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Regulation of Hypoxia Inducible Factor-1 α by NF- κ B.

Patrick van Uden*, Niall S. Kenneth*, and Sonia Rocha

College of Life Sciences
Wellcome Trust Centre for Gene Regulation and Expression
MSI/WTB/JBC Complex
Dow Street
University of Dundee
Dundee, DD1 5EH
Scotland,
United Kingdom
Tel: +44 1382 385 792
Fax +44 1382 388 072

Corresponding author Rocha, S. (s.rocha@dundee.ac.uk)

*-these authors contributed equally to this work

Running title: NF- κ B regulates HIF-1 α .

Abstract:

The Hypoxia Inducible Factor (HIF) is the main transcription factor activated by low oxygen tensions. HIF-1 α (and other alpha subunits) is tightly controlled mostly at the protein level, through the concerted action of a class of enzymes called Prolyl Hydroxylases (PHD1, 2 and 3). Most of the knowledge on HIF derives from studies following hypoxic stress, however, HIF-1 α stabilisation is also found in non-hypoxic conditions through an unknown mechanism. Here, we demonstrate that NF- κ B is a direct modulator of HIF-1 α expression. The HIF-1 α promoter is responsive to selective NF- κ B subunits. siRNA studies for individual NF- κ B members revealed differential effects on HIF-1 α mRNA levels, indicating that NF- κ B can regulate basal HIF-1 α expression. Finally, when endogenous NF- κ B is induced by TNF- α treatment, HIF-1 α levels also change in a NF- κ B dependent manner. In conclusion, we find that NF- κ B can regulate basal, TNF- α , and in certain circumstances the hypoxia induced HIF-1 α .

HIF-1/TNF/NF- κ B/Hypoxia

Introduction

Hypoxia Inducible Factor (HIF) plays key roles in development, physiological processes and pathological conditions as its presence affects survival, cell cycle progression and metabolism [1, 2]. Although, first identified as the main transcription factor activated under low oxygen tensions, HIF is a key transcription factor activated by cytokines, oncogenes and reactive oxygen species (ROS) under normoxic conditions [3]. HIF is a heterodimeric factor composed of α and β subunits (also called Aryl Hydrocarbon Nuclear Translocator, ARNT) [1]. While, HIF-1 β is constitutively expressed and not regulated by oxygen levels, HIF- α subunits are tightly controlled at the protein level. This occurs, predominantly, through the concerted action of a class of enzymes called Prolyl Hydroxylases (PHD1, 2 and 3) that catalyse prolyl hydroxylation of HIF- α subunits, which incessantly targets them for VHL-dependent 26S proteosomal degradation [4]. PHD enzymes require molecular O₂, 2-oxoglutarate, iron ions (Fe²⁺), and ascorbic acid, to be fully active, and are inhibited in hypoxic conditions [4].

Four PHDs have been identified so far, but only three have been functionally characterised in terms of HIF modulation, PHD1, 2 and 3 [4]. PHD2 is thought to be the dominant PHD in controlling HIF-1 α following hypoxia [5]. Furthermore, all three PHDs can be regulated by HIF and by oxygen levels [6-8]

In addition to the HIF- α stabilisation mechanism controlled by prolyl hydroxylation, hydroxylation of Asn 803 in HIF-1 α or Asn 851 in HIF-2 α , prevents interaction with the CH1 domains of histone acetyltransferases (p300 / CBP) that act as transcriptional co-activators. This modification alters the ability of HIF-1/ HIF-2 to transactivate their target genes [9].

While most knowledge regarding HIF-1 has been derived from studies following hypoxic stress, HIF-1 stabilisation has also been found in non-hypoxic settings, such as relatively well oxygenated regions of tumours and in diseases such as rheumatoid arthritis (RA) and diabetes [10, 11]. However, the mechanisms for HIF stabilisation under those conditions have not been elucidated. Many of the stimuli that induce HIF-1 in normoxia are known to activate a number of other transcription factors such as NF- κ B. It is therefore plausible that crosstalk between these two transcription factors can occur. In addition, the HIF-1 α and PHD1 promoters contain NF- κ B binding sites, which have not been functionally characterised. Importantly, HIF-1 α stabilisation and induction through H₂ O₂ was recently shown to be NF- κ B dependent under normoxic conditions [12].

NF- κ B is the collective name for a transcription factor that exists as either a hetero- or homo- dimer and is formed by a family of subunits called RelA (p65), RelB, cRel, p50 and its precursor p105 (NF- κ B1), and p52 and its precursor p100 (NF- κ B2). Some dimers are more prevalent than others and they shuttle between the cytoplasm and nucleus but are predominantly sequestered in the inactive state in the cytoplasm, inhibited by members of the I κ B family [13]. Upon stimulation, by compounds such as TNF- α , oncogenes or UV light, a kinase signalling cascade results in phosphorylation of I κ B, signalling ubiquitination mediated proteasomal degradation, resulting in NF- κ B release and translocation into the nucleus [13, 14]. The activated NF- κ B dimer then binds to target DNA sequences in the nucleus where it regulates transcription of NF- κ B target genes. Although, NF- κ B activation is extremely fast, the cyclic nature of NF- κ B activity, in combination with post

translational modifications and hyper- and hypo- acetylated histones at target genes are indicative of further levels of regulatory complexity [15]

There have been several studies demonstrating cross-talk between the NF- κ B and HIF signalling pathways, including shared target genes, but a direct link has yet to be elucidated. In this study we show that several NF- κ B subunits are bound to the HIF-1 α promoter. NF- κ B depletion results in reduced basal level of HIF-1 α mRNA. In addition, we demonstrate that TNF- α induced NF- κ B can increase HIF-1 α mRNA, protein and activity levels, leading to transactivation of target genes in normoxia. Changes in HIF-1 α levels following hypoxia are only seen when the NF- κ B pathway is completely blocked. These data demonstrate that NF- κ B can directly modulate HIF-1 α pathway, and that this modulation is sufficient to alter HIF target gene expression *in vivo*.

