Evolutionary Conserved Regulation of HIF-1β by NF-κB

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Abstract

Hypoxia Inducible Factor-1 (HIF-1) is essential for mammalian development and is the principal transcription factor activated by low oxygen tensions. HIF-α subunit quantities and their associated activity are regulated in a post-translational manner, through the concerted action of a class of enzymes called Prolyl Hydroxylases (PHDs) and Factor Inhibiting HIF (FIH) respectively. However, alternative modes of HIF-α regulation such as translation or transcription are under-investigated, and their importance has not been firmly established. Here, we demonstrate that NF-κB regulates the HIF pathway in a significant and evolutionary conserved manner. We demonstrate that NF-κB directly regulates HIF-1β mRNA and protein. In addition, we found that NF-κB–mediated changes in HIF-1β result in modulation of HIF-2α protein. HIF-1β overexpression can rescue HIF-2α protein levels following NF-κB depletion. Significantly, NF-κB regulates HIF-1β (tango) and HIF-α (sima) levels and activity (Hph/fatiga, ImpL3/ldha) in Drosophila, both in normoxia and hypoxia, indicating an evolutionary conserved mode of regulation. These results reveal a novel mechanism of HIF regulation, with impact in the development of novel therapeutic strategies for HIF–related pathologies including ageing, ischemia, and cancer.

Introduction

Hypoxia Inducible Factor-1 (HIF-1) is a transcription factor which is part of a stress response mechanism that is initiated in the presence of low oxygen tensions. Moreover, HIF has been demonstrated to play key roles in development, physiological processes and pathological conditions as its presence affects cell cycle progression, survival, and metabolism [1,2]. The α subunits are mostly controlled post translationally, through the concerted action of a class of enzymes called Prolyl Hydroxylases (PHD1, 2 and 3). The proline hydroxylation of HIF-α, subsequently target the α subunit for VHL-dependent 26S-proteosomal degradation [3]. The oxygen dependent mechanism of HIF-α control is conserved in organisms such as worms [4–6] and flies [4,7–9], with homologues of HIF-α, HIF-1β and PHD being identified in these organisms. Multiple studies have thus demonstrated the importance of the O_2 and PHD-dependent control mechanism in an evolutionary context.

Although predominantly studied following hypoxic stress, HIF-α stabilisation is also found in non-hypoxic conditions through largely uncharacterised mechanisms [10,11]. However, recent studies have demonstrated that control of the HIF-1α gene by NF-κB provides an important, additional and parallel level of regulation over the HIF-1α pathway [12–15]. In the absence of NF-κB, the HIF-1α gene is not transcribed and hence no stabilisation and activity is seen even after prolonged hypoxia exposures [14,15]. NF-κB is the collective name for a transcription factor that exists as either a hetero- or homo-dimeric complex. The family of Rel homology domain containing genes (NF-κB) is composed of RelA (p65), RelB, cRel, p50 and its precursor p105 (NF-κB 1), and p52 and its precursor p100 (NF-κB 2). These subunits are predominantly sequestered in the inactive state in the cytoplasm, by members of the IκB family [16]. Upon activation, by compounds such as TNF-α, oncogenes or UV light; a kinase signaling cascade results in the IKK mediated phosphorylation of IκB and its subsequent poly-ubiquitin mediated proteasomal degradation. This allows for NF-κB release and translocation into the nucleus and binding to target gene promoters and enhancers [16–18]. Aberrantly active NF-κB has been associated with a number of human diseases, stimulating the pharmaceutical industry's interest in finding potential applications for NF-κB inhibition [19]. NF-κB homologues have been found in a number of species from sea squirt, frogs to flies (www.NF-kB.org). Significantly, NF-κB function in the immune system was greatly propelled by studies in Drosophila, where genetics helped delineate NF-κB's contribution to this important process [20,21].

Here, we have investigated the role of NF-κB in the control of the HIF pathway. We find that NF-κB controls HIF-1β and HIF-α levels in response to cytokine stimulation in both human and mouse cells. Importantly, we demonstrate that NF-κB controls HIF levels and activity in Drosophila. These results indicate that in likeness to the oxygen dependent regulation of HIF, NF-κB dependent control is an important and evolutionary conserved pathway. Furthermore, it places the HIF system as a downstream effector of NF-κB biological functions.

Results

TNF-α increases HIF-1β and HIF-2α protein expression

HIF is a major transcription factor responding to changes in oxygen levels within the cell [1]. However, it is also induced in normal oxygen conditions in response to cytokines and oncogene

Author Summary

The mechanisms by which cells and organisms respond to oxygen are of extreme importance for development and also for certain pathologies such as cancer, ageing, and ischemia. These are mediated by a family of transcription factors called hypoxia inducible factor (HIF), a factor that coordinates expression of a great number of genes. Significantly, these processes are evolutionary conserved from worms to humans. It is known that regulation of HIF occurs to a great extent through protein degradation. However, other important mechanisms of HIF control are currently being investigated. In this study, we have uncovered a novel mechanism of HIF regulation that relies on the action of another transcription factor family called NF-κB. We have found that NF-κB controls the levels of HIF-1α and HIF-1β genes by direct regulation. Furthermore, through its control of HIF-1β, NF-κB indirectly controls HIF-2α. Importantly, we find that this mechanism is conserved in *Drosophila* and mice. These results suggest an alternative avenue for therapeutic intervention in the HIF pathway, which has important implications for many human diseases.

activation [14]. A few studies have demonstrated that one of the mechanisms behind HIF activation in normoxia, occurs via increases in transcription of the HIF-1α gene [12–15]. We investigated how the HIF system as a whole responds to cytokine stimulation. For this purpose, a time course analysis of nuclear extracts, from cells previously exposed to TNF-α, was performed, and the levels of HIF-1β and HIF-2α were analysed by western blot (Figure 1A). We observed an increase in the nuclear levels of both HIF-1β and HIF-2α in response to TNF-α (Figure 1A). This could be explained by either an increase in nuclear translocation of these proteins, or an increase in the total levels. To discern between these options, whole cell lysates were also analysed for the levels of these proteins in response to TNF-α. TNF-α treatment resulted in increased total protein levels of both HIF-1β and HIF-2α (Figure 1B) in both cell lines tested.

TNF-α increases HIF-1β but not HIF-2α transcript levels

To investigate the mechanism behind HIF-1β and HIF-2α protein increases, the mRNA levels of these two genes following TNF-α exposure were analysed. qRT-PCR analysis indicated that in HEK293 cells or in HeLa cells, TNF-α treatment does not result in any significant increases of HIF-2α mRNA (Figure 1C). However, we found that HIF-1β transcript levels were induced in response to TNF-α in both cell types (Figure 1C).

IKK and NF-κB are required for TNF-α induction of HIF-1β mRNA

TNF-α activates the classical activation pathway that requires the IKK complex [17]. To test if TNF-α induced HIF-1β mRNA was due to IKK-dependent activation of NF-κB, cells were pre-treated with the IKK inhibitor Bay 11 7082 for 30 minutes, prior to TNF-α treatment. Total RNA was extracted and HIF-1β mRNA was measured using qRT-PCR. When IKK was inhibited TNF-α induced HIF-1β mRNA was greatly reduced (Figure 2A). Once again, we did not observe any effects of TNF-α on HIF-2α mRNA in the presence or absence of IKK activity. A significant reduction in the NF-κB subunit and RelA target, p100 mRNA in response to TNF-α, was also seen when IKK was inhibited (Figure 2A).

As IKK has NF-κB independent functions [22], we investigated the role of NF-κB in the control of HIF-1β. Using siRNA mediated depletion of RelA, TNF-α treatment was performed and the effects on the levels of HIF-1β and HIF-2α were analysed by qRT-PCR. The siRNA treatment was very effective at reducing the levels of RelA (Figure 2B). Importantly, depletion of RelA significantly impaired TNF-α mediated induction of HIF-1β and p100 mRNA (Figure 2B). HIF-2α mRNA levels were unaffected by RelA depletion in untreated and TNF-α treated cells (Figure 2B).

Since, RelA depletion only partially prevented TNF-α induction of HIF-1β, we assessed if other NF-κB subunits were also involved in this process. For this purpose, we targeted all NF-κB subunits concurrently, using a siRNA oligonucleotide directed against the Rel homology domain. This siRNA reduced the levels of all subunits of the NF-κB family (Figure S1A). Using RT-qPCR we analysed the levels of HIF-1β in both HEK293 and HeLa cells (Figure 3A). Importantly, depletion of all NF-κB subunits prevented TNF-α mediated induction of HIF-1β in both cell systems (Figure 3A). In addition it prevented the induction of HIF target genes such as GLUT3 and PGK1 (Figure 3B). As expected we did not observe any significant change in HIF-2α mRNA levels (Figure S1B). Consistent with our previous work, HIF-1α mRNA was also significantly decreased in the absence of NF-κB (Figure S1C) [14,15]. These results support our finding with the IKK inhibitor and RelA depletion in addition to confirming an appreciable level of redundancy within the NF-κB pathway. Taken together these results suggest that IKK-dependent activation of NF-κB, mediates the induction of HIF-1β mRNA following TNF-α treatment.

