (**g**) in control and day-1 post-hypoxic (Hypoxia) wild-type RTECs that had or had not been transfected with the *gp96* siRNA or negative control siRNA. (**h**, **i**) DCF fluorescence recordings of ROS production (**h**), and percentage of apoptotic cells (**i**) in 24 h post-hypoxic (Hypoxia) wild-type, *Tlr4*<sup>-/-</sup>, and *Tlr2*<sup>-/-</sup> RTECs transfected or not with the *gp96* siRNA. Values are means ± S.E.M. from (n) individual cell recordings or cell counts performed on 2 to 3 separate cell cultures from 2 (**h**, **i**) to 3 (**f**, **g**) different kidneys for each condition tested. \* *P* < 0.05 between groups.

**Figure 3** TRAF2 controls phosphorylated ASK1 and JNK in post-hypoxic renal tubule cells. (**a**) Lysates from non-hypoxic (C) or 24 h post-hypoxic (H) wild-type RTECs were subjected to IP using an antibody against ASK1. The IP material was then subjected to Western blot analysis, and proteins were detected with an anti-TRAF2 antibody. (**b**) Immunoblot (left panel) and densitometric analyses (right panel) (representative of 2 separated experiments) of TRAF2 and NOX4, and corresponding β-actin, and phosphorylated (p-) over total ASK1 and JNK in day-1 post-hypoxic (Hypoxia) wild-type RTECs transfected or not with a specific *Traf2* siRNA or negative control siRNA. (**c**, **d**) DCF fluorescence recordings of ROS production (**c**), and percentage of apoptotic cells (**d**) in control and day-1 post-hypoxic (Hypoxia) wild-type RTECs that had or had not been transfected with a specific *Traf2* siRNA or negative control siRNA. Values are means ± S.E.M. from (n) individual cell recordings or cell counts performed on 2 separate cell cultures from 2 different kidneys for each condition. \* *P* < 0.05 between groups.

**Figure 4** Renal ischemia/reperfusion injury induces selective overexpression of NOX4 interacting with TLR4 in wild-type mice kidneys. (**a**, **b**) Immunoblot analysis of NOX1 (**a**) and NOX4 (**b**) in colon (C) and kidney (K) homogenates that had or had not been incubated with

an excess of the peptide used for rabbit immunization (K+pep). (c-f) Lysates from non-hypoxic (C) or 24 h post-hypoxic (H) wild-type (WT) RTECs or *Tlr4*<sup>-/-</sup> RTECs were subjected to IP using an antibody against TLR4 (c, d, f) or gp96 (e). The IP material was then subjected to Western blot analysis, and proteins were detected either with anti-NOX4 (c, e, f), anti-TLR4 (e), and/or anti-gp96 (d, f) antibodies. As controls, Western blotting were performed on non-immunoprecipitated cell lysates (None). (g) Schematic representation of the TLR4/gp96/NOX4 complex. (h) Immunoblot analysis of NOX1, NOX4, and  $\beta$ -actin in control (C) and day (D)-1, D-2 and D-7 post-ischemic wild-type and *Tlr4*<sup>-/-</sup> kidney homogenates. Bars are the ratio values (means  $\pm$  S.E.M. from 3 to 5 separate experiments) of densitometric analyses of NOX1 or NOX4 over  $\beta$ -actin labeled bands. \*  $P < 0.05$  versus control (C) values. (i) Patterns of NOX1 (arrows) and NOX4 (arrowheads) immunostaining in non-ischemic (Control, upper panels) and day-2 post-ischemic (I/R Day-2, lower panels) WT kidneys. As a control, no positive NOX4 immunostaining was detected in the day-2 post-ischemic kidney section incubated with an excess of the peptide used for immunization (i, inset). Bar = 10  $\mu$ m.

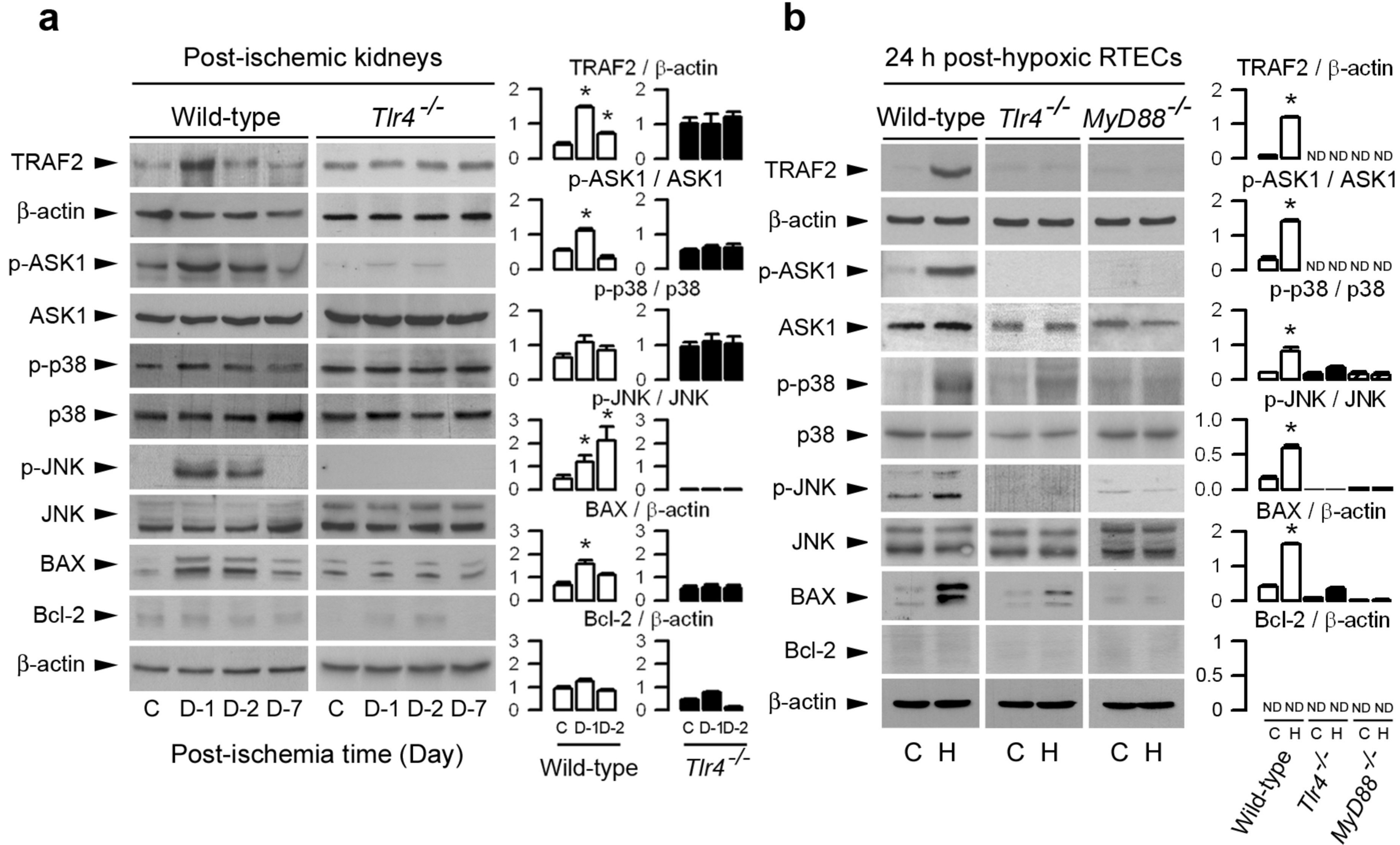
**Figure 5** The 28-kDa NOX4 interacts with TLR4 and controls apoptosis in post-hypoxic renal tubule epithelial cells. (a, b) RT-PCR analysis of the *Nox4* mRNA isoforms and corresponding *GAPDH* mRNA expression (a), and immunoblot analysis of the 65-kDa (b, upper panels) and 28-kDa NOX4 protein (b, middle panels) isoforms and corresponding  $\beta$ -actin (b, lower panels) in primary cultures of 24 h post-hypoxic wild-type RTECs that had or had not been transfected with specific *Nox4* siRNAs, which target sequences encoding either both the full length 65-kDa and the 28-kDa isoform (*Nox4*<sub>65-28</sub>) (left panels) or only the 28-kDa NOX4 isoform (*Nox4*<sub>28</sub>) (right panels). As controls, post-hypoxic RTECs were also transfected with a negative control siRNA (data are representative of 3 separate experiments). (c) DCF fluorescence recordings of ROS production (left panel) and percentage of apoptotic cells (right panel) in non-hypoxic (Control) and day-1 post-hypoxic (Hypoxia) wild-type RTECs

transfected or not with the *two Nox4* siRNAs, or with negative control siRNA. Values are means  $\pm$  S.E.M. from (n) individual cell recordings or cell counts performed on 2 separate cell cultures for each condition tested. \*  $P < 0.05$  between groups.