Materials and Methods

Tissue culture, Medium and hypoxia treatments

U2OS osteosarcoma and human embryonic kidney (HEK293) cell lines were obtained from the European Collection of Cell Cultures. IKK wildtype, IKK α -/-, IKK β -/- and IKK α/β -/- MEFS were a kind gift from Inder Verma (La Jolla, USA). All cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Lonza) supplemented with 10% fetal calf serum (GIBCO), 50 U/mL penicillin (BioWhittaker), and 50 μ g/mL streptomycin (BioWhittaker) for no more than 30 passages. For hypoxia treatments, cells were placed in 1% O₂ using an InVivo 300 workstation (Ruskin, UK).

siRNA transfection and sequences:

siRNA duplex oligonucleotides were synthesized by MWG and transfected using Interferin (Polyplus) as per manufacturers instruction.

Control 5' -aac agu cgc guu ugc gac ugg- 3' (Anderson et al, 2003)

RelA 5' -gcu gau gug cac cga caa g- 3' (Anderson et al, 2003)

RelB 5' -aau ugg aga uca ugc acg agu- 3'

cRel siRNA sequence acquired from (Schumm et al, 2005)

p50/p105 5' -aag ggg cua uaa ucc ugg acu- 3'

p52/p100 5' -aag aug aag auu gag cgg ccu- 3' (Schumm et al, 2005)

HIF1 α 5' -cug aug acc agc aac uug a- 3'

IKK α 5'-gca ggc ucu uuc agg gac a-3'

IKK β 5'-cag gug agc aga uug cca u-3'

DNA constructs

Individual NF- κ B subunit expression vectors were a kind gift from N. Perkins (Dundee, UK). HIF-1 α promoter luciferase constructs were a kind gift from C. Michiels (Namur, Belgium).

Antibodies:

HIF1 α , (R&D Systems), RelA (p65), RelB, cRel, p50 (NF- κ B1), Chk1, and p52 (NF- κ B2) (SantaCruz), Glut1/3 (Neomarkers), HIF-2 α (Novus Biologicals), HIF-1 β , IKK α and IKK β (Cell Signalling), β -Actin, PCNA (Sigma), PHD2 (abcam), Bcl-xL (Merck Biosciences). Enhanced chemiluminescence (PIERCE) was used for detection.

QRT-PCR

Real-time quantitative RT-PCR, qRT-PCR, was carried out in a 25 μ l total reaction mixture with 1 μ l extracted RNA sample (10pg – 100 ng), 12.5 μ l One-Step Reverse Transcriptase qPCR master mix Plus for SYBR Green I (Eurogentec), forward primer 0.2 μ M, reverse primer 0.2 μ M, EuroScript RT 0.125 U/mL, and RNase free dH₂O 10.875 μ l. Amplification and detection were performed using Rotor-Gene 3000 (Corbett Research) and IQ5 Icyler (BioRad) detection system under the following conditions: an initial reverse transcription at 48°C for 30 min, followed by PCR activation at 95°C for 10 min and 45 cycles of amplification (15 s at 95°C and 1 min at 58°C). During amplification, detector monitored real-time PCR amplification by quantitative analysis of the fluorescence emissions. Sample values obtained with specific primer sets were normalized to β -actin primer set values.

QRT-PCR primer sequences

β -actin For -gtg gga gtg ggt gga ggc

Rev -tca act ggt ctc aag tca gtg

18S	For –aaa cgg cta cca cat cca ag Rev –cgc tcc caa gat cca act ac
GLUT1	For- gat tgg ctc ctt ctc tgt gg Rev-tca aag gac ttg ccc agt tt
GLUT3	For- caa tgc tcc tga gaa gat cat aa Rev-aaa gcg gtt gac gaa gag t
HIF-1 α	For- cat aaa gtc tgc aac atg gaa ggt Rev-att tga tgg gtg agg aat ggg tt
HIF-2 α	For- gcg cta gac tcc gag aac at Rev-tgg cca ctt act acc tga ccc tt

Chromatin Immunoprecipitation (ChIP)

Proteins were cross-linked with formaldehyde for 10 minutes. After adding 0.125 mol/L glycine, cell lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCL, pH8.1, 1mM PMSF, 1mg/ml leupeptin, 1mg/ml aprotinin) was added, followed by sonication and centrifugation. The supernatant was precleared with sheared salmon sperm DNA and protein A Sepharose beads (Sigma). Supernatant incubated with specific antibodies overnight, and then with protein A sepharose beads for 1 hour. After extensive wash step the complexes were eluted with buffer (100 mmol/L NaHCO₃, 1% SDS) and incubated with proteinase K. DNA was purified using QIAquick polymerase chain reaction purification kit (Qiagen). Polymerase chain reaction was performed with primers for the HIF-1 α promoter (forward: 5' -gaa cag aga gcc cag cag ag-3' ; reverse: 5' -cct gag gtg gag gcg ggt tc-3') flanking the NF- κ B binding site (-197/188 bp) at 64°C annealing and 72°C extension for 32 cycles. This primer set spans from -536 till -137 bp from the transcription start site. HIF-1 α control primer set forward: 5' -tgc tca tca gtt gcc act tc- 3'; reverse: 5' -aaa aca ttg cga cc acct tc- 3'. This primer set is located in the gene itself, 24937 bp away from the transcription start site. HIF-1 α target genes promoters VEGF forward 5' -ac gtt cct tag tgc tgg cgg gta ggt ttg a-3'; reverse 5' -gc acc aag ttt gtg gag ctg aga acg gg-3', CA9 forward 5' -gac aaa cct gtg aga ctt tgg ctc c-3'; reverse 5' -agt gac agc agc agt tgc aca gtg-3', DEC1 forward 5' -cac gtg aga ctc atg tga tga agc c-3'; reverse 5' -aag ccg agg agt aat gga gag gct-3'.