HIF-1β is a novel NF-κB target

TNF-α treatment induced HIF-1β protein and mRNA in an IKK-NF-κB dependent manner (Figure 1, Figure 2, Figure 3). As NF-κB is a transcription factor, we wanted to determine if this control was direct. Therefore, we analysed the HIF-1β promoter for possible NF-κB binding sites, using MatInspector (www.genomatix.de). Our analysis revealed that there are three putative NF-κB binding sites, which are located at –36 bp, 59 bp, and 354 bp from the transcription start site. We designed primer sets encompassing these sites as well as a negative control region within the first intronic region of the gene. Chromatin Immunoprecipitation assays using antibodies specific for NF-κB subunits were used in extracts derived from cells untreated or treated with TNF-α for 24 h. PCRs were performed using two sets of primers and products were resolved on agarose gels. The results show that RelA is actively recruited to site 1 within the HIF-1β promoter following TNF-α treatment, while no binding was observed when the control region of the HIF-1β gene was analysed (Figure 4A). Furthermore, recruitment of an active marker of transcription, polymerase II, was also observed at the HIF-1β promoter regions where the NF-κB binding sites are located, following TNF-α treatment (Figure 4B).

As we had observed an active recruitment of NF-κB to the HIF-1β promoter following TNF-α treatment, we sought to formally demonstrate this occurred in an IKK-dependent manner. As such, cells were treated with the IKK inhibitor Bay 11 7082, prior to treatment with TNF-α and ChIPs were performed. TNF-α-dependent recruitment of RelA to the HIF-1β promoter was impaired in cells where IKK activity was reduced (Figure 4C). In addition, we could detect an IKK-dependent increase in binding of p50, c-Rel and p52 to the HIF-1β promoter (Figure S2A, S2B, S2C).
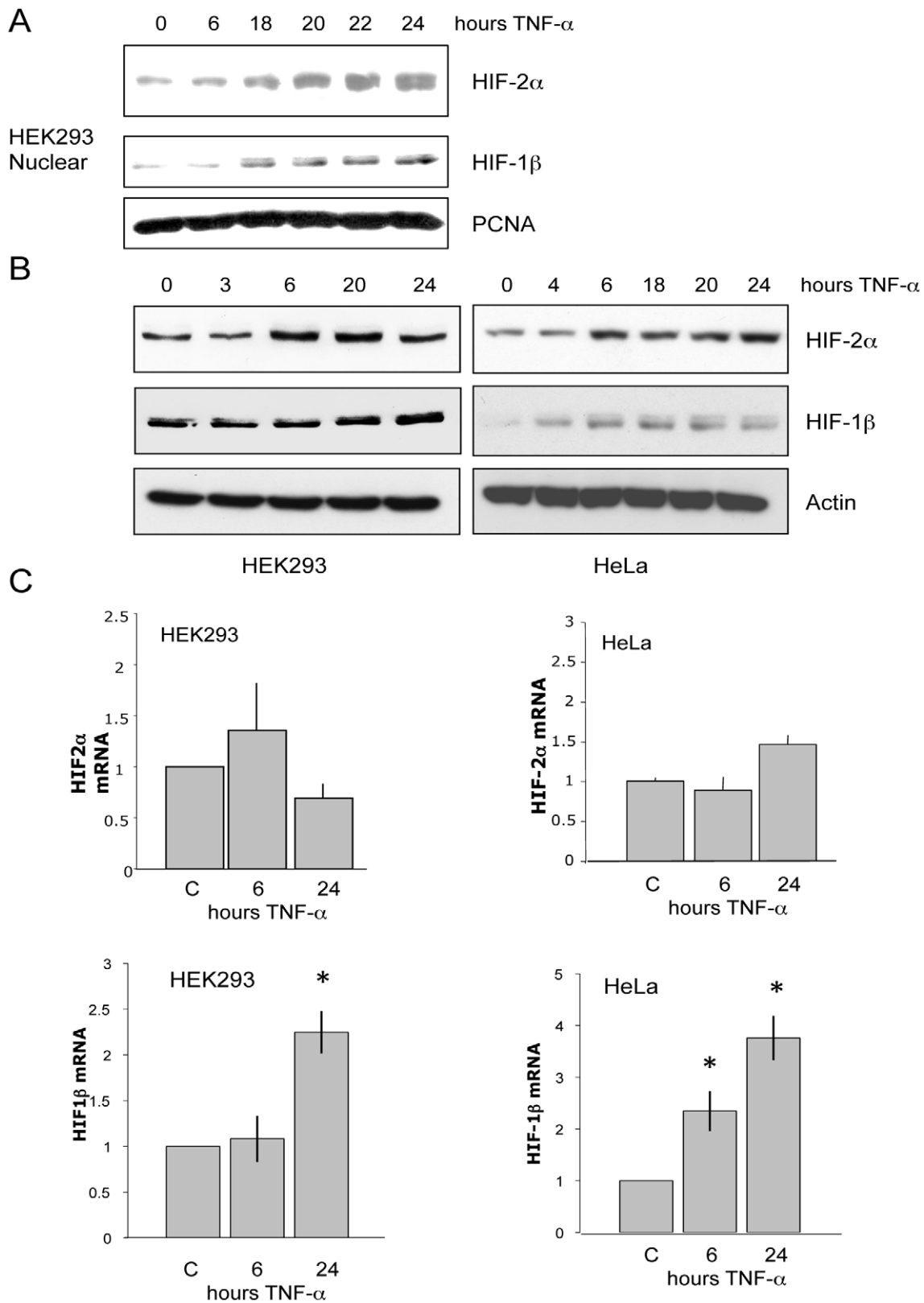
Figure 1. TNF-α induces HIF-1β and HIF-2α protein but only HIF-1β mRNA. (A) HEK293 were treated with 20 ng/mL TNF-α for the indicated times prior to nuclear extraction. Levels of HIF-2α and HIF-1b were analysed by Western Blot. PCNA was used as a loading control. (B) HEK293 and HeLa cells were treated as in *A*, prior to lysis. Whole cell lysates were analysed by Western Blot. (C) HEK293 and HeLa cells were treated with 20 ng/mL TNF-α for the indicated periods of times prior to total RNA extraction. Following cDNA synthesis, qPCR was performed for the levels of HIF-2α and HIF-1β.
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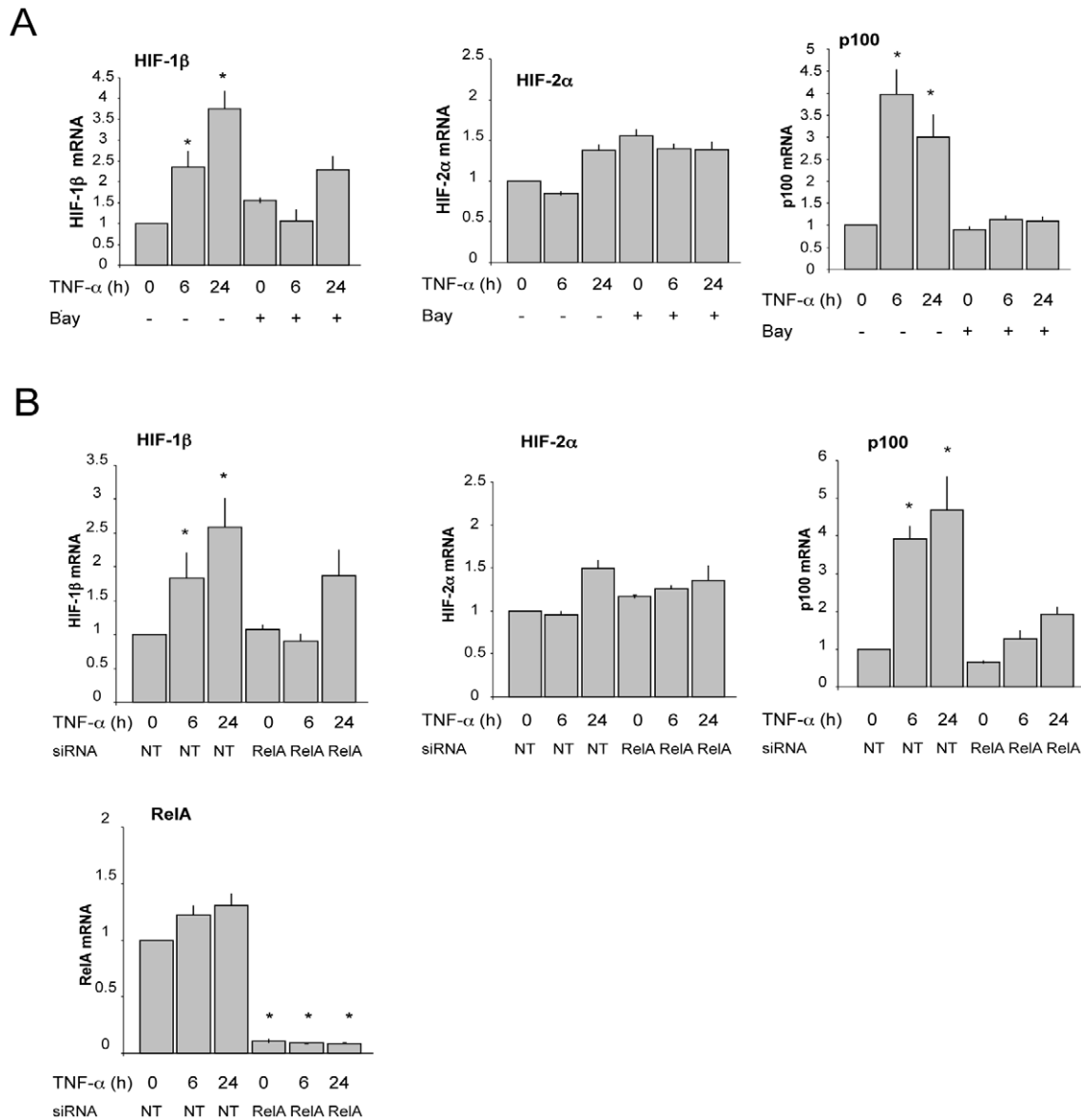
Figure 2. TNF-α–induced HIF-1β mRNA is IKK- and RelA-dependent. (A) HEK293 cells were pre-treated with the IKK inhibitor Bay 11 7082 prior to treatment with 20 ng/mL TNF-α for the indicated times. Total RNA was extracted, converted to cDNA, and qPCR was performed for the levels of HIF-1β, HIF-2α and p100. (B) HEK293 cells were transfected with control and RelA siRNA oligonucleotides for a total of 72 hours. Where indicated cells were treated with 20 ng/mL TNF-α prior to total RNA extraction. qPCR was performed for the levels of HIF-1β, HIF-2α, p100 and RelA.
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To determine the functional significance of the IKK-dependent recruitment of NF-κB to the HIF-1β promoter region, we examined if TNF-α induced polymerase loading was affected by IKK inhibition (Figure 4D). When the NF-κB pathway is inhibited, RNA polymerase II recruitment to the HIF-1β promoter is severely impaired (Figure 4D), indicating that NF-κB is a necessary factor for the formation of the RNA polymerase II transcriptional complex. These data show a direct regulation of the HIF-1β promoter by NF-κB.