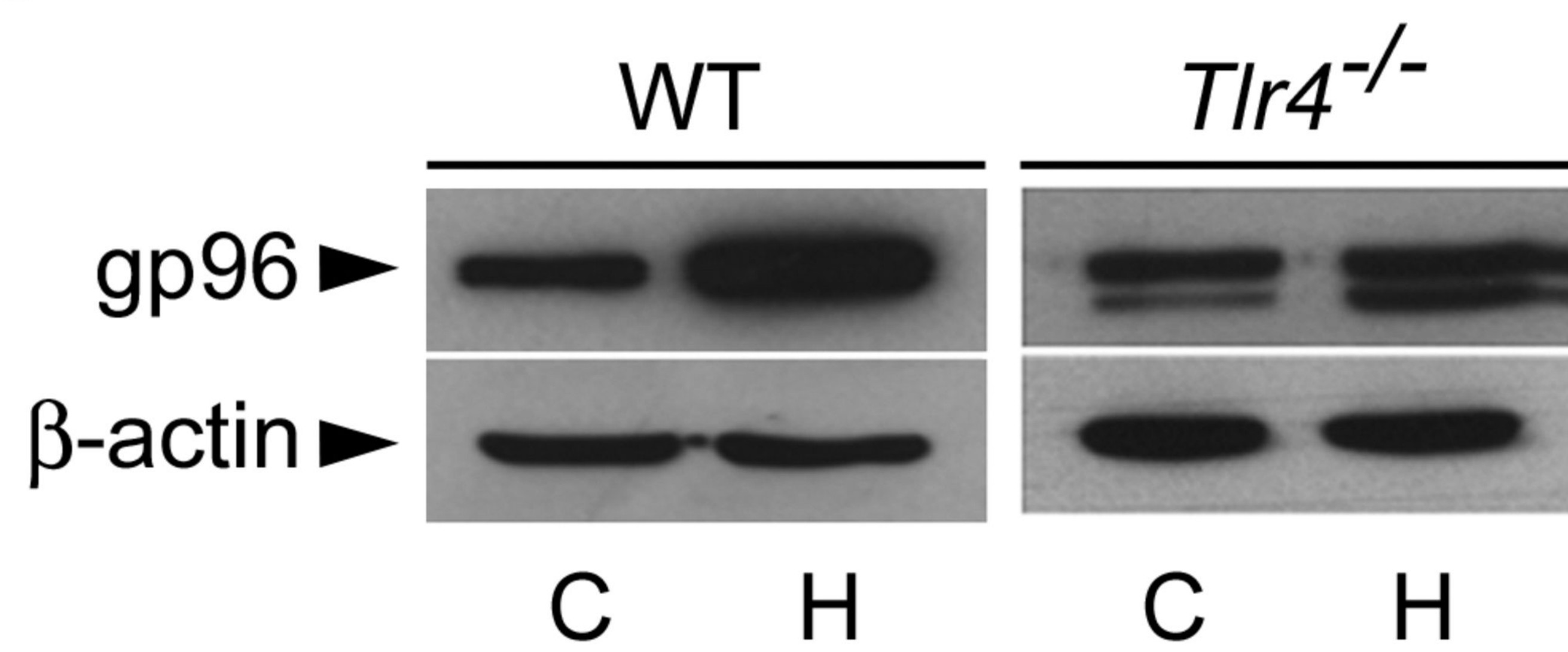
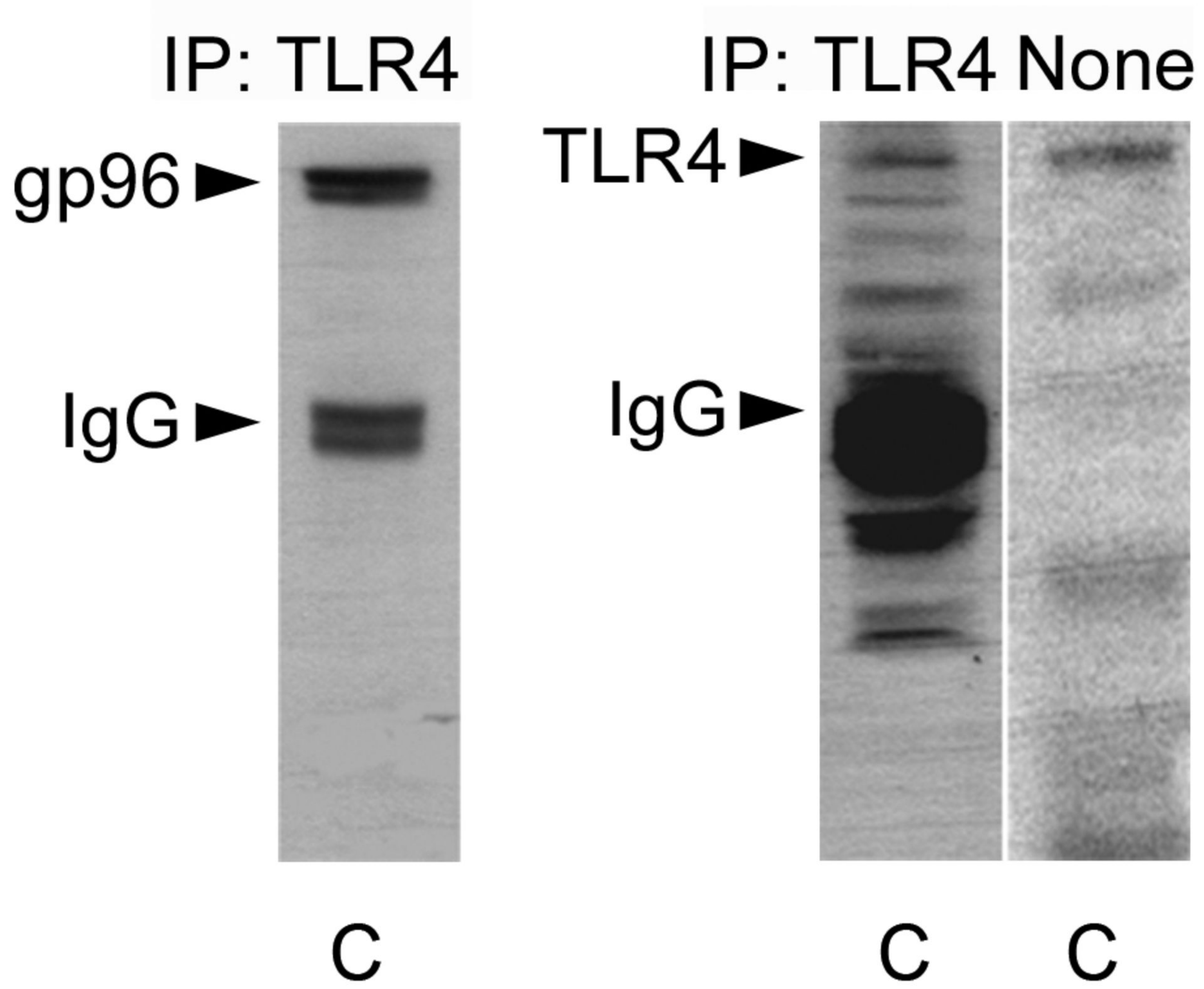
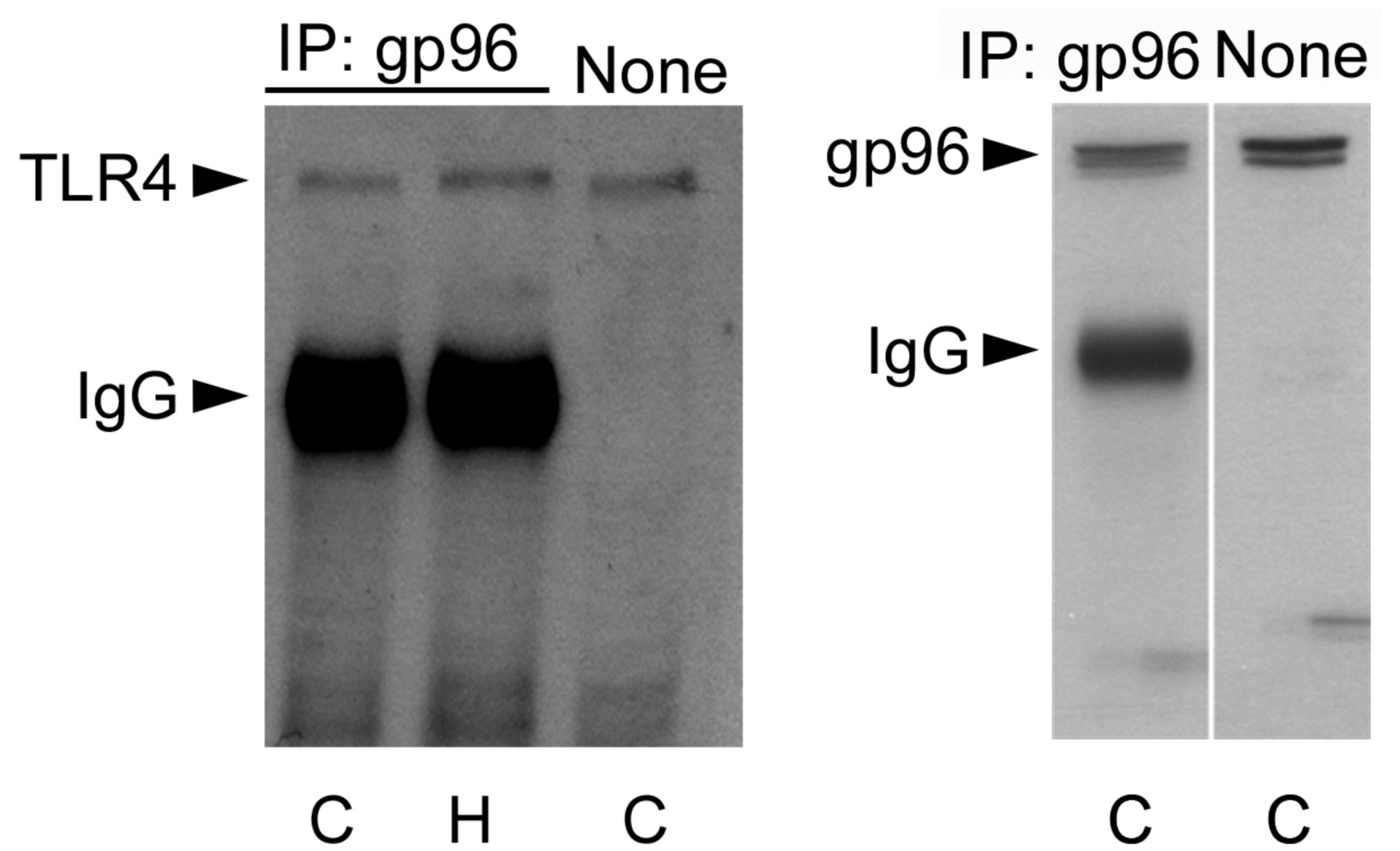
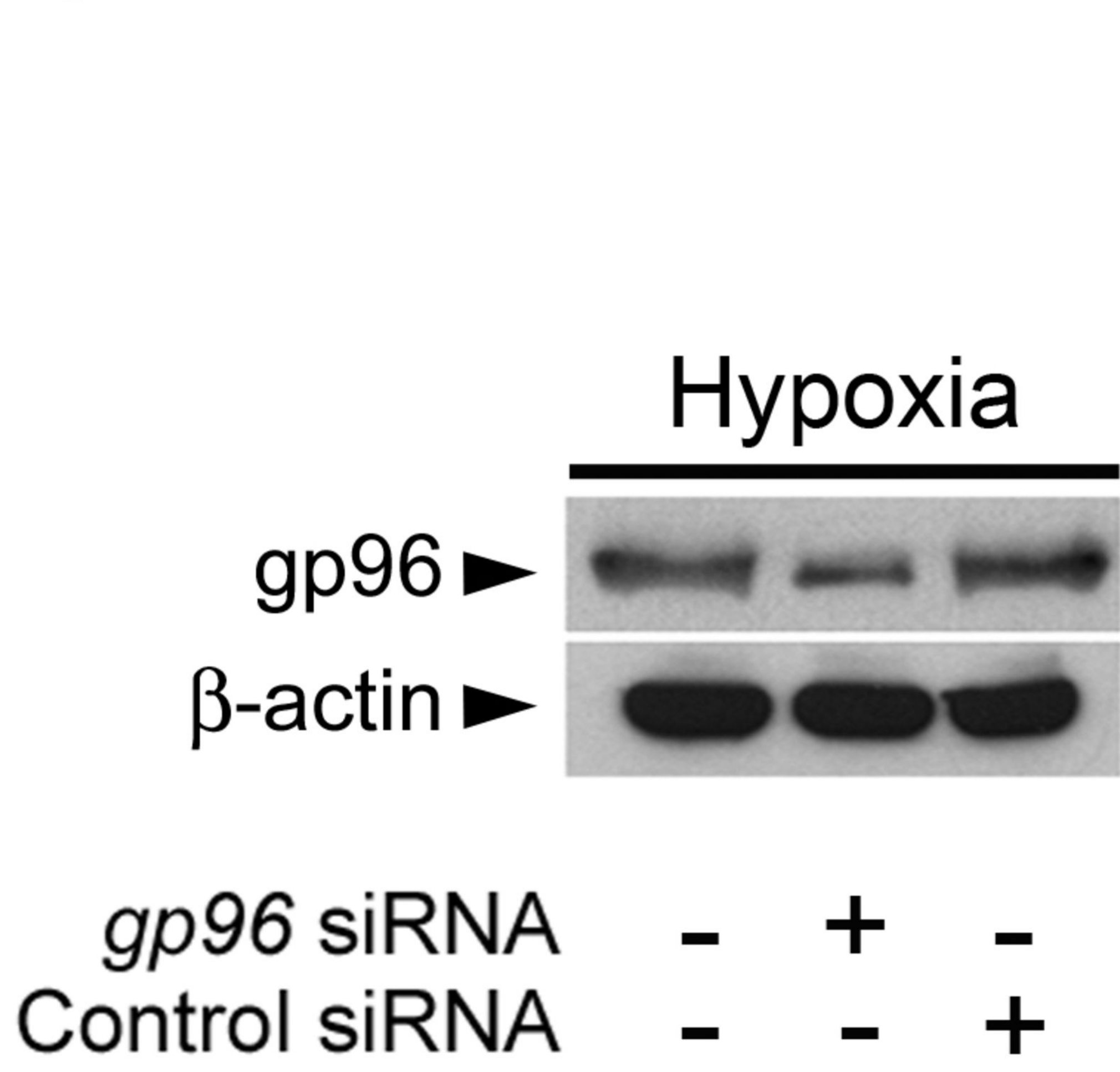
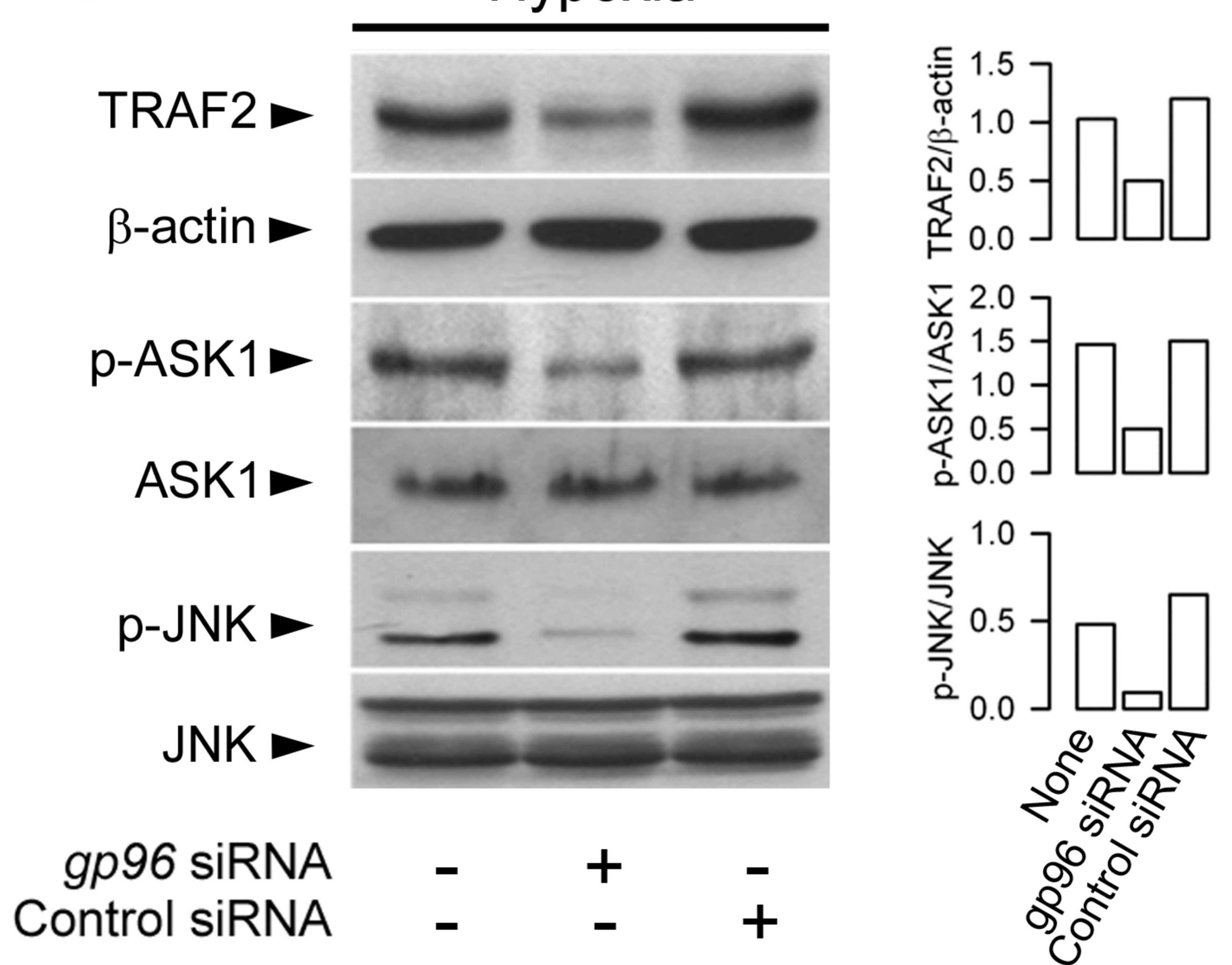
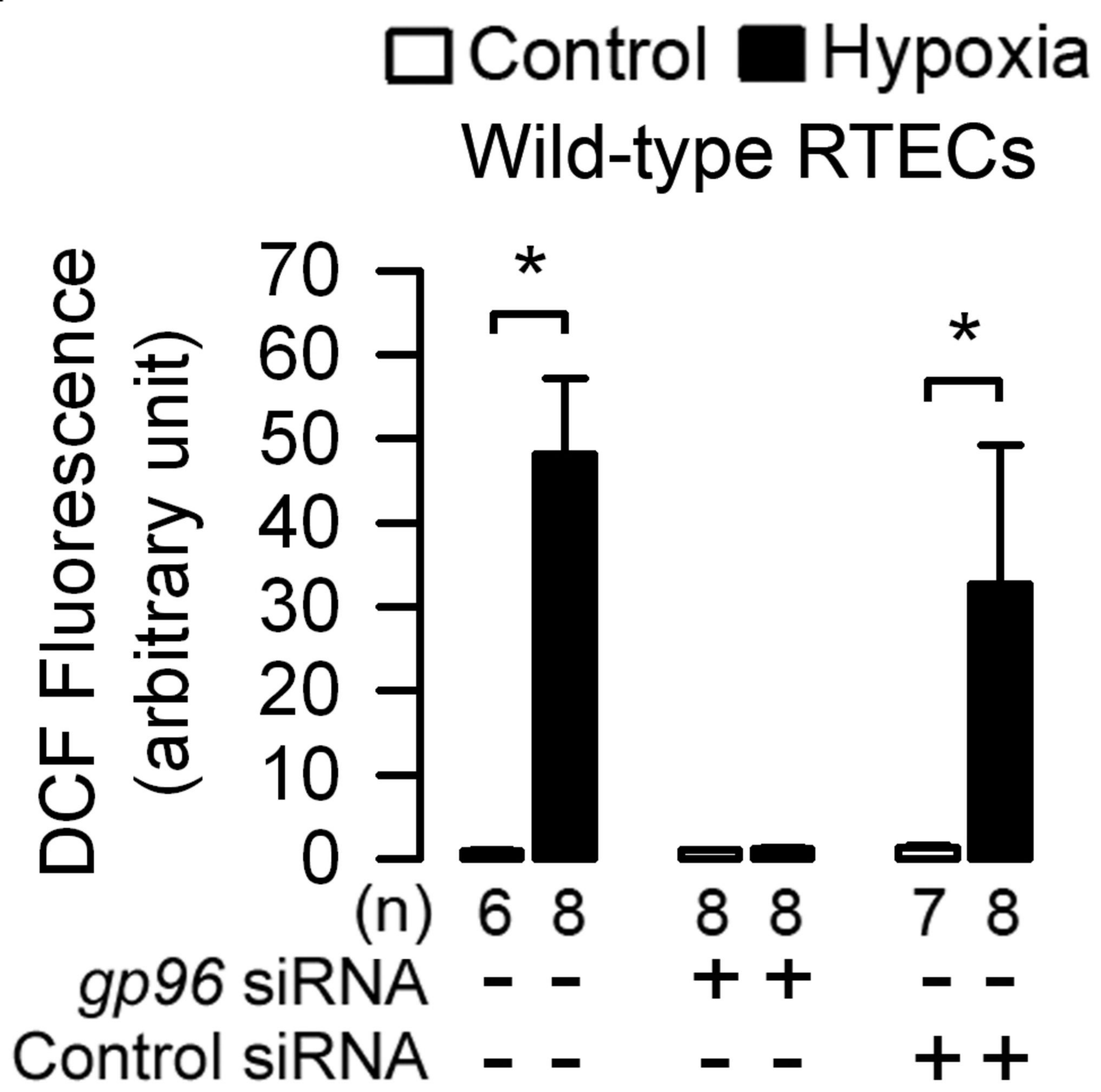
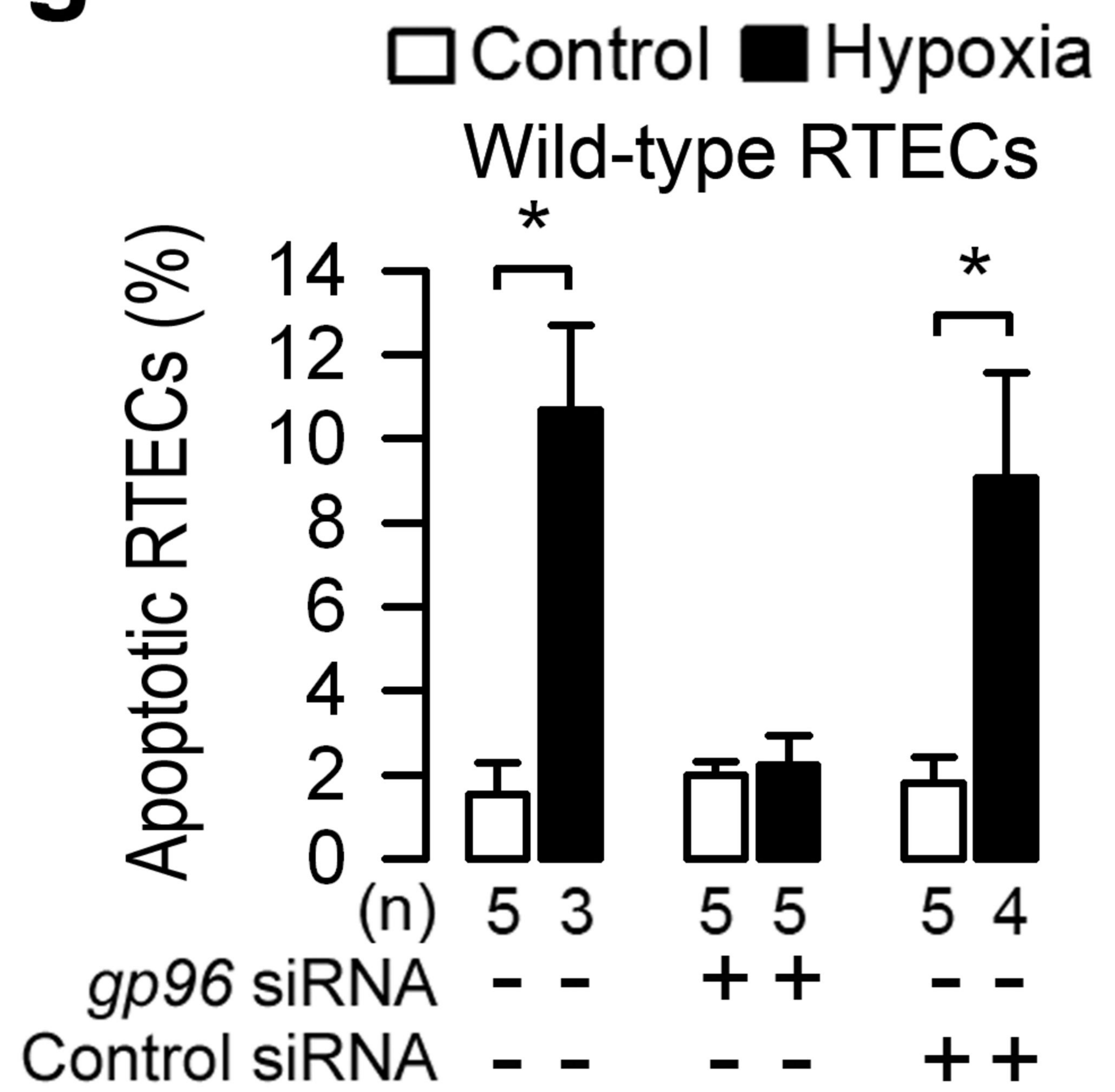
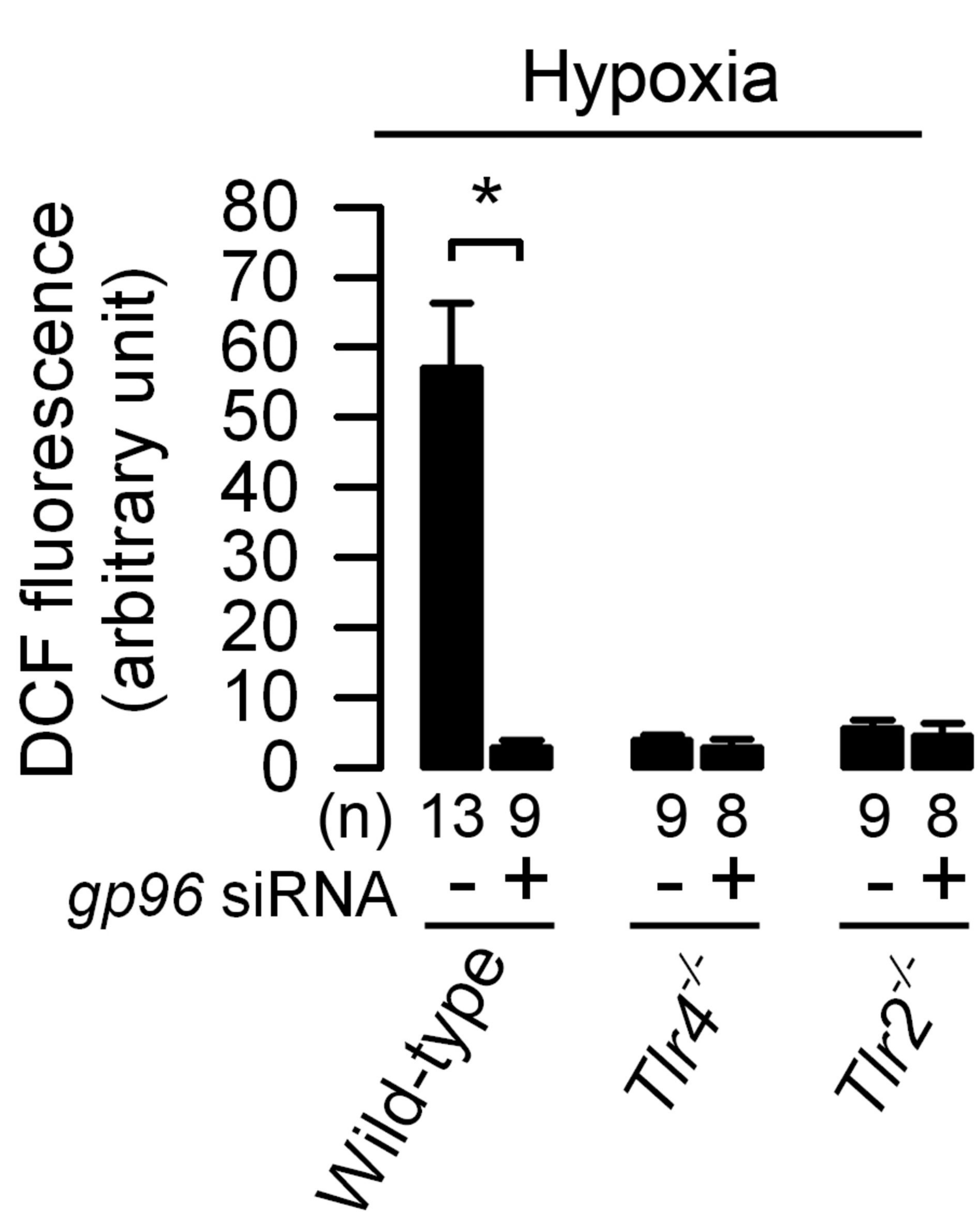
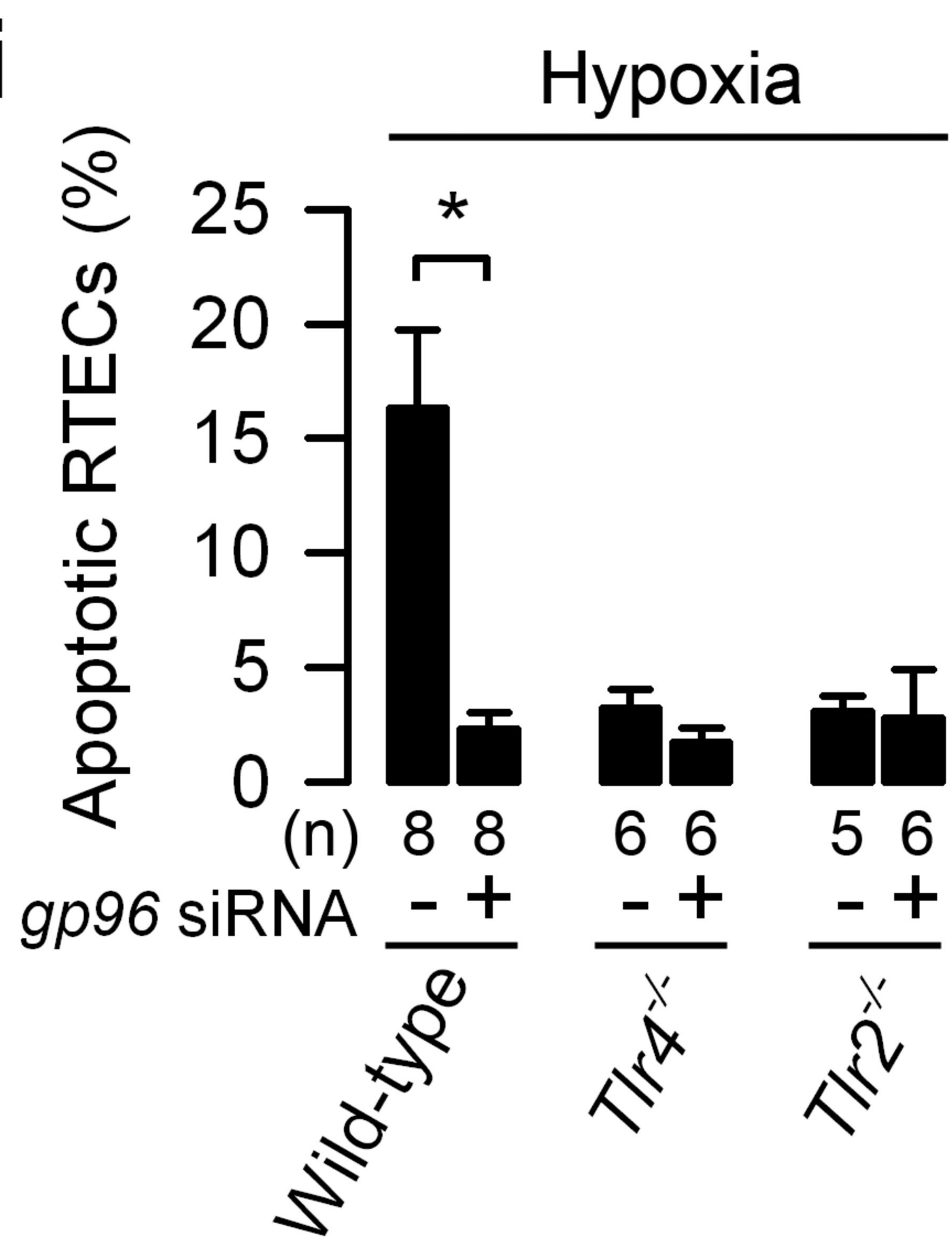
**Figure 6** Reciprocal control of 28-kDa NOX4 and JNK on ROS production and