Other experimental procedures

Luciferase assays, whole cell protein, nuclear extracts and EMSA analysis were performed as previously described ((Rocha *et al.*, 2005 and references therein). RNA was extracted using a nucleospin RNA II kit (Machery-Nagel). EMSA probes for HIF-1 α κ B (gcg tgg gct ggg gtg ggg act tgc cgc ctg cgt cgc).

Results

Differential control of the HIF-1 α promoter by NF- κ B subunits

The HIF-1 α promoter was originally cloned in 1996 [16] and a study by the Michiels group, identified several binding sites for transcription factors such as AP1 and NF- κ B [17]. Given the advance in our knowledge of NF- κ B consensus sites [14], we reanalysed the HIF-1 α promoter (<http://www.genomatix.de/products/MatInspector>). This analysis identified a putative NF- κ B binding site located -197/188 bp from the initiation site (Figure 1a). Using HIF-1 α promoter reporter constructs either with or without the putative NF- κ B binding site [17], we investigated the contribution of individual NF- κ B subunits in normoxia (Figure 1a, b). All NF- κ B subunits could activate the HIF-1 α promoter, with the highest effect being observed with p50 and the lowest with p52. In contrast, none of the NF- κ B subunits activated a truncated version of the HIF-1 α promoter construct, which lacks the NF- κ B site (Figure 1b). To investigate if NF- κ B could bind the putative site on the HIF-1 α promoter, we performed EMSA assays using nuclear extracts derived from HEK293 which had been transfected with the individual NF- κ B subunits (Figure 1c). We could detect intense binding by p50, and weaker binding by p65, c-Rel and p52. No visible binding could be observed with RelB, although the expression levels were comparable (Figure 1e). Binding to the canonical site in the HIV promoter was used to assess NF- κ B binding ability (Figure 1d). These results suggest that NF- κ B is able to regulate HIF-1 α promoter activity.

NF- κ B modulates basal HIF-1 α mRNA levels

To investigate if endogenous NF- κ B can modulate the HIF-1 α promoter, siRNA oligonucleotides directed towards the different NF- κ B subunits were used. The siRNA sequences have been previously validated [18, 19]. Q-RT-PCR analysis demonstrated that endogenous NF- κ B regulated basal HIF-1 α mRNA levels (Figure 2a). Interestingly, reduction of p50 levels had no effect on HIF-1 α mRNA levels, possibly indicating a compensatory action by p52 or other NF- κ B subunits. The co-operation between p50 and p52 is best depicted in the genetic knockout mice, where p50 and p52 single deletions show no developmental defects, double deletion of these genes results in severe impairment in bone development [20, 21]. Endogenous p65, RelB and p52 have the highest effect on HIF-1 α mRNA levels (Figure 2a). Despite inducing a slight reduction in HIF-1 α mRNA, c-Rel depletion did not induce statistically significant effects (Figure 2a). To assess if NF- κ B is directly regulating HIF-1 α mRNA levels chromatin immunoprecipitation (ChIP) analysis was performed. Several of the NF- κ B subunits could be found at the HIF-1 α promoter, however, we could not detect any NF- κ B binding in a control region of the gene (Figure 2b). This demonstrates that NF- κ B modulates the basal level of HIF-1 α mRNA directly.

NF- κ B modulates HIF-1 α protein levels in normoxia

HIF-1 α is predominantly regulated at the protein level following hypoxia [22]. Since the studies presented here were conducted at normal oxygen levels, prolyl-hydroxylase activity is not readily inhibited. To investigate if NF- κ B could also modulate HIF-1 α protein levels, NF- κ B overexpression and siRNA-mediated knockdowns were used. Given, that HIF-1 α protein is rapidly degraded at normal oxygen, only small changes could be expected. It was possible to observe increases in protein levels, when all of the NF- κ B subunits were overexpressed (Figure 3a).

However, RelA and c-Rel induced the highest increase followed by p50, p52 and RelB. Changes in HIF-1 α protein were best visualised when the proteasome inhibitor MG132 was used (Figure 3a). Conversely, a small reduction of HIF-1 α protein levels could be observed when RelA, RelB, p52 were depleted. A small effect was also evident when c-Rel was depleted but no effects could be detected with p50 depletion (Figure 3b). In either overexpression or siRNA mediated knockdown of NF- κ B subunits, HIF-1 β levels remained stable (Figure 3a, b).

TNF- α induced NF- κ B modulates HIF-1 α expression

Activation of NF- κ B is best associated with inflammatory responses [13]. It is rapidly activated following cytokines such as TNF- α or bacterial products such as LPS. In fact, these stimuli have both been described to activate HIF-1 α as well, although the mechanism has not been fully elucidated [23, 24]. Given that prolonged TNF- α exposure is present in many inflammatory diseases and certain cancers, we investigated if HIF-1 α was induced under such conditions (Figure 4). We exposed cells to TNF- α for several hours and analysed nuclear fractions (Figure 4a). It was possible to detect nuclear accumulation of all NF- κ B subunits and also a significant increase in HIF-1 α (Figure 4a). Furthermore, this prolonged TNF- α treatment also induced increases in two HIF target genes Glut1 and Glut3 (Figure 4b). To test if the observed increases in HIF-1 α , Glut1 and Glut3 protein resulted from higher mRNA levels, q-RT-PCR was performed for the indicated times following TNF- α treatment. Interestingly, it was possible to observe an increase in HIF-1 α , Glut3 and to a lesser extent Glut1 mRNA with no change in HIF-2 α mRNA (Figure 4c). These results indicate that the HIF-1 α and Glut3 protein increases result from increased gene transcription while HIF-2 α levels increase possibly due to protein stability effects. Furthermore, siRNA mediated depletion of HIF-1 α or HIF-2 α demonstrated that TNF- α induced Glut3 is HIF-1 α dependent (Figure 4d, Supp. Figure 1), indicating that TNF- α induced HIF-1 α is active transcriptionally.