## IKK and NF-κB are required for TNF-α induced HIF-1β and HIF-2α protein

Our results indicate that changing NF-κB activity and levels results in altered HIF-1β mRNA. We wanted to determine if this was also seen at the protein level. HeLa cells were treated with

IKK inhibitor prior to treatment with TNF-α. Analysis of the whole cell lysates demonstrated that in the absence of IKK activity, TNF-α treatment failed to induce HIF-1β expression (Figure 5A). These results support our mRNA analysis, where IKK inhibition resulted in impaired TNF-α induced HIF-1β (Figure 2A). We also analysed HIF-2α protein levels. Interestingly, inhibition of IKK also prevented TNF-α induced HIF-2α protein. These results suggest that IKK is controlling HIF-2α through a protein stabilisation mechanism and not as a result of increased HIF-2α mRNA production (Figure 2A and Figure 5A).

To further demonstrate the involvement of NF-κB in the control of HIF-1β and HIF-2α proteins, NF-κB subunits were depleted by siRNA (Figure 5B). Under conditions of NF-κB impairment, TNF-α treatment did not increase HIF-1β, HIF-1α or, HIF-2α protein levels in HeLa or HEK293 cells (Figure 5B).
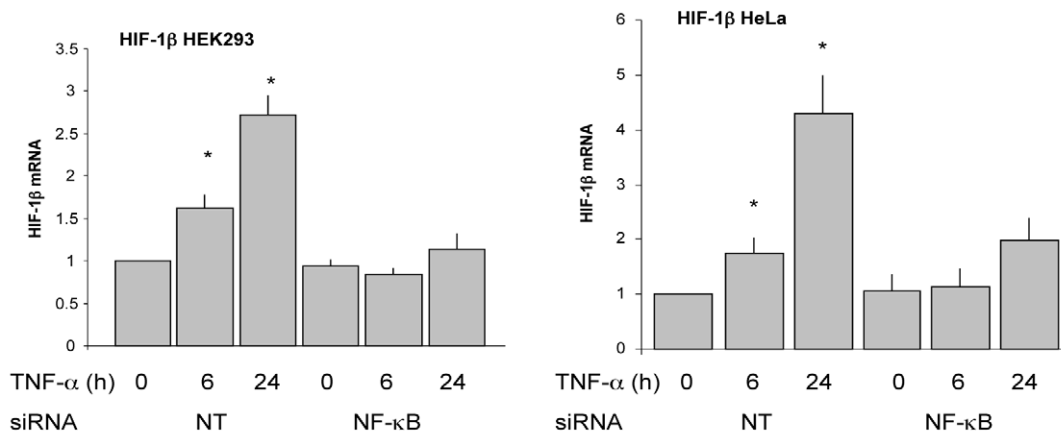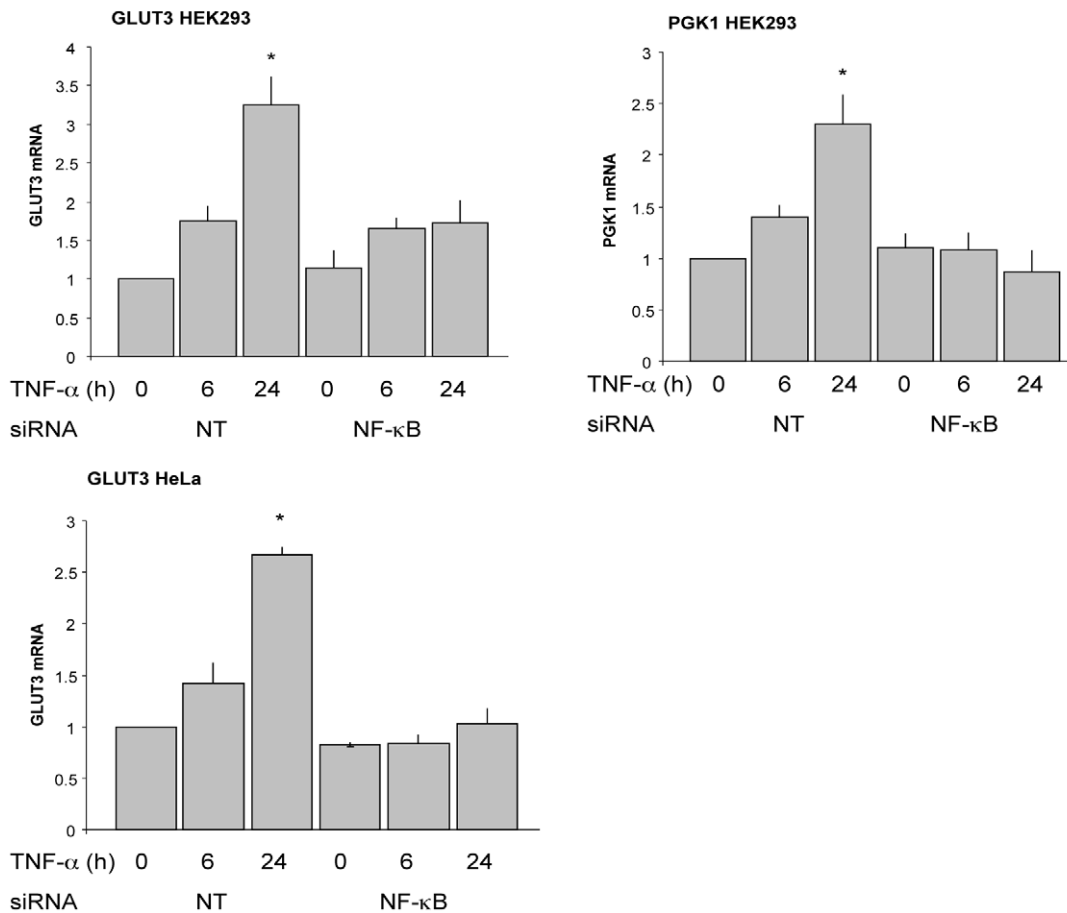
Figure 3. TNF-α–induced HIF mRNA levels and activity are NF-κB–dependent. (A) HEK293 and HeLa cells were transfected with control and pan-NF-κB siRNA oligonucleotides for a total of 72 hours. Where indicated cells were treated with 20 ng/mL TNF-α prior to total RNA extraction. qPCR was performed for the levels of HIF-1β. (B) Cells were treated and processed as in *A*, and qPCR was performed for the indicated genes.
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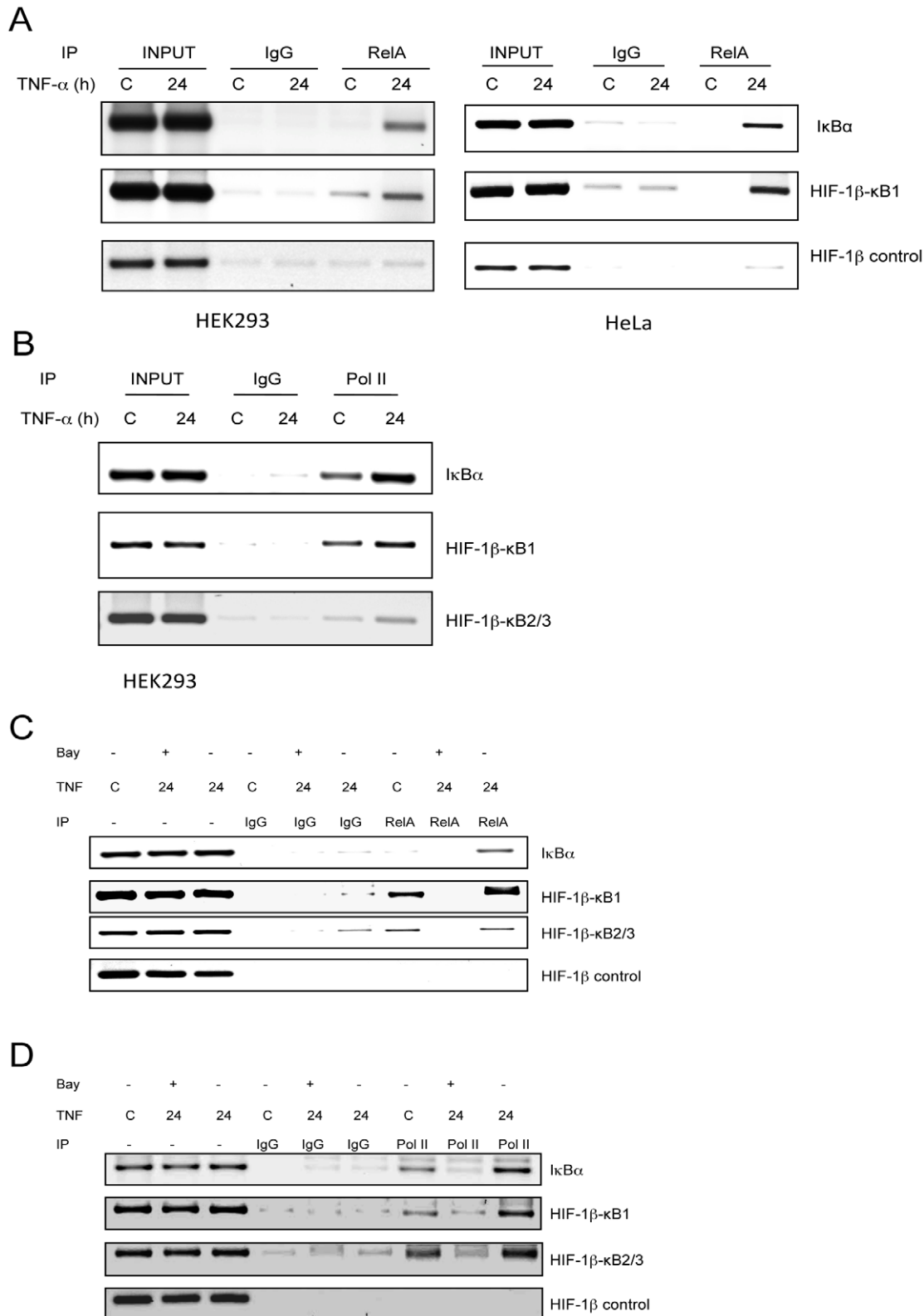
Figure 4. TNF-α induces RelA and RNA polymerase II recruitment to the HIF-1β promoter. (A) HEK293 and HeLa cells were treated with 20 ng/mL TNF-α for 24 hours prior to crosslinking and lysis. ChIPs were performed using the indicated antibodies and purified DNA was amplified using PCR primers for the HIF-1β promoter and control regions. The IκB-α promoter was used as a positive control. (B) ChIPs were performed for RNA polymerase II and purified DNA processed as in A. (C) HeLa cells were pre-treated with the IKK inhibitor Bay 11 7082 for 30 minutes prior to TNF-α treatment. Cells were crosslinked and lysed 24 hours later. ChIPs were performed for the levels of RelA. (D) ChIPs were performed for RNA polymerase II and purified DNA processed as in A. IκB-α promoter was used as a positive control.
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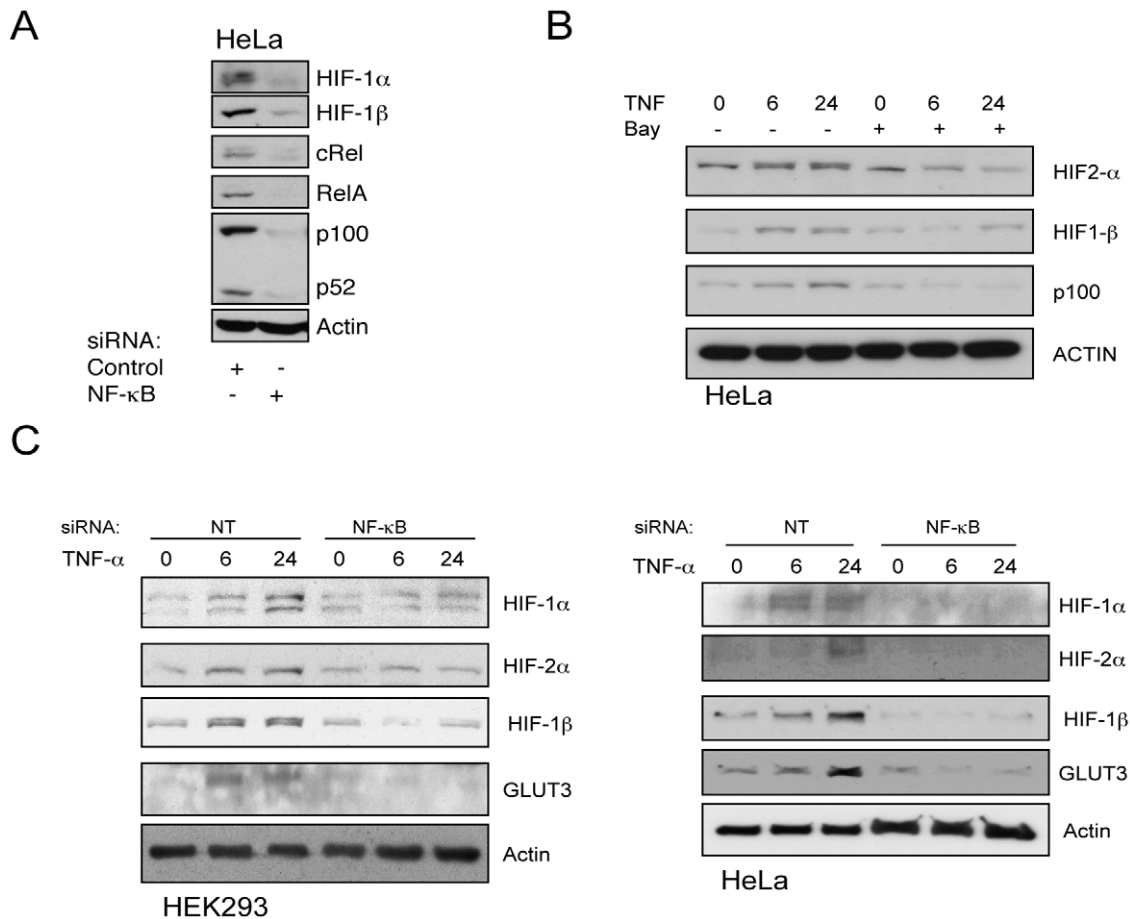
Figure 5. TNF-α–induced HIF-1β and HIF-2α proteins are IKK- and NF-κB–dependent. (A) HeLa cells were transfected with control or pan-NF-κB siRNA oligonucleotides and cultured for a total of 48 hours. Whole cell lysates were analysed by Western blot for the proteins mentioned. (B) HeLa cells were pre-treated with the IKK inhibitor Bay 11 7082 for 30 minutes prior to TNF-α treatment for the indicated periods of time prior to lysis. Whole cell lysates were analysed by Western blot for the proteins mentioned. (C) HEK293 and HeLa cells were transfected with control or pan-NF-κB siRNA oligonucleotides and cultured for a total of 72 hours. Where indicated, cells were treated with 20 ng/mL TNF-α prior to lysis. Whole cell lysates were analysed by Western blot for the proteins mentioned.
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Taken together these results indicate that IKK-NF-κB control HIF-1β, HIF-1α and HIF-2α proteins.