apoptosis in post-hypoxic renal tubule cells. **(a)** Immunoblot analysis (upper panel) and densitometric values (lower panel) (means  $\pm$  S.E.M. from 3 to 5 separate experiments) of NOX4, NOX1, and corresponding  $\beta$ -actin in control (C) and day-1 post-hypoxic (H) wild-type (WT), *Tlr4*<sup>-/-</sup>, and *MyD88*<sup>-/-</sup> RTECs. **(b)** Immunoblot (left panel) and densitometric analyses (right panel) (representative from 3 separate experiments) of TRAF2 and corresponding  $\beta$ -actin, p- and total ASK1 and JNK in control and day-1 post-hypoxic (Hypoxia) wild-type RTECs that had or had not been transfected with the *NOX4*<sub>28</sub> siRNA or negative control siRNA. **(c-e)** Immunoblot and densitometric analyses (representative from 2 separate experiments) of the 28-kDa NOX4 and corresponding  $\beta$ -actin, p- and total c-jun **(c)**, DCF fluorescence recordings **(d)**, and percentage of apoptotic cells **(e)** in control (C) and day-1 post-hypoxic (H) wild-type RTECs incubated with (+) or without (-) 2  $\mu$ M SP600125 JNK inhibitor which was added 30 min before adding mineral oil and during the 24 h following hypoxia induction. Values are means  $\pm$  S.E.M. from (n) individual cell recordings or cell counts on 3 to 5 cell cultures from 2 to 3 different kidneys in each group tested. \*  $P < 0.05$  between groups.