TNF- α induced HIF-1 α activity is NF- κ B dependent

To test if the observed changes in HIF-1 α were NF- κ B dependent, siRNA for the different NF- κ Bs was combined with TNF- α treatment. Depletion of all of the subunits with the exception of p105/p50, resulted in an impairment of TNF- α induced HIF-1 α expression (Figure 5a). In addition, depletion of HIF-1 α results in impaired TNF- α induction of PHD2 (Supp. Figure 2), indicating that this is a HIF-1 α dependent event. Furthermore, we can detect an inducible recruitment of HIF-1 α to the promoters of some of its target genes (Figure 5b).

To verify that TNF- α was inducing changes at the HIF-1 α promoter, ChIPs were performed. It was possible to observe, following 18 hours of TNF- α treatment, an active recruitment of RelA and p52 to the HIF-1 α promoter (Figure 5c). Importantly, TNF- α treatment did not induce NF- κ B binding to a control region of HIF-1 α gene. Furthermore, depletion of RelA, RelB, c-Rel and p52 reduced TNF- α induced HIF-1 α -mediated PHD2 induction (Figure 5d). These results indicate that TNF- α induced changes in HIF-1 α levels and activity, are NF- κ B dependent.

Complete block of the NF- κ B activation pathway prevents Hypoxia induced HIF-1 α

Given that NF- κ B can control basal HIF-1 α mRNA levels, we investigated if blocking NF- κ B could prevent hypoxia induced HIF-1. We took advantage of genetic knockout cells and also used siRNA for the upstream kinase complex that regulates

NF- κ B. We could observe that when IKK α or IKK β were depleted, either by genetic knockout or siRNA mediated silencing, there was very little effect on HIF-1 α levels (Figure 6a, b), suggesting that protein stabilisation can compensate for any impairment in the canonical or non-canonical pathways. However, when both IKK α and β were depleted, HIF-1 stabilisation in response to hypoxia was severely impaired (Figure 6c). This suggests that HIF-1 α gene transcription is mediated by several subunits of NF- κ B and these derive from both pathways of activation. These results support our mRNA and CHIP data on how NF- κ B controls the HIF pathway. Given that hypoxia does not induce increases in HIF-1 α mRNA, it was not surprising that no changes in NF- κ B recruitment to the HIF-1 α promoter were observed following hypoxia treatment (Figure 6d). Once again, we could not detect any NF- κ B binding to a control region of the HIF-1 α gene (Figure 6d). These results suggest that any impairment seen in HIF-1 α stabilisation following hypoxia, in the absence of NF- κ B, is due to changes in basal mRNA levels and not due to a lack of an active induction of mRNA.

Discussion

Chronic inflammation is self-perpetuating and has been shown to distort the microenvironment as a result of aberrantly active transcription factors. Consequent alterations in growth factor, chemokine, cytokine, and ROS balance within the cellular milieu, provide the axis of growth and survival needed for de-novo development of cancer and metastasis [25]. As such, we hypothesised that novel interactions between two key transcription factors, NF- κ B and HIF, existed in this process.

In this report we demonstrate that HIF-1 α basal mRNA levels are controlled by several NF- κ B subunits (Figure 1, 2, 3, 5, 6). In addition, we show that TNF- α , and to some extent hypoxia, induced NF- κ B regulates HIF-1 α levels and activity, triggering the transcription of target genes such as Glut3 and PHD2 (Figure 4, 5, 6).

The findings described in this report have numerous implications for a number of pathologies where NF- κ B and HIF-1 are deregulated, such as rheumatoid arthritis or cancer. However, despite these implications, NF- κ B regulation of HIF-1 α could be stimulus specific or even cell type specific. The evidence for this comes from the genetic knockout models available. While HIF-1 α deletion results in embryonic lethality at day 9-11 [26, 27] with defects evident as early as 7 days, combined genetic deletion of IKK α and IKK β results in lethality at day 12 [28]. Single deletions of IKK α , IKK β or RelA result in lethality at much later stages [28]. In HIF-1 α $-/-$ mice, defects are seen in neural tube formation, cardiovascular malformations and increases cell death in the cephalic mesenchyme. Of interest, IKK α/β double null mice also have defects in neurulation, but these seem to be associated with increased apoptosis in the neuronal epithelium. It would be interesting to re-analyse these mice embryos for HIF-1 α expression in the different tissues.

The results presented here also demonstrate that TNF- α induces HIF-1 activity despite the presence of oxygen. TNF- α can produce reactive oxygen species in cells [29] and these have been shown to inhibit PHD activity [30, 31]. Although we have not investigated this possibility, our results on HIF-1 α stabilisation and activity would support it. Precise ROS measurements and PHD activity assays would answer these questions.