Changes in HIF-1β alter HIF-2α protein levels and result in AHR inhibition

TNF-α induces increases in HIF-1α [14], HIF-1β, and HIF-2α (Figure 1, Figure 5). We have demonstrated that HIF-1α and HIF-1β increases are both transcriptional ([14], Figure 2, Figure 3, Figure 4, Figure 5, Figure 6). However, we could not establish a direct link between NF-κB and HIF-2α. To investigate the mechanism behind HIF-2α stabilisation, we hypothesised that this was due to increased levels of its binding partner HIF-1β. HIF-1β increases could provide HIF-2α protection from proteolytic degradation. To test this hypothesis, we depleted HIF-1β directly, treated with TNF-α and analysed HIF-2α levels. In the absence of HIF-1β, TNF-α treatment did not result in any visible increases of HIF-2α protein in both HEK293 and HeLa cells (Figure 6A). These results suggest that HIF-1β levels can modulate HIF-2α subunit protein levels. Similar results were also obtained for HIF-1α, in U2OS cells exposed to hypoxia (Figure S3). To further investigate this mechanism, we performed gain of function experiments, where we overexpressed HIF-1β in the absence of any additional treatment. We could observe a dose dependent

increase of HIF-1β as expected (Figure 6B). Importantly, this was also evident in the levels of HIF-2α protein (Figure 6B). Taken together these results indicate that HIF-1β is necessary and sufficient for the stabilisation of HIF-2α.

We have found that NF-κB mediates the TNF-α induction of HIF-1β mRNA and protein. In addition, gain and loss of function experiments, presented in Figure 6A and 6B, demonstrate that modulation of HIF-1β levels has a direct impact on HIF-2α protein levels. To specifically demonstrate that NF-κB induction of HIF-2α protein is dependent on HIF-1β levels, we performed NF-κB depletion by siRNA and simultaneously increased HIF-1β by exogenous overexpression. Cell extracts were analysed for both HIF-1β and HIF-2α levels by western blot (Figure 6C). Our analysis revealed that HIF-1β overexpression is sufficient to rescue the effects of NF-κB depletion on HIF-2α levels (Figure 6C).