**Figure 7** Effects of silencing the NOX4 28-kDa isoform on p38 activation and consequence of p38 inhibition on ROS production and apoptosis in post-hypoxic renal tubule cells. **(a)** Immunoblot (left panel) and densitometric analyses (right panel) (representative from 2 separate experiments) of p- and total p38 in non-hypoxic (Control) and day-1 post-hypoxic (Hypoxia) wild-type RTECs that had or had not been transfected with the *NOX4*<sub>28</sub> siRNA or negative control siRNA. **(b)** Immunoblot and densitometric analyses (representative from 2

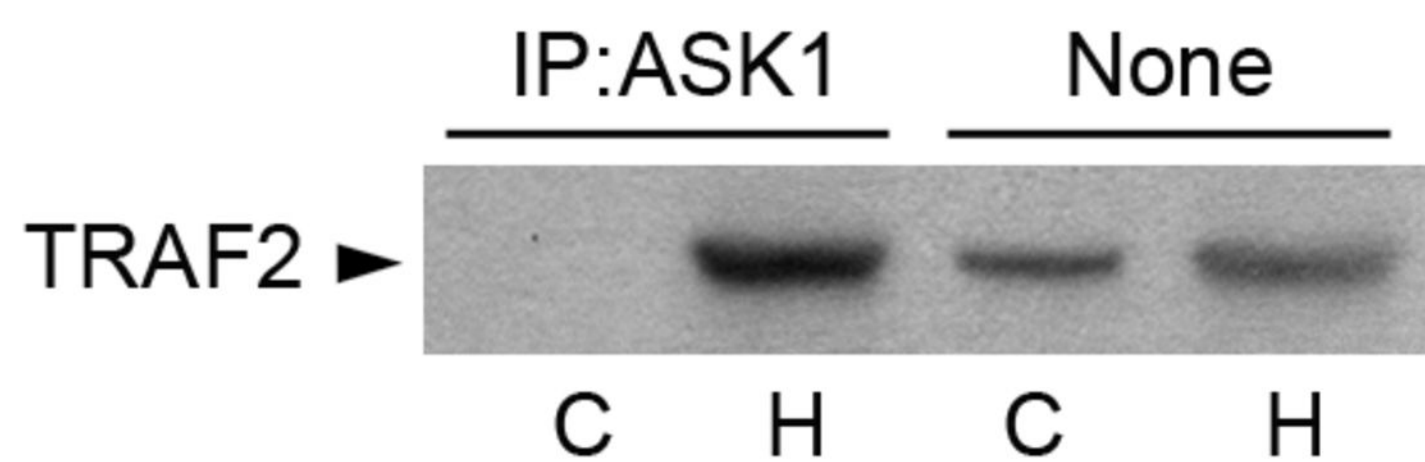
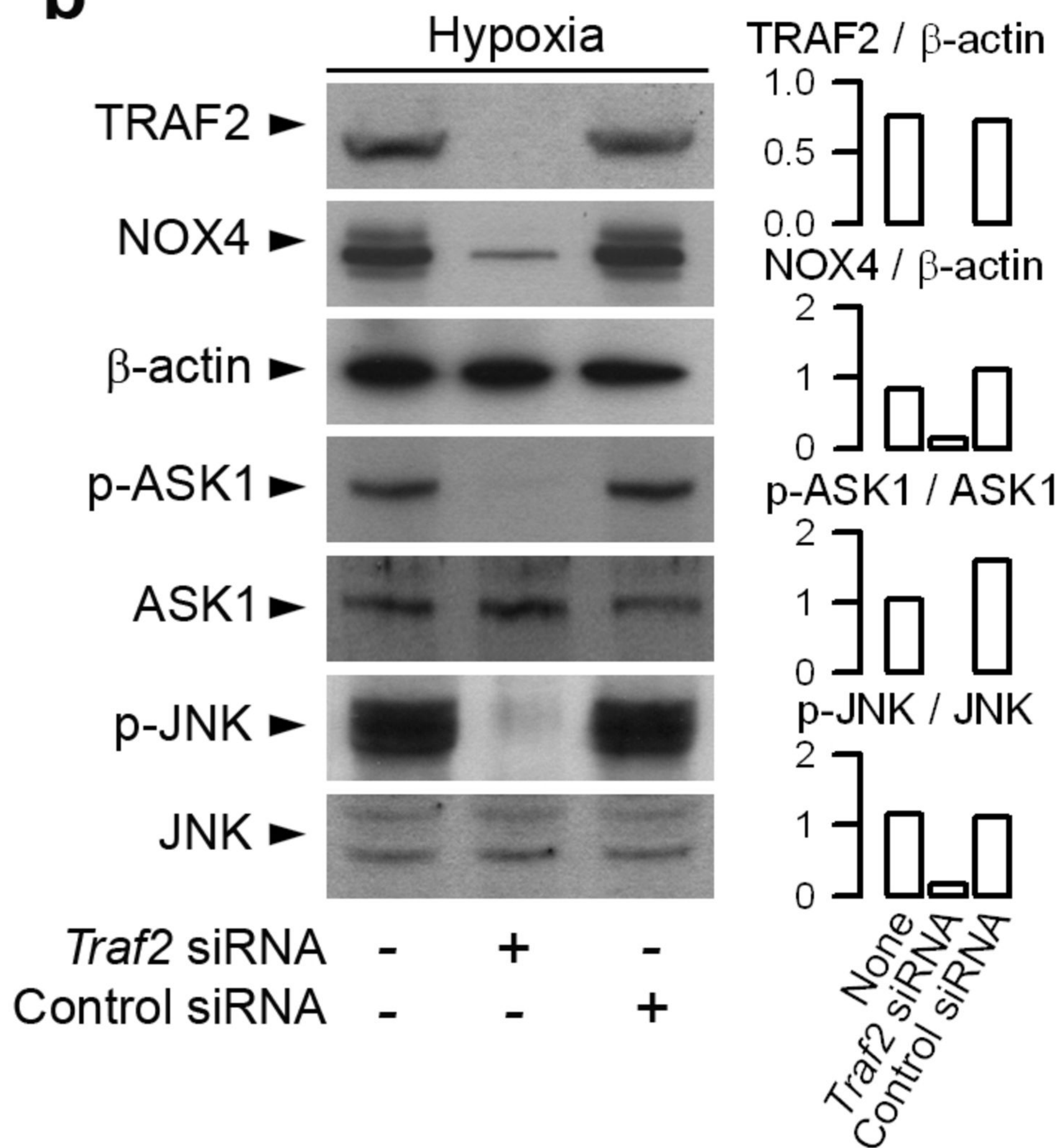
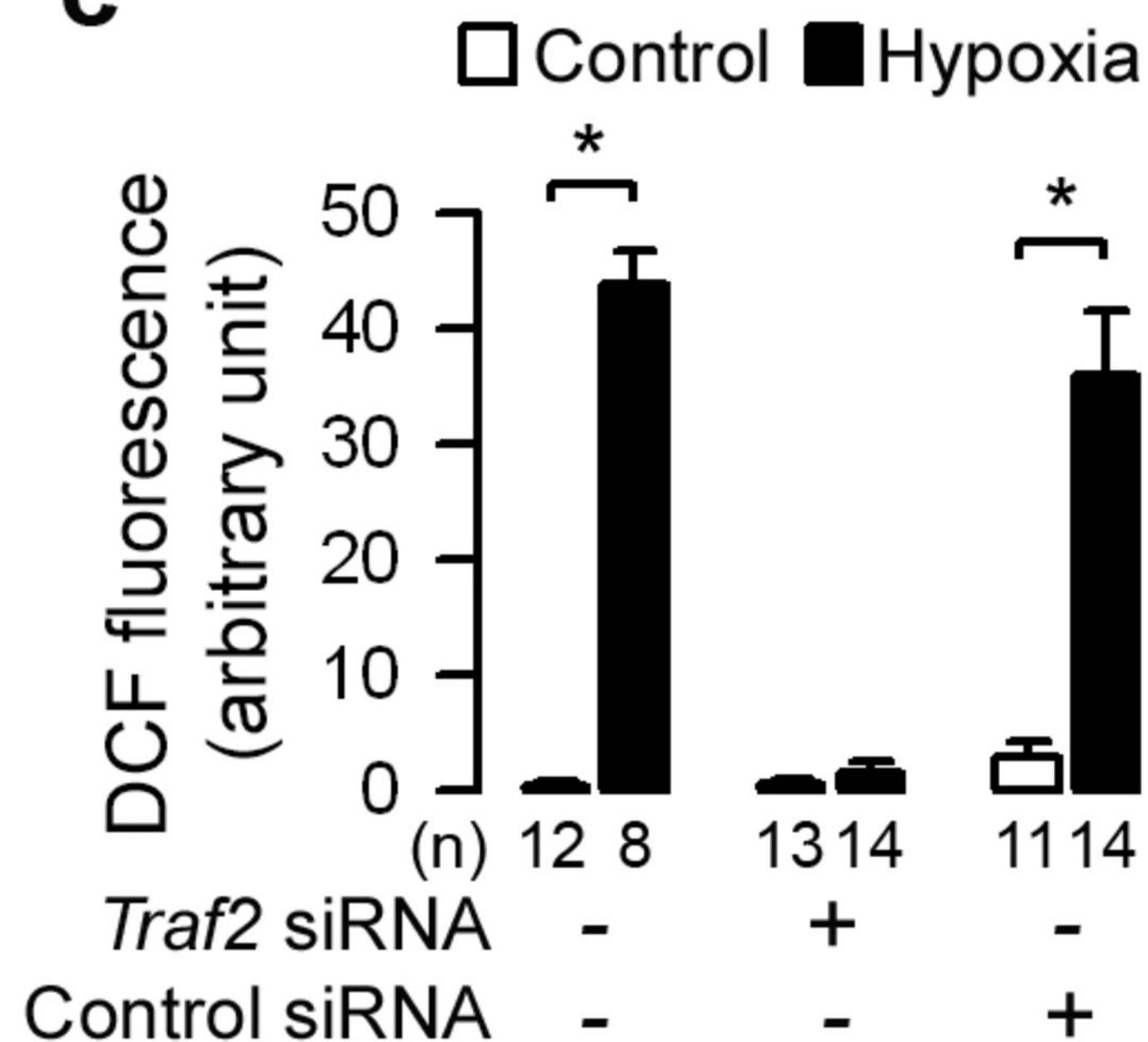
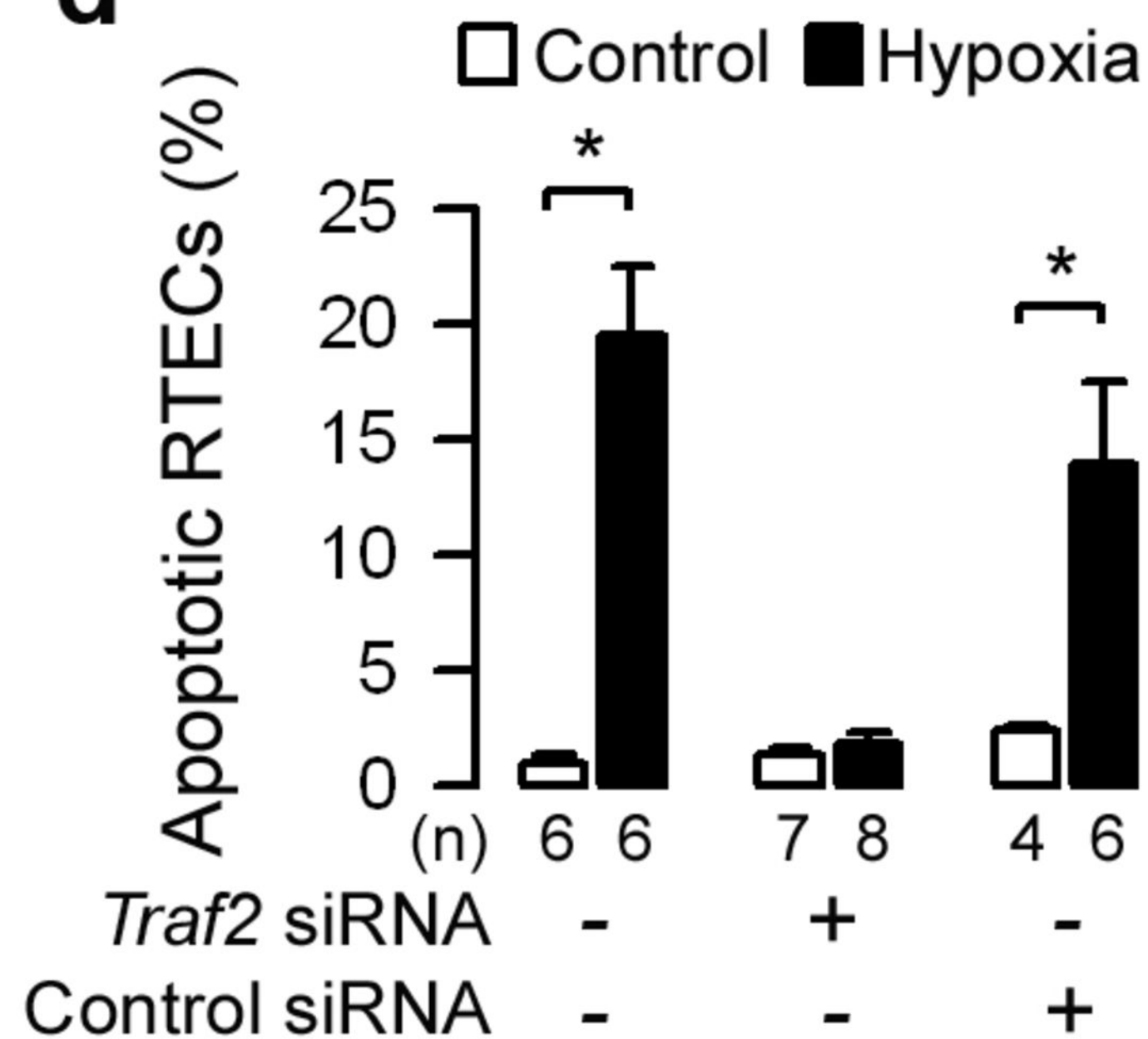
separate experiments) of p- and total MAPKAPK2 in control (C) and day-1 post-hypoxic (H) wild-type RTECs incubated (+) or not (-) with 10  $\mu$ M SB203580 p38 inhibitor as described in the legend of Figure 6. (c) DCF fluorescence recordings in non-hypoxic control (C) and day-1 post-hypoxic (H) wild-type RTECs incubated without (-) or with (+) 10  $\mu$ M SB203580. (d) Percentage of apoptotic cells in non-hypoxic control (C) and day-1 post-hypoxic (H) wild-type RTECs incubated without (-) or with (+) 10  $\mu$ M SB203580 alone or with 2  $\mu$ M SP600125. Values are means  $\pm$  S.E.M. from (n) individual cell recordings or cell counts on 2 to 3 cell cultures from 2 different kidneys. \*  $P < 0.05$  between groups.