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Figure Legends

Figure 1. NF- κ B subunits can activate the HIF-1 α promoter. a). Schematic diagram of the HIF-1 α promoter luciferase constructs. b) HEK293 cells were co-transfected with 1 μ g of HIF-1 α promoter luciferase constructs and 1 μ g of each of the NF- κ B subunits. Luciferase activity was measured 48 hours post-transfection. Graph depicts mean plus standard deviation of a minimum of 3 independent experiment performed in duplicate. Y-axis shows fold activation above control plasmid. c) NF- κ B subunits bind to the κ B site in the HIF-1 α promoter. HEK293 cells were transfected with 2 μ g of each of the NF- κ B subunits, nuclear extracts prepared and DNA binding activity was measured by EMSA using a specific probe for the HIF-1 α promoter. d) nuclear extracts prepared as in c) and DNA binding activity was measured using a canonical NF- κ B target promoter, HIV. e) Nuclear extracts were analysed by western blot for the individual NF- κ B subunits.

Figure 2. Endogenous NF- κ B subunits control basal HIF-1 α mRNA levels. a) HEK293 cells were transfected with the indicated siRNA oligonucleotides and qRT-PCR was performed. Graph depicts relative levels of HIF-1 α mRNA normalised over Actin mRNA levels. Mean and standard deviation were calculated from a minimum of three independent experiments. Statistic t-test analysis was performed and * represent $p < 0.050$, while ** represents $p < 0.010$. RelA, $p = 0.012$; RelB, $p = 0.012$; c-Rel, $p = 0.076$; p50, $p = 0.348$; p52, $p = 0.035$; HIF-1 α , $p = 0.007$. b) Chromatin immunoprecipitation analysis using the indicated antibodies and PCR analysis using specific primers for the HIF-1 α promoter and HIF-1 α control region was performed.

Figure 3. NF- κ B subunits control basal levels of HIF-1 α protein. a) HEK293 cells were transfected with the indicated DNA constructs and whole cell lysates were performed 48 hours post transfection. Three hours prior to harvest, half of the samples were incubated with 50 μ M of MG132. Western blot analysis for the indicated proteins was then performed on these extracts. b) HEK293 cells were transfected with the indicated siRNA oligonucleotides and whole cell lysates were performed 48 hours post transfection. Three hours prior to harvest, half of the samples were incubated with 50 μ M of MG132. Western blot analysis for the indicated proteins was then performed on these extracts.

Figure 4. TNF- α treatment induces NF- κ B and HIF-1 α . a) HEK293 cells were treated with 10ng/mL TNF- α for the indicated times, nuclear extracts were prepared and these extracts were analysed by western blot for the indicated proteins. PCNA was used as a loading control. b) Cells were treated as in A and cell extracts were analysed by western blot for the indicated HIF-1 α targets. c) Cells were treated as in a), but mRNA was extracted and qRT-PCR was performed for the indicated gene transcripts. Graph depicts relative levels specific mRNA transcripts normalised over Actin mRNA levels. Mean and standard deviation were calculated from a minimum of three independent experiments. d) Cells were transfected with the indicated siRNA oligonucleotides and treated with 10ng/mL TNF- α , 24 hours prior to total mRNA extraction. qRT-PCR was performed as in c).

Figure 5. TNF- α induces HIF-1 α activity in a NF- κ B dependent manner. a) HEK293 cells were transfected with the indicated siRNA oligonucleotides, treated with

10ng/mL of TNF- α 24 hours prior to harvest, and whole cell lysates prepared. Extracts were analysed by Western Blot using the indicated antibodies. b) Chromatin immunoprecipitation analysis using the indicated antibodies and PCR of specific regions of the HIF-1 α target genes, VEGF, CA9, and DEC1. c) As in b) but HIF-1 α promoter and control regions were analysed. d) Cells were treated as in a), and Western Blot analysis was performed using the indicated antibodies. PCNA was used as a loading control.

Figure 6. Complete inhibition of NF- κ B impairs hypoxia induced HIF-1 α levels. a) U2OS cells were transfected with the IKK α or IKK β siRNA oligonucleotides and exposed to 1% O₂ for the indicated times prior to harvest. Whole cell lysates were analysed by Western Blot. b) Wildtype, IKK α ^{-/-}, IKK β ^{-/-} mouse embryo fibroblasts were exposed to 1% O₂ for the indicated periods of time and whole cell lysates prepared. Extracts were analysed by Western Blots. c) Wildtype and IKK α/β ^{-/-} mouse embryo fibroblasts were exposed to 1% O₂ for the indicated periods of time and whole cell lysates prepared. Extracts were analysed by Western Blots. d) U2OS cells were left untreated or treated with 4 hour 1% O₂ prior to harvest and chromatin immunoprecipitation analysis using the indicated antibodies was performed. PCR analysis using specific primers for the HIF-1 α promoter and HIF-1 α control region was performed.

Figure 1 van Uden et al.