Apart from binding HIF-α subunits, HIF-1β also binds the transcription factor Aryl Hydrocarbon Receptor (AHR) [23]. AHR is responsible for activating genes involved in detoxification and xenobiotic metabolism [23]. To determine if TNF-α induced HIF-1β and consequentially HIF-1α and HIF-2α had any functional significance in the modulation of AHR, we analysed if HIF-1β changed binding partners under these conditions. We co-immunoprecipitated HIF-1β and determined the levels of
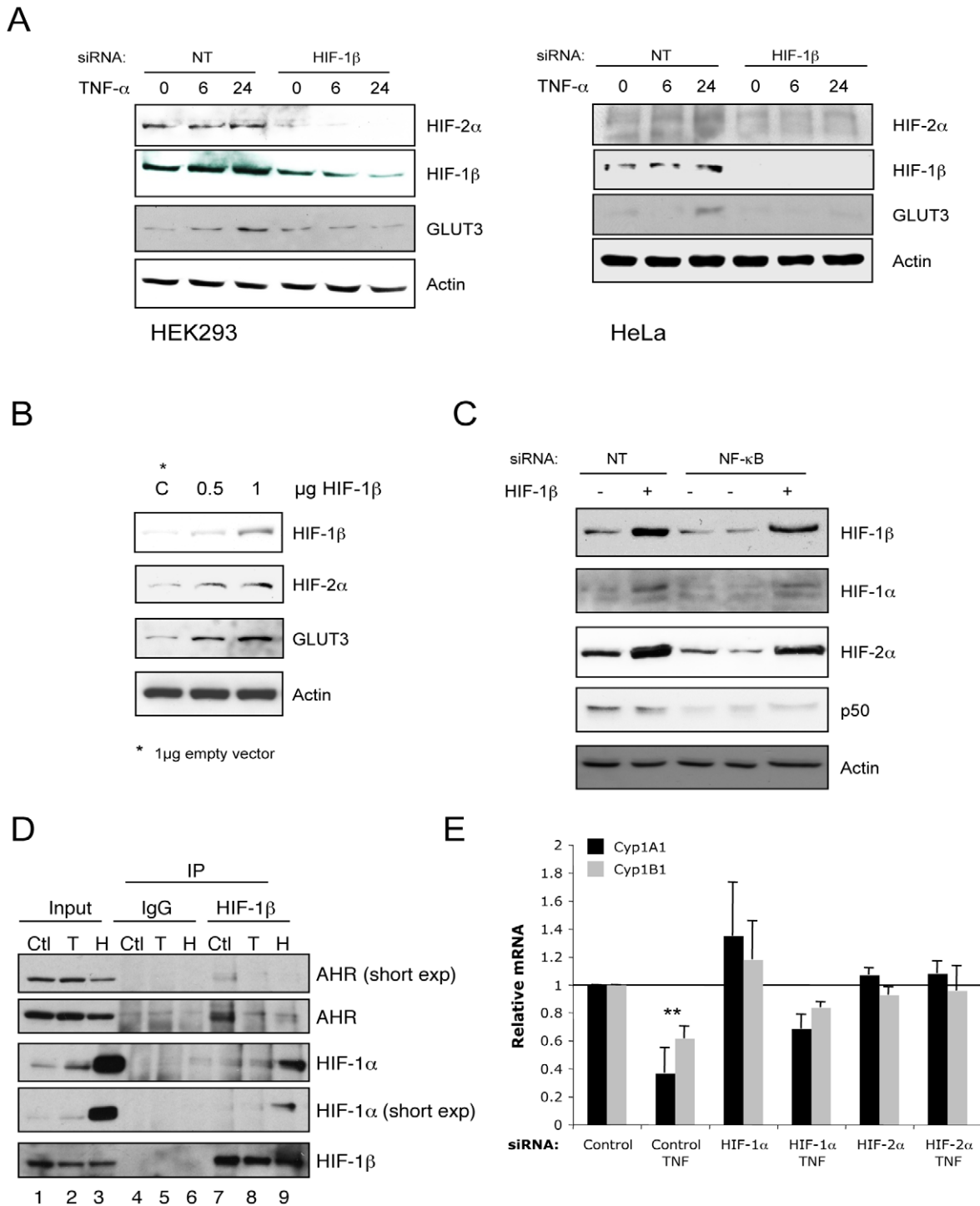
Figure 6. HIF-1β is required for TNF-α–induced HIF-2α, which represses AHR function. (A) HEK293 and HeLa cells were transfected with control or HIF-1β siRNA oligonucleotides and cultured for a total of 72 hours. Where indicated cells were treated with 20 ng/mL TNF-α prior to lysis. Whole cell lysates were analysed by Western blot for the proteins mentioned. (B) HEK293 cells were transfected with increasing amount of HIF-1β expression plasmid for 48 hours prior to lysates. Whole cell lysates were analysed by Western blot for the indicated proteins. (C) HEK293 cells were transfected with control or pan-NF-κB siRNA oligonucleotides and cultured for 72 hours. Where indicated cells were co-transfected with HIF-1β expression plasmids. Whole cell lysates were analysed by Western blot for the mentioned proteins. (D) HeLa cells were left untreated (Ctl) treated with 20 ng/mL TNF-α (T) or 1% O_2 (H) for 24 hours prior to lysis. 200 μg of protein were used to immunoprecipitate HIF-1β. Levels of associated proteins were assessed by western blot using the indicated antibodies. Input corresponds to 10% of starting material. (E) HeLa cells were transfected with control, HIF-1α or HIF-2α siRNA oligonucleotides for a total of 48 hours. Where indicated cells were treated with 20 ng/mL TNF-α prior to total RNA extraction. qPCR was performed for the levels of the indicated genes.
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associated HIF-1α and AHR (Figure 6D). Under non-stimulated conditions, HIF-1β is significantly associated with AHR (Figure 6D, lane 7), however following TNF-α or hypoxia HIF-1β dissociates from AHR (Figure 6D, lane 8 and 9). This suggests that in response to TNF-α or hypoxia AHR function is reduced. To test this hypothesis, we assessed if TNF-α treatment had any effect on the levels of two AHR target genes, CYP1A1 and CYP1B1 (Figure S4A). Our analysis revealed that TNF-α treatment resulted in a time-dependent reduction in the levels of these two genes. Given that TNF-α induces HIF activity and a dissociation of HIF-1β from AHR, we assessed if the levels of CYP1A1 and CYP1B1 could be rescued by HIF-α depletion (Figure 6E). HIF-α depletion was effectively achieved (Figure S4B). Interestingly, the levels of CYP1A1 and CYP1B1 were not reduced by TNF-α treatment when HIF-2α was depleted. Knockdown of HIF-1α resulted in a partial recovery of these two genes (Figure 6E). Taken together these results indicate that TNF-α induced HIF-1β results in higher HIF-α activity and reduced AHR function, suggesting an impaired xenobiotic metabolism.

NF-κB mediated control of the HIF pathway is conserved in mouse and Drosophila

Alignment of the human and mouse HIF-1β promoter region revealed that these are well conserved (Figure S2C). Therefore, we hypothesised that in a murine model HIF-1β was likely to show a similar dependence on NF-κB. To test this hypothesis, Mouse Embryonic Fibroblasts (MEFs) were treated with TNF-α, and HIF-1β levels were analysed (Figure 7A). HIF-1β levels were increased after 24 hours of treatment. In addition, we treated MEFs with an IKK inhibitor (BAY11 7082) for 24 hours. Whole cell lysates were analysed by western blot and the results show that when NF-κB is inhibited, HIF-1β protein level is reduced (Figure 7B). In addition, analysis of IKKβ or IKKα/β null MEFs also revealed a reduction in HIF-1β levels when compared with wildtype MEFs (Figure 7C). Finally, we performed depletion of IKK subunits by siRNA in MEFs (Figure 7D). While depletion of IKKα and IKKβ did not alter the levels of HIF-1β, knockdown of IKKγ resulted in a visible decrease of the HIF-1β protein (Figure 7D). These results suggest that NF-κB dependent transcription of HIF-1β is conserved in mice.
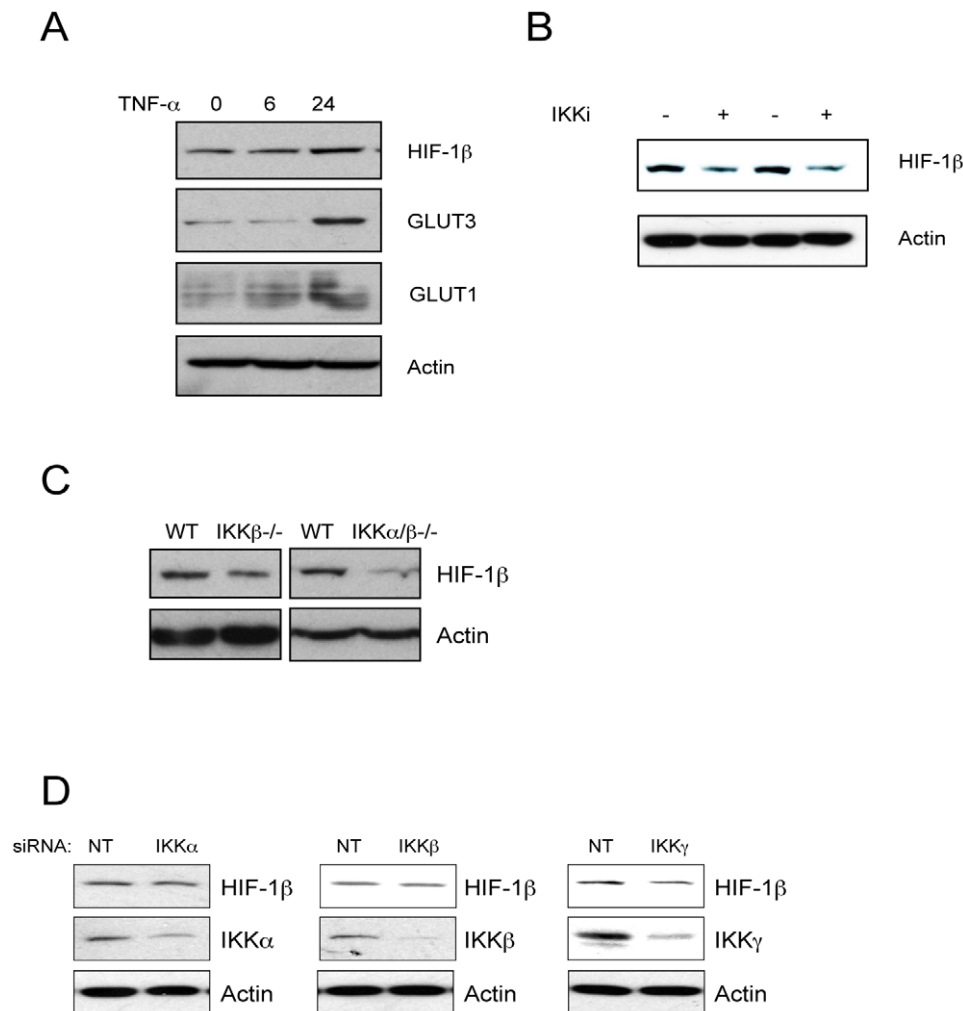
Figure 7. NF-κB–mediated control of the HIF system is conserved in mice. (A) Mouse embryo fibroblasts (MEFs) were treated with 20 ng/mL TNF-α for the indicated period of time prior to lysis. Whole cell lysates were analysed by Western blot. (B) MEFs were treated with the IKK inhibitor for 24 hours prior to lysis. Whole cell lysates were analysed by Western blot for the levels of HIF-1β. (C) Whole cell lysates were obtained from IKK wildtype (WT), IKKβ-/- or IKKα/β-/- MEFs and analysed by western blots. (D) MEFs were transfected with siRNA oligonucleotides for IKKα, IKKβ or IKKγ and the levels of HIF-1β were analysed by western blot.
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NF-κB and HIF are also conserved in insects [4,20]. The *Drosophila* genome encodes three NF-κB family members, Dorsal, Dif and Relish [21], and HIF homologues are encoded by the genes *similar* (*sima*; HIF-1α) and *tango* (HIF-1β) [4]. To test if the NF-κB-mediated regulation of the HIF pathway is also conserved and hence important in *Drosophila*, we analysed the levels of HIF-1α and HIF-1β mRNA, as well as the HIF target PHD/Fatiga (encoded by *Hph* in *Drosophila*) levels in control and flies mutant for loss-of-function alleles of *dorsal* (Figure 8A). Our analysis revealed that in dorsal mutant flies, both *sima* and *tango* mRNA levels are significantly reduced compared to wildType controls (Figure 8A), while an unrelated gene, *Iswi*, is unaffected in dorsal mutants. Importantly, the levels of the HIF target and PHD2 homologue *Hph/fatiga* are also reduced (Figure 8A), indicating that the changes observed in mRNA are also functionality translated into lower target gene activation.