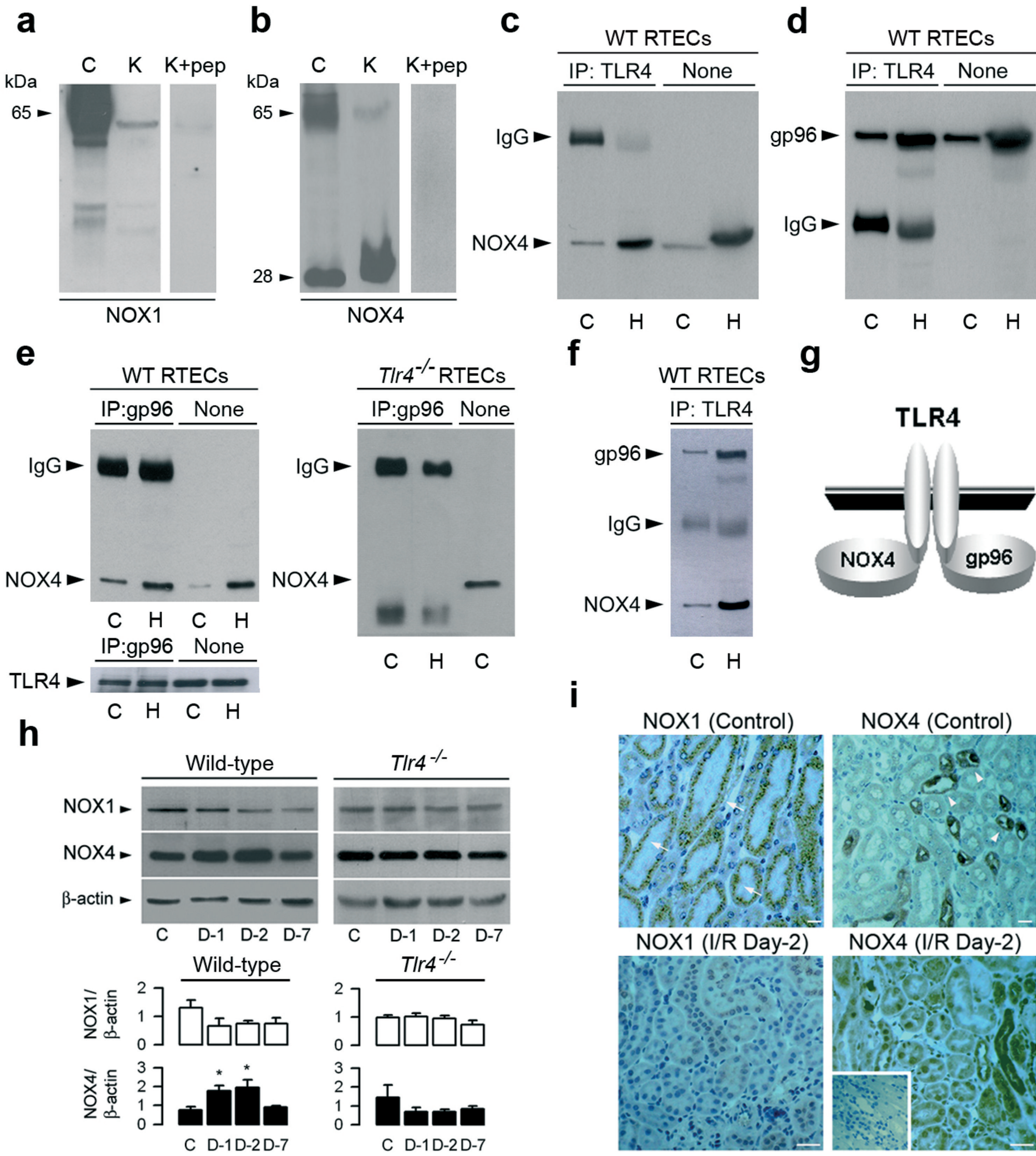


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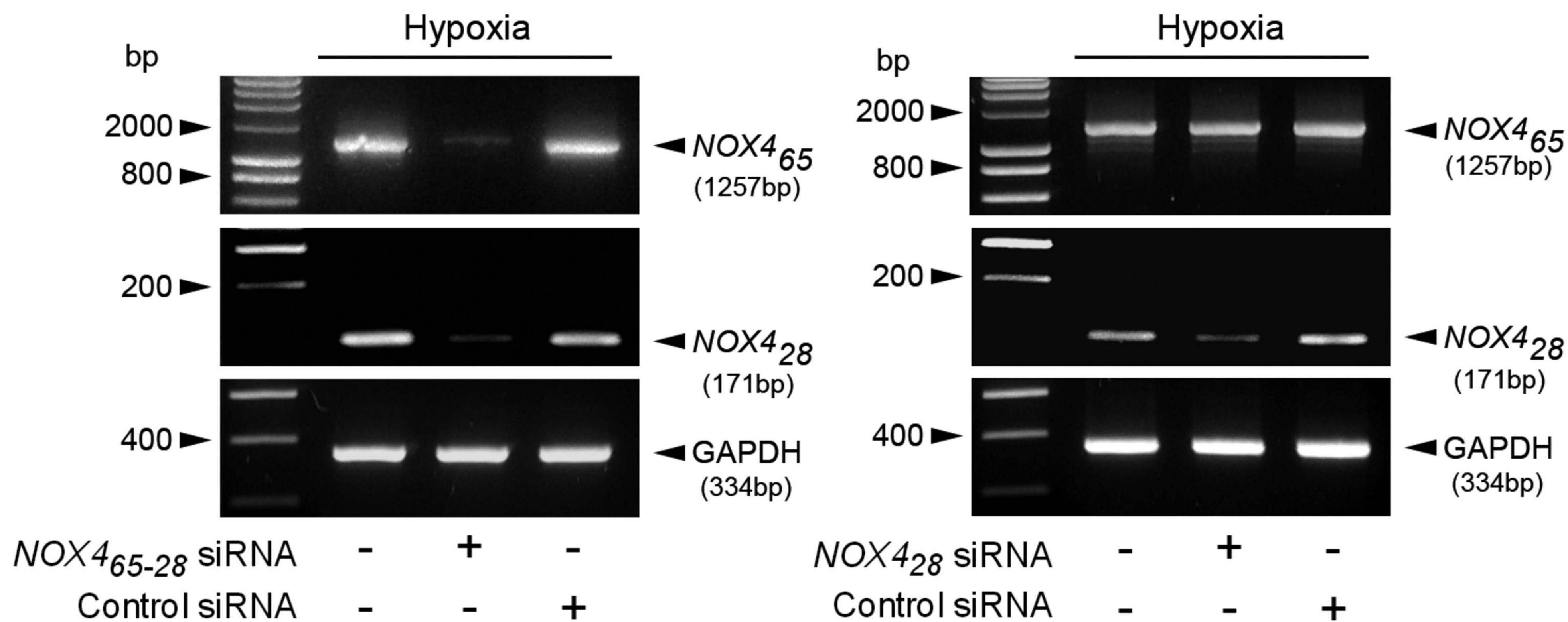
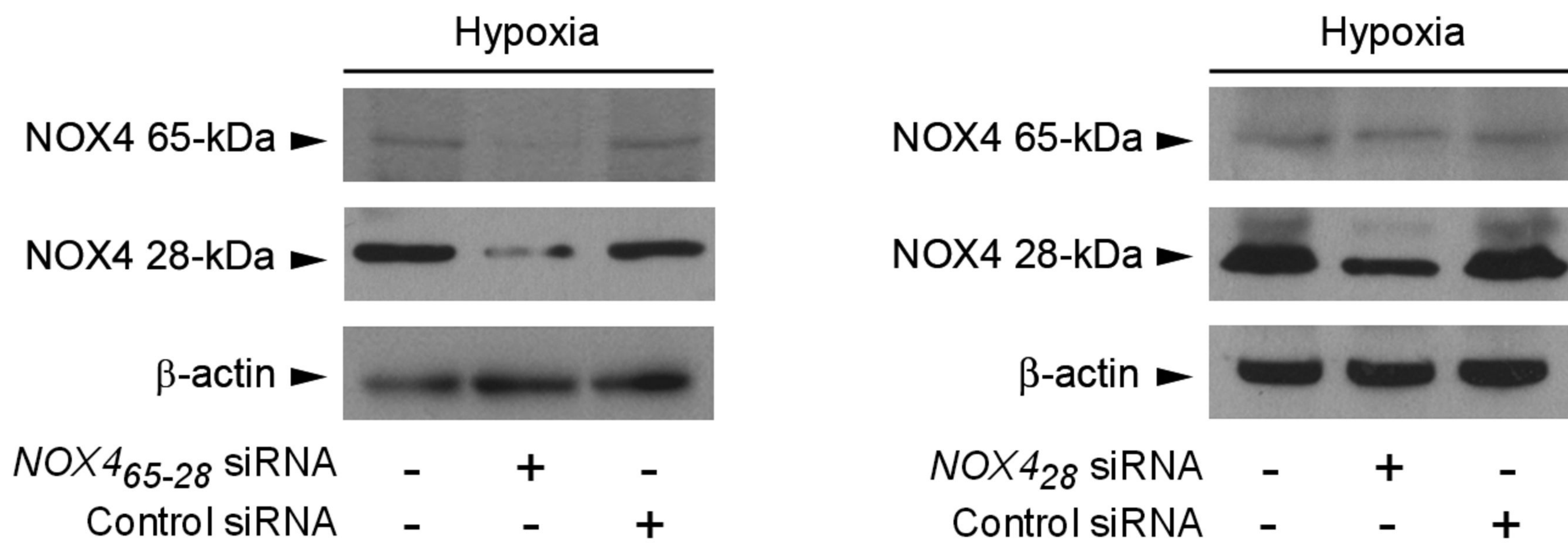


**a****b****c****d**







**a****b****c**