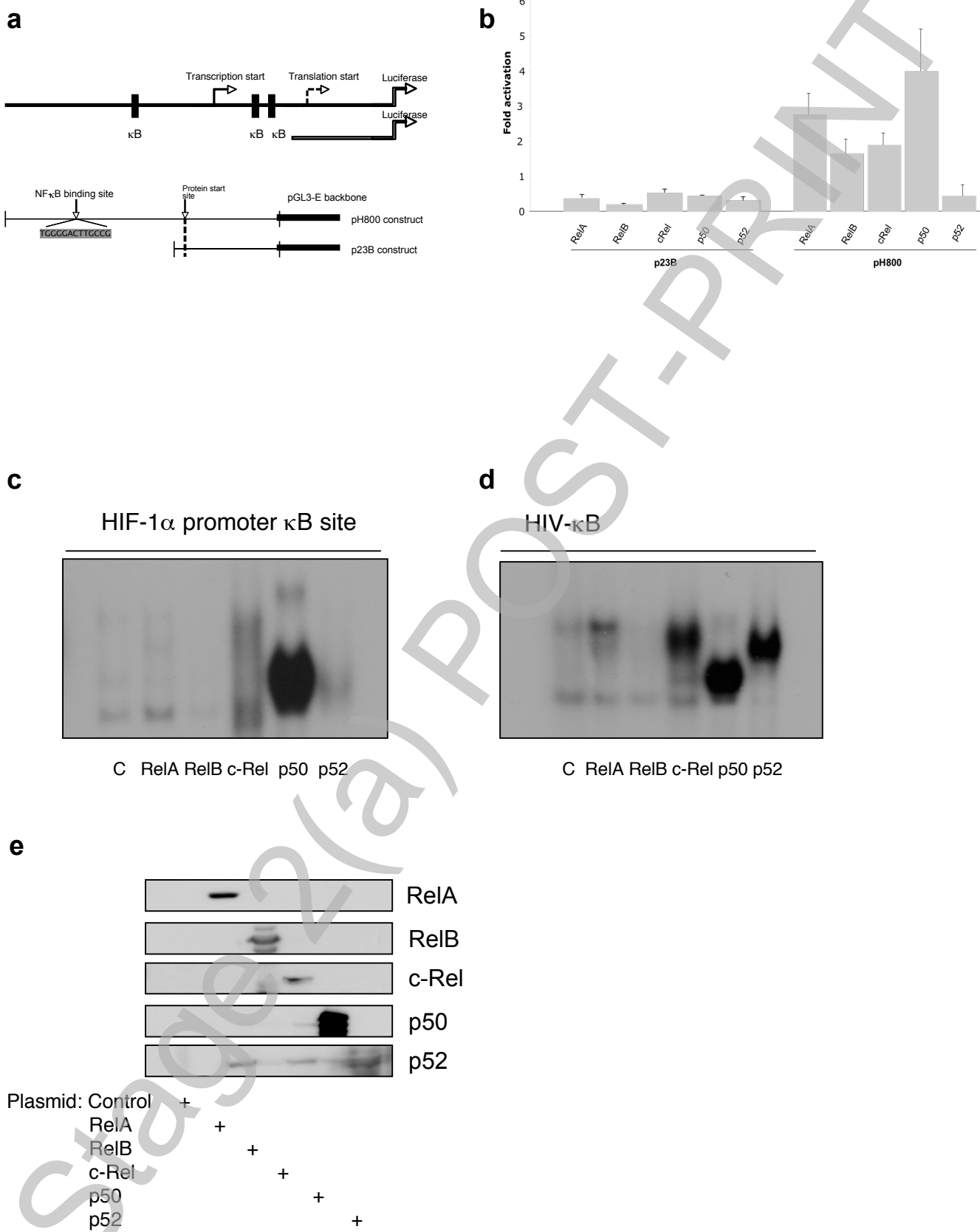


Figure 2 van Uden et al.

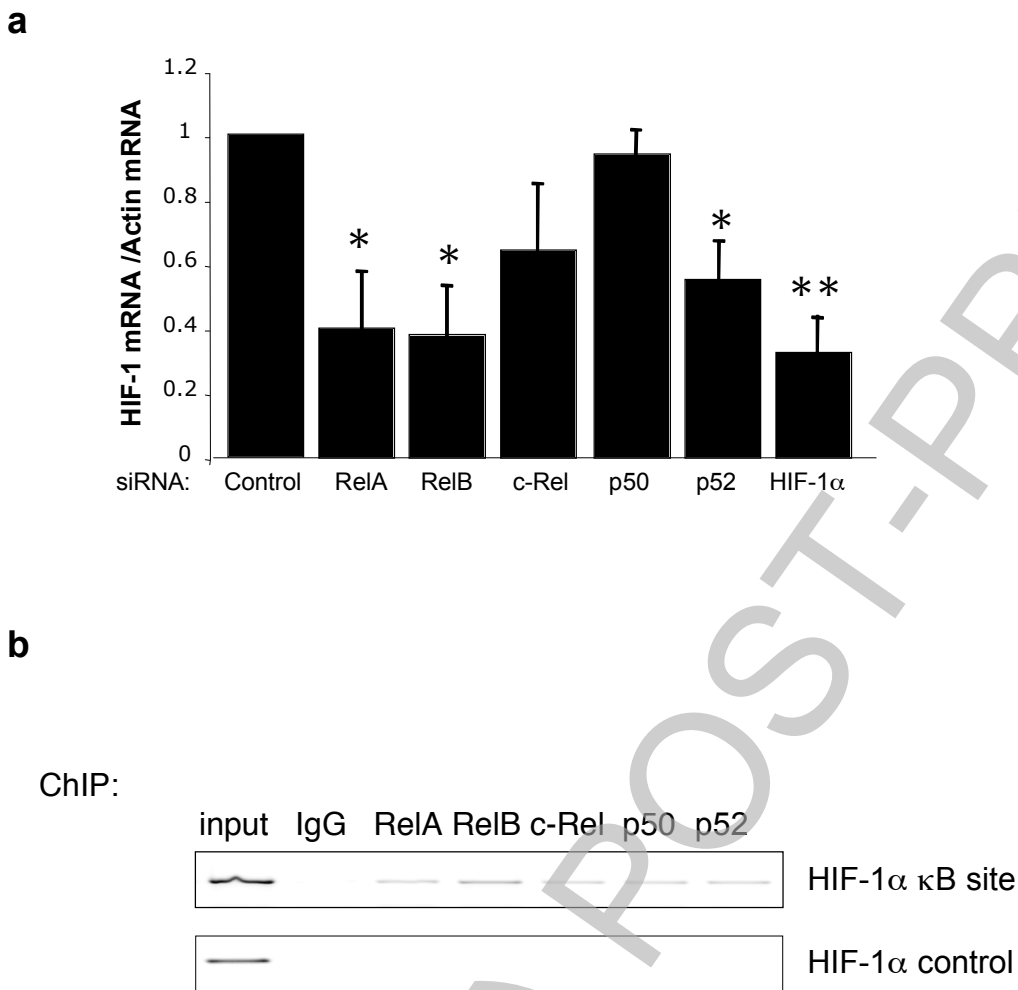


Figure 4 van Uden et al.