Given that *Drosophila* possesses two additional NF-κB members (Dif and Relish), we next determined if these also contributed to the control of HIF levels and activity in the fly. While loss of *dif*, resulted in reduced levels of *sima* and *tango*, it had no effect in the levels of the target genes *Hph/fatiga* and *ImpL3/ldha* (Figure 8B). On the other hand, loss of *relish* resulted in higher levels of *sima* and *tango* and also Sima targets *Hph/fatiga* and *ImpL3/ldha*, suggesting that relish acts as a repressor of these genes.

To further analyse the role of NF-κB in the control of the HIF system in *Drosophila*, we performed gain of function experiments using ubiquitous overexpression of *dorsal*, and flies hemizygous for a loss of function allele of *cactus*, the fly homologue of IκB. In both of these genotypes, we observed an increase in the levels of *sima* and *tango* and their target genes *Hph/fatiga* and *ImpL3/ldha* (Figure 8C). These data suggest that activated Dorsal is able to induce the expression of HIF in the fly.

A recent study using *Drosophila* S2 cells has demonstrated that hypoxia induces increases in *sima* mRNA [24]. Given our findings, we next assessed if in adult flies, NF-κB was responsible for hypoxia induced *sima* mRNA and activity. For this purpose, we exposed adult flies to 5% O_2 for 24 hours prior to mRNA extraction. We could confirm that like in S2 cells, hypoxia induces *sima* mRNA production in adult flies (Figure 8D). Interestingly, we also observed a significant increase in *tango* mRNA (Figure 8D). Importantly, these responses were abolished in *dorsal* loss of function flies (Figure 8D). Significantly, levels of the HIF target genes, *Hph/fatiga* and *ImpL3/ldha*, following hypoxia exposure, were significantly reduced in the absence of *dorsal* (Figure 8E). These results demonstrate that Dorsal is required for basal and hypoxia-induced HIF levels and activity in *Drosophila*.

All together our data demonstrate a novel control mechanism over the HIF system. By controlling HIF-1β, NF-κB is able to indirectly induce HIF-2α and thus has multiple levels of control over the HIF pathway (Figure 8F). Importantly, this is an evolutionary conserved mechanism, observed from flies to humans.

Discussion

In this report we demonstrate an evolutionary conserved and important mode of regulation of the HIF pathway by the transcription factor family NF-κB. We show that NF-κB can control the HIF pathway through several different mechanisms. Our results demonstrate that, in response to TNF-α, NF-κB controls the HIF-1β promoter directly (Figure 4). Depletion or inhibition of NF-κB activity results in low levels of HIF-1β and importantly, HIF-α proteins (Figure 6, Figure 7). Interestingly, we have identified a mechanism by which NF-κB can control HIF-2α

levels and activity. We show that HIF-1β is necessary and sufficient for HIF-α stability in normoxia and hypoxia (Figure 6). Significantly, we show that NF-κB-mediated regulation of the HIF system is conserved in mice and *Drosophila* (Figure 7, Figure 8).

It is well established that HIF-α subunits are tightly regulated in the cell. However, very few studies have investigated how HIF-1β is controlled, or if it responds to stimulation. Yet, HIF-1β importance for development, homeostasis and disease is underscored by the numerous studies where HIF-1β is deleted, systemically or conditionally [25-30]. Systemic deletion of HIF-1β results in very early embryonic lethality with defects in placental, yolk sac and hemapoetic vascularization [30]. Conditional deletion of HIF-1β has demonstrated important roles in skin and liver [25,26,28].

HIF-1β is also known as Aryl Hydrocarbon Nuclear Translocator (ARNT), and apart from binding HIF-α subunits it is also an important binding partner for other transcription factors such as Aryl Hydrocarbon Receptor (AHR), a central regulator of xenobiotic metabolism [23]. As such, HIF-1β plays a role in both hypoxic and xenobiotic responses [31]. Importantly, several studies have demonstrated that ARNT has higher affinity for the HIF-α subunits, hence hypoxia induces a displacement of ARNT from AHR [32]. Furthermore, hypoxia or HIF-α stabilisation leads to inhibition of AHR activity [33,34].

From our results, it would be predicted that NF-κB activation by TNF-α, apart from increasing HIF-1β and HIF-α subunits, would lead to the inhibition of AHR response. TNF-α and NF-kB have been shown previously to repress the expression of certain AHR targets such as CYP1A1, however, the mechanism behind such repression is not fully understood [35,36]. We have thus investigated this possibility. Our results demonstrate that TNF-α induces the displacement of ARNT from AHR to HIF-α (Figure 6D). Furthermore, we show that as published previously [35,36], TNF-α induces repression of AHR targets such as CYP1A1 and CYP1B1 (Figure 6E and Figure S4A). Importantly, this can be prevented by depletion of HIF-2α and, to some extent, by depletion of HIF-1α. These results indicate that hypoxia or inflammation will lead to a lowering of AHR activity and hence reduced xenobiotic metabolism.

Our results demonstrate a mechanism by which NF-κB can indirectly regulate HIF-2α protein levels. These events take place in normoxia, indicating a PHD independent mechanism. A few reports have analysed PHD-independent HIF-α degradation pathways. Amongst these, HSP90 and RACK1 compete for HIF-1α binding and hence modulate stability [37–39]. In these studies, HIF-1α degradation despite being PHD-VHL-independent is still proteasome-dependent [38,39]. Similarly, GSK3 mediated degradation of HIF-1α is also VHL-independent but relies on the proteasome [40]. These are a few examples of direct regulation of the HIF-α subunits. Our analysis revealed that HIF-1β protects HIF-α subunits from degradation (Figure 6). HIF-1β disruption by both curcumin and EF24 results in proteasome independent degradation of HIF-1α even in hypoxia. How curcumin regulates HIF-1β is still a matter of debate. While some studies indicate the involvement of ubiquitin and the proteasome [41], other studies demonstrate that curcumin disrupts HIF-1β independent of the proteasome [42]. Of note, curcumin is known to inhibit NF-κB in many different cellular backgrounds [43], however, whether HIF-1β mRNA levels decrease in curcumin treated cells has not been investigated.

HIF-2α levels have been shown to be high in macrophages at normoxic levels and also in tumour areas, where near normal levels of oxygen are present [44]. The mechanism behind the increased HIF-2α levels in these areas has not been delineated thus
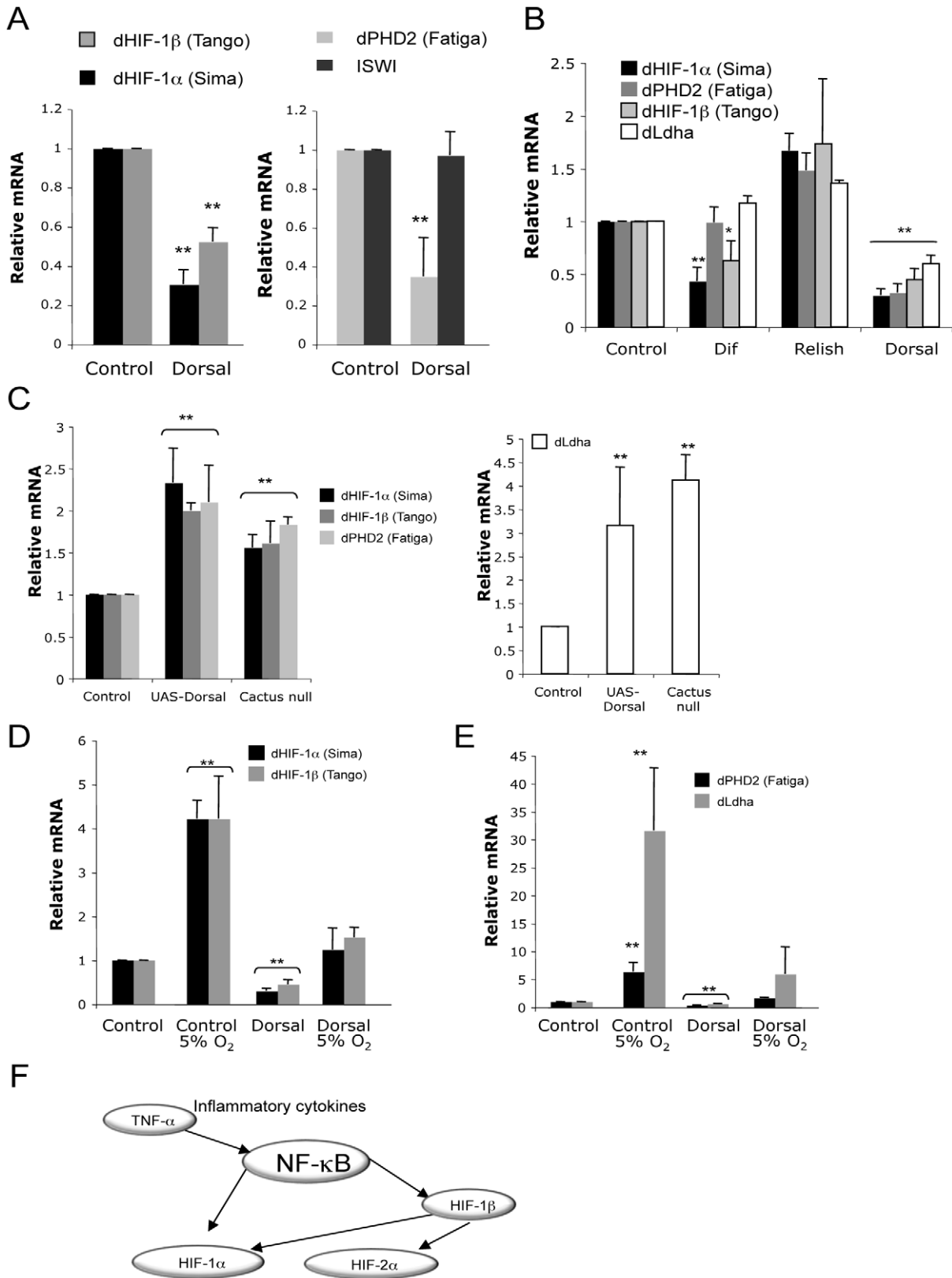
Figure 8. NF-κB–mediated control of the HIF system is conserved in *Drosophila*. (A) Total RNA was extracted from control and Dorsal null flies, and qPCR was performed for the levels of Drosophila HIF-1α, HIF-1β, PHD and ISWI. (B) Total RNA was extracted from control Dif, Relish and Dorsal null flies, and qPCR was performed for the levels of the indicated genes. (C). Total RNA was extracted from control, UAS-Dorsal or Cactus null flies, and qPCR was performed for the levels of the indicated genes. (D) Total RNA was extracted from control and Dorsal null flies exposed or not to 5% O_2 for 24 hours. qPCR was performed for the levels of Drosophila HIF-1α, HIF-1β. (E) As in *D*, but qPCR was performed for the levels of Drosophila PHD and Ldha. (F) Proposed model of NF-κB modulation of the HIF pathway. NF-κB directly regulates HIF-1α and HIF-1β genes. NF-κB induced HIF-1β mediates HIF-2α stability.

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far. While it is possible, that PHD inhibition might take place, our results demonstrate that NF-κB-mediated increases in HIF-1β, induce HIF-2α protein, provide an alternative explanation. However, the levels of HIF-1β under the conditions presented in the published studies, have not been investigated.

NF-κB and the HIF pathways are conserved in flies [4,20]. While the VHL-PHD-dependent regulation has been shown to be conserved in *Drosophila* [4], the relevance and conservation of NF-κB-mediated control over HIF has not been previously established. Our analysis of *dorsal* mutant flies (c-Rel homologue), demonstrates that Dorsal controls the levels and activity of HIF also in *Drosophila* (Figure 8). We also found that *dorsal*, *sima* and *tango* are expressed in the head of the fly, a tissue we used for our analysis (Figure S5).

Loss of function of *dif*, although reducing the levels of *sima* and *tango*, does not alter Sima activity (Figure 8B), suggesting that the observed reduction in mRNA levels of the HIF subunits is not severe enough to have a functional effect at the protein level. Loss of Relish, an additional NF-κB family member in the fly, resulted in higher levels of *sima*, *tango* and its targets (Figure 8B). This suggests that Relish acts a repressor of these genes. In mammalian systems, it is well documented that p50 homodimers can act as repressor forms of NF-κB [45,46], this is consistent with our analysis of Relish mutants.

Our results also demonstrate that activation of NF-κB, assessed by loss of function of Cactus and overexpression of Dorsal, will lead to increased levels and activity of the HIF system (Figure 8C). This raises the intriguing questions of whether in flies HIF is also a part of the immune system and whether NF-κB mutations alter the HIF response in hypoxia. These questions should thus be addressed in future studies.

Recently, a study in *Drosophila* S2 cells demonstrated that the levels of sima mRNA were hypoxia inducible [24]. Given these findings, we determined the role of NF-κB in regulating this event. Our results demonstrate that in adult flies, as observed in S2 cells, hypoxia induces *sima* mRNA. Interestingly, we also observed increases in *tango* mRNA levels. More importantly, we found that these increases were dependent on Dorsal (Figure 8D). Functionally, inhibition of Dorsal led to significant reduction in the levels of hypoxia-induced Sima-dependent genes (Figure 8E). This suggests that NF-κB also plays a role in the regulation of hypoxia induced HIF in *Drosophila*. Furthermore, it reveals that, as we had observed in mammalian cells [47], NF-κB is activated following hypoxia in the fly.

In summary, our results provide new evidence of the extensive control of the HIF pathway by the Rel homology domain containing transcription factor family, NF-κB, which is often found deregulated in a number of human pathologies, including cancer, autoimmune diseases and diabetes.

Materials and Methods

Cells

Human embryonic kidney cells HEK293 and human cervix carcinoma HeLa cells were obtained from the ATCC. Mouse Embryonic Fibroblasts were a kind gift from Prof. Ron Hay. All cells were maintained at 5% CO_2 in Dulbecco's modified Eagle's medium (Lonza) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 1% penicillin-streptomycin (Lonza), and 1% L-glutamine (Lonza).

Treatments

TNF-α was obtained from Peprotech, dissolved in PBS and used at a final concentration of 20 ng/mL, subsequent to medium change. IKK inhibitor BAY 11-7082 and MG132 were purchased from Merck Biosciences, dissolved in DMSO and used at a final concentration of 20 μM.

Hypoxia treatments were performed in an InVIVO 300 hypoxia workstation (Ruskin, UK).

Plasmids

HIF-1β/ARNT expression plasmid was a kind gift from Prof. O. Hankinson (UCLA, USA) and was transfected into cells using the Calcium phosphate technique.

siRNA

siRNA oligonucleotides were purchased from MWG and used in a final concentration of 36 nM. siRNA oligonucleotides were transfected using the Calcium phosphate method (HEK293 and HeLa) or using interferin from Polyplus according to manufacturer's instructions.

siRNA sequences

Human
Control-5′ -aac agu cgc guu ugc gac ugg- 3′ [48]
RelA-5′ -gcu gau gug cac cga caa g- 3′ [48]
NF-κB-5′-AAG GUG CAG AAA GAG GAC A-3′
HIF-1β-5′-GGU CAG CAG UCU UCC AUG A-3′
HIF-1α-5′- CUG AUG ACC AGC AAC UU-3′ [15,49]
HIF-2α-5′- CAG CAU CUU UGA CAG U-3′
Mouse
IKKα, GCAGAAGAUUAUUGAUCUA [50]
IKKβ, UGACGUGAAGCAUCUAGUA [50]
IKKγ, GGAUUCGAGCAGUUAGUGA [50]

Drosophila strains and RNA preparation

Fly culture and husbandry was performed after standard protocols. The amorphic *dorsal* alleles, *dl^1* and *dl^4* [51] were crossed to obtain transheterozygous flies lacking *dorsal* function. To generate *cactus* loss of function flies, the amorphic *cact^1* [52] allele was crossed to *Df(2L)r10* and *cact^1* hemizygous adult flies were collected. For overexpression of *dorsal*, *P[matα4-GAL-VP16]* [53] was used to drive expression of P[UAS-dl.H]2. Adult flies homozygous for the loss of function alleles *Dif^1* and *rel^E20* were viable and fertile and maintained as stock [54,55]. As control an isogenized *white^1118* stock was used. Hypoxia treatment was performed for 24 hours at 25°C. For RNA preparations, groups of 70 adult flies were frozen in liquid nitrogen and heads were separated using a sieve. Total RNA was extracted using RNAeasy extraction kit (Qiagen, Germany).