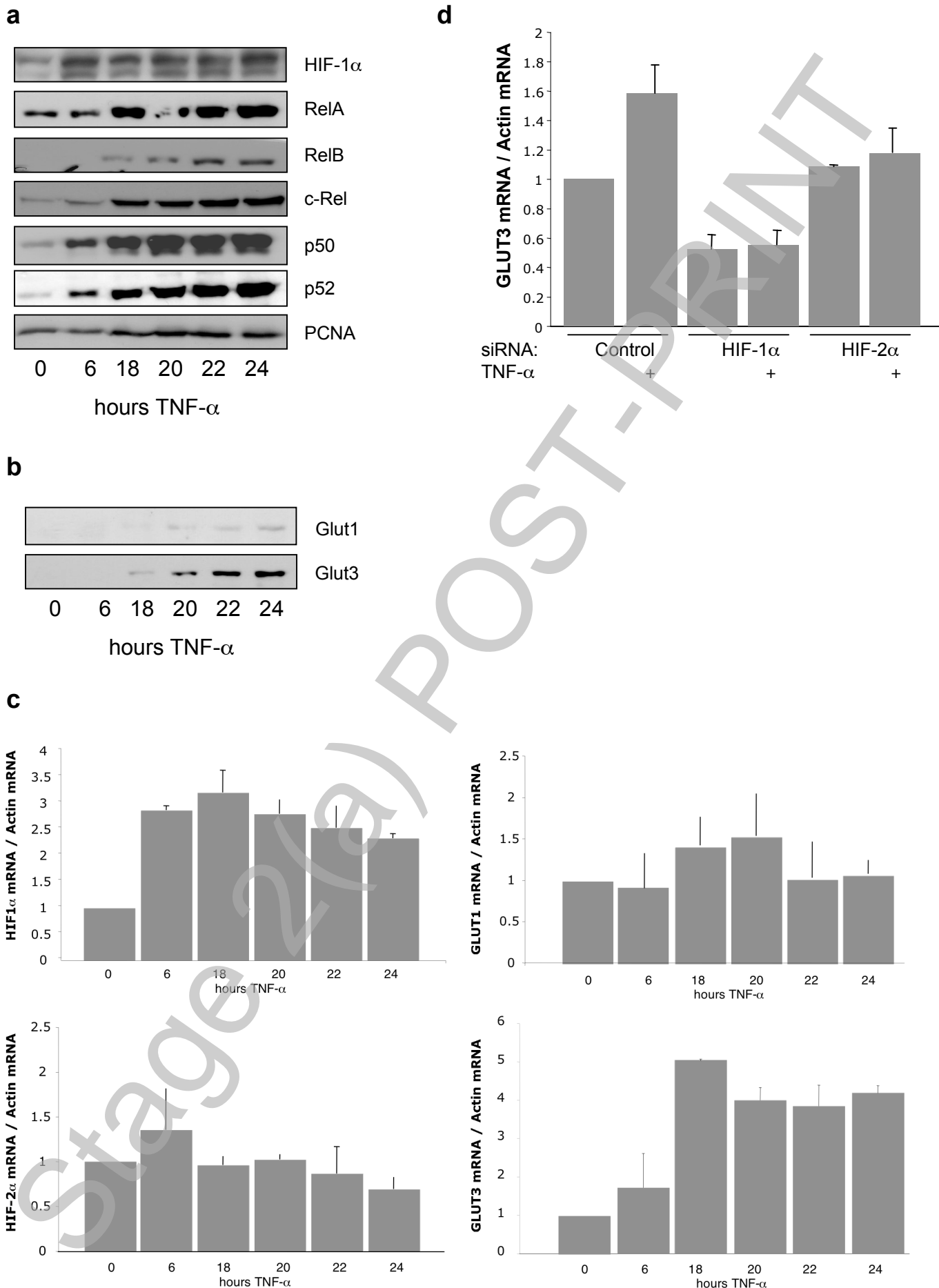
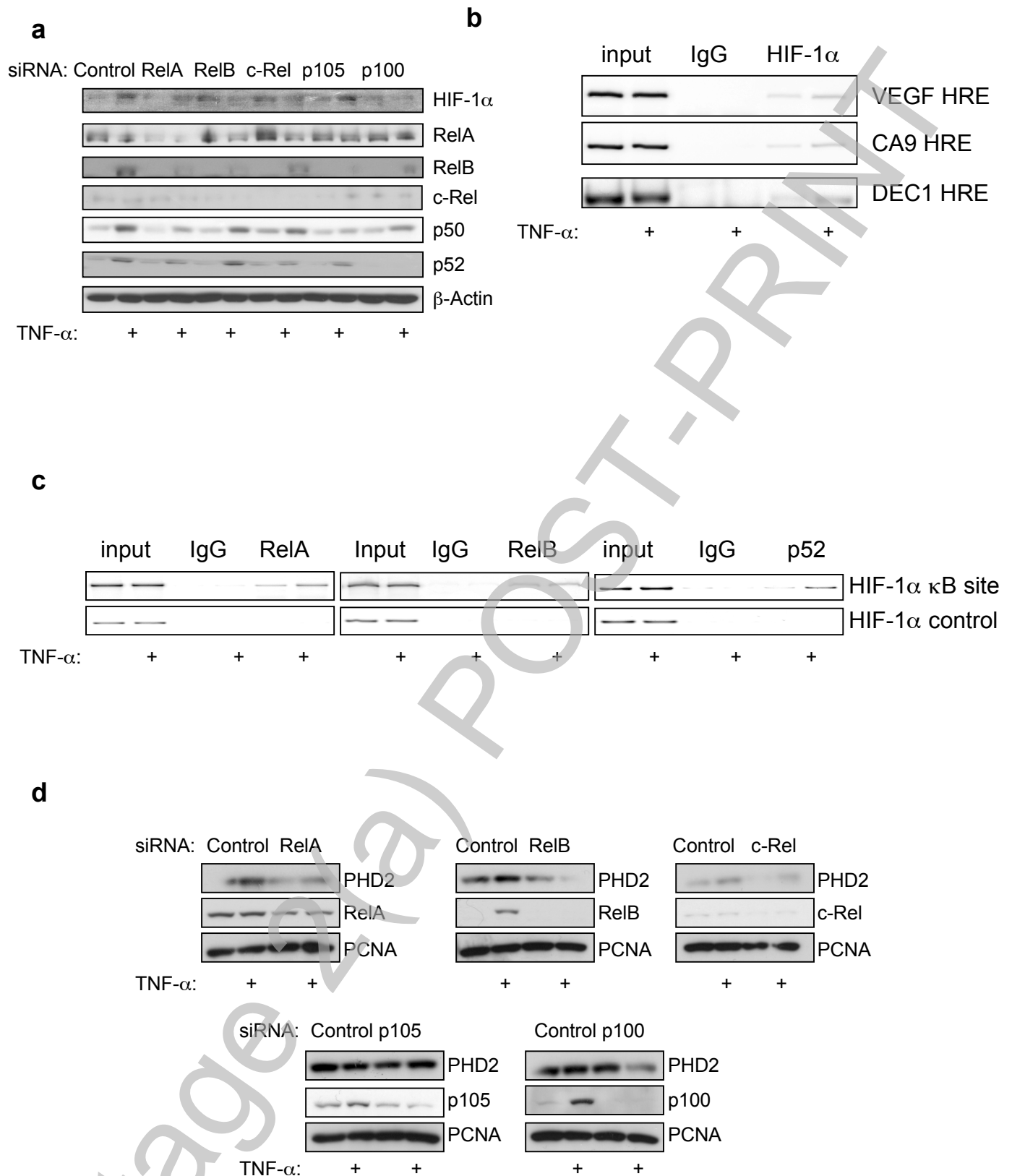


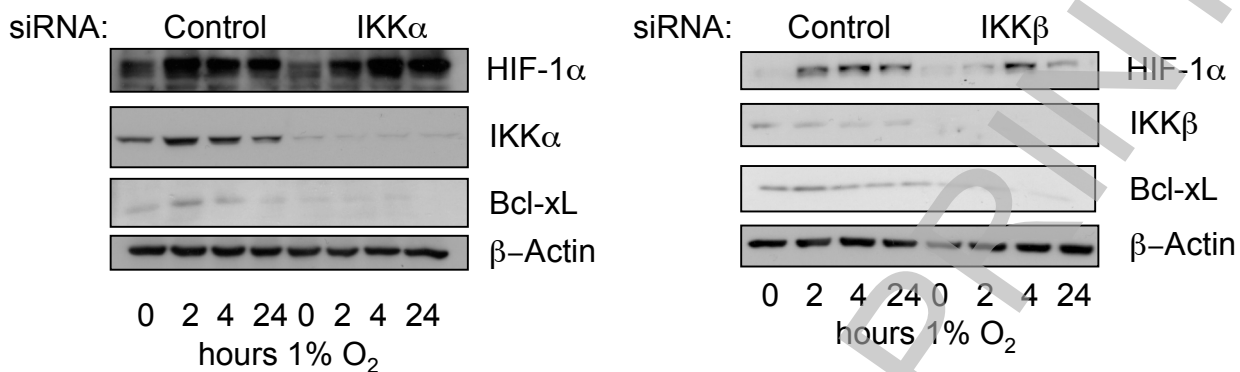
Figure 5 van Uden et al.



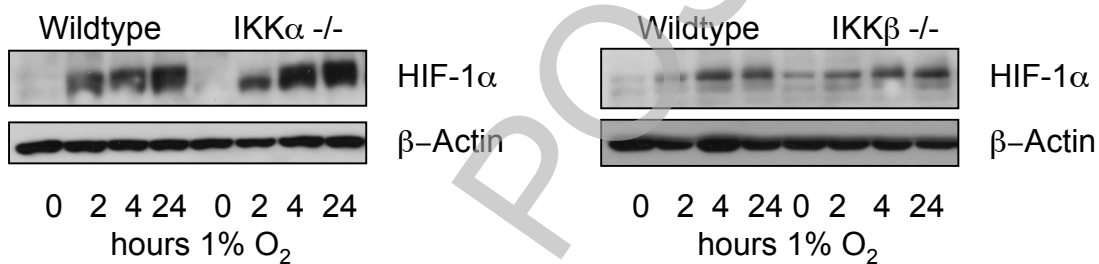
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Figure 6 van Uden et al.

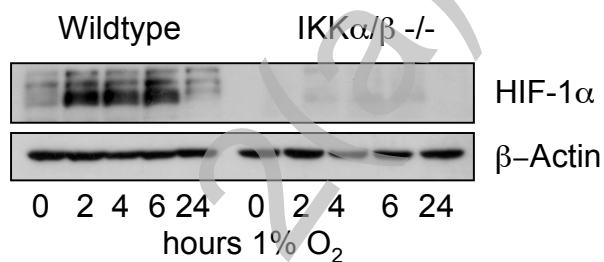
a



b



c



d