Quantitative PCR analysis

Total RNA was extracted with the Nucleospin RNA II isolation system (Macherey Nagel; 740955) or Invisorb spin cell RNA (Invitek), according to the manufacturer's directions. RNA was converted to cDNA using Quantitect Reverse Transcription Kit (Qiagen). cDNA was used in semi-quantitative PCR. For quantitative PCR, Brilliant II Sybr green kit (Stratagene/Agilent), including specific MX3005P 96 well semi-skirted plates, were used to analyse samples on the Mx3005P QPCR platform (Stratagene/Agilent). Actin was used as a normalising gene in all experiments.

RT-qPCR sequences

Actin
For-CTGGGAGTGGGTGGAGGC
Rev-TCAACTGGTCTCAAGTCAGTG
GLUT3

For- CAA TGC TCC TGA GAA GAT CAT AA
Rev-AAA GCG GTT GAC GAA GAG T
HIF-1α
For- CAT AAA GTC TGC AAC ATG GAA GGT
Rev-ATT TGA TGG GTG AGG AAT GGG TT
HIF-2α
For- GCG CTA GAC TCC GAG AAC AT
Rev-TGG CCA CTT ACT ACC TGA CCC TT
HIF-1β
For-CAA GCC CCT TGA GAA GTC AG
Rev-GAG GGG CTA GGC CAC TAT TC
PGK1
For-AAG TGA AGC TCG AAA GCT TC TAT
Rev-AGG GAA AAG ATG CTT CTG GG
RelA
For-CTGCCGGGATGGCTTCTAT
Rev-CCGCTTCTTCACACACTGGAT
p100
For-AGCCTGGTAGACACGTACCG
Rev-CCGTACGCACTGTCTTCCTT
CYP1A1
For-GAGGCCAGAAGAAACTCCGT
Rev-CCCAGCTCAGCTCAGTACCT
CYP1B1
For-CTGCACTCGAGTCTGCACAT
Rev-TATCACTGACATCTTCGGCG
Fly primers.
drActin
For-GCGTTTTGTACAATTCGTCAGCAACC
Rev-GCACGCGAAACTGCAGCCAA
drHIF-1α (Sima)
For-AGCCCAATCTGCCGCCAACC
Rev-TGGAGGCCAGGTGGTGGGAC
drHIF-1β (Tango)
For-CGGCTGCTCATACGCCCGAG
Rev-GGCCAGCATGTGCGTCTGGT
drPHD (Fatiga)
For-TGGCCCGCCGAGGTAGACAA
Rev-CAGGCCCGTCTCCATCCCCA
drISWI
For-ACAGGTTGCACACTC GATTTTCAGT
Rev-CGGATGAACTGGTGGCCGCA
dLhda
For- CAGTTCGCAACGAACGCGCA
Rev- CAGCTCGCCCTGCAGCTTGT

Whole-cell protein lysates

Briefly, medium was aspirated and cells were washed once in PBS and then resuspended in modified high salt whole cell extraction buffer (20 mM HEPES pH7.6, 400 mM NaCl, 1 mM EDTA, 25% glycerol, 0.1% NP-40, 1 mM dithiothreitol (DTT), 1 mM PMSF, 5 mM NAF, 500 μM Na_3VO_4, and 1 protease inhibitor cocktail tablet (Roche) per 10 mL buffer). The cells were lysed by incubating them at 4°C on a rotating wheel for 20 minutes before centrifugation at maximum speed for 15 minutes at 4°C.

Western blotting

Following SDS-PAGE, gels were transferred onto polyinylidine difluoride (PVDF) membrane using a semi-dry transfer system. Unless otherwise stated, western blots were incubated in primary antibody at 4°C overnight, followed by a one hour incubation in an HRP-linked secondary antibody solution and developed using Enhanced chemo-luminescence (ECL) solution (PIERCE).

Immunoprecipitations

200 μg of whole cell lysates were used per immunoprecipitation condition. Protein lystates were incubated overnight with 1 μg of antibody in a rotating platform at 4C. 20 μl of packed protein-G-sepharose beads were used to recover the immune-complexes, by incubation for 2 hours at 4C in a rotating platform. Following 3 washes with PBS, complexes were eluted from the beads using 30 μl of SDS-loading buffer and boiled for 5 minutes prior to being run in SDS-PAGE gels.

Antibodies

Antibodies used were: anti-HIF-1α (MAB1536, R&D Systems and sc-10790, Santa Cruz), anti-HIF-1β (3718, Cell Signalling and sc-5580, Santa Cruz), HIF-2α (NB100-122, Novus Biologicals), anti-100/p52 polyclonal (sc-848, Santa Cruz), anti-RelA (sc-372, Santa Cruz), anti-p105/p50 (sc-7178, Santa Cruz)), anti-RelB (sc-48366, Santa Cruz), anti-cRel (sc-71, Santa Cruz) anti-PCNA (P8825, Sigma), anti-Actin (A5441, Sigma), anti-Pol II (sc-47701, Santa Cruz), anti-Glut3 (53520, AnaSpec), anti-AHR (SA-210, Enzo Life Sciences).

Chromatin immunoprecipitation (ChIP)

Cells were grown in a 150 mm plate to 70% confluency and cross-linked with 1% formaldehyde at room temperature for 10 minutes. Glycine was added to a final concentration of 0.125M for 5 m at room temperature. Cells were washed twice with 10 mL of ice-cold phosphate-buffered saline (PBS) and then scraped into 2 mL ice cold harvest buffer (PBS, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/mL leupeptin, 1 mg/mL aprotinin) before being centrifuged at 1000rpm in a Beckman & Coulter centrifuge at 4°C for 5 minutes. The supernatant was removed and the pellet was resuspended in 400 μL of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 1 mM PMSF, 1 mg/mL leupeptin, 1 mg/mL aprotinin) and left on ice for 10 minutes. Samples were then sonicated at 4°C eight times. Each sonication cycle lasted 15 seconds with a 30 second gap between each sonication using the 130W Sonics Vibracell. Supernatants were recovered by centrifugation at 12,000rpm in an eppendorf microfuge for 10 minutes at 4°C before being diluted 10 fold in dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1). Samples were then pre-cleared for 2 hours at 4°C with 2 mg of sheared salmon sperm DNA and 20 mL of protein A-Sepharose (50% slurry). At this stage, 10% of the material was kept and stored at –80°C as input material. Immunoprecipitations were performed overnight with specific antibodies (1–2 mg depending on antibody), with the addition of BRIJ-35 detergent to a final concentration of 0.1%. The immune complexes were captured by incubation with 30 mL of protein A-Sepharose (50% slurry) and 2 mg of single stranded sheared salmon sperm DNA (Sigma) for 1 hour at 4°C. The immunoprecipitates were washed sequentially for 5 m each at 4°C in Wash Buffer 1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), Wash Buffer 2 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), and Wash Buffer 3 (0.25M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1). Beads were washed twice with Tris-EDTA (TE) buffer and eluted with 100 mL of Elution Buffer (1% SDS, 0.1M NaHCO3). Eluates were purified using a DNA purification kit (NBS Biologicals). Samples were then amplified using semi-quantitative PCR and analysed.

ChIP PCR sequences

HIF-1β Control
For- TTC CAC CCA TCC CCC TAT TTT

Rev- ATG CAA AGC TGT TCA ATA GCA TAA
HIF-1β κB1
For-CCA GCG GGC AGA GGG TTA
Rev-ATC CCG GGG ACT TGG GTA GA
HIF-1β κB2/3
For-CGC GGA ATC AAA GAT GGC
Rev-GCC CTC CCT TCA CTG GAC
IκB-α
For-CTT AGA AGT CTG GGG AAA GCA AAT
Rev-GTA ATC CTG TCC CTC TGC AAG T

Statistical analysis

ANOVA and Student's t-tests were performed on the means, and p values were calculated. *, $p \leq 0.050$; **, $p \leq 0.010$ and ***, $p \leq 0.001$.

Additional methods

Additional materials and methods can be found in Text S1.

Supporting Information

Figure S1 Depletion of NF-κB results in reduction in HIF-1α but not HIF-2α mRNA levels. (A) U2OS and HEK293 cells were transfected with control and pan-NF-κB siRNA oligonucleotides for 48 hours prior to lysis. Whole cell lysates were analysed by immunoblot using the indicated antibodies. (B) HEK293 cells were tranfected with control and pan-NF-κB siRNA oligonucleotides for 48 hours prior to lysis. Where indicated cells were also treated with 20 ng/mL TNF-α prior to total RNA extraction. qPCR was performed for the levels of HIF-2α. (C) Cells were treated and processed as in B, but qPCR was performed for HIF-1α.
Found at: doi:10.1371/journal.pgen.1001285.s001 (0.34 MB TIF)

Figure S2 IKK inhibition prevents NF-κB recruitment to the HIF-1β promoter. HeLa cells were pre-treated with the IKK inhibitor Bay 11 7082 for 30 minutes prior to TNF-α treatment. Cells were crosslinked and lysed 24 hours later. ChIPs were performed for the levels of cRel (A), and p52 (B), present at the HIF-1β promoter. IκB-α promoter was used as a positive control. (C) Schematic diagram of the HIF-1β promoter with

sequence alignment of the κB sites from human, gorilla, and mouse.
Found at: doi:10.1371/journal.pgen.1001285.s002 (0.29 MB TIF)

Figure S3 HIF-1β is required for hypoxia induced HIF-1α. U2OS cell stably expression control and HIF-1β shRNA constructs were exposed to 1% O2 for the indicated periods of time prior to lysis. Whole cell lysates were analysed by immunoblot for the levels of the indicated proteins.
Found at: doi:10.1371/journal.pgen.1001285.s003 (0.21 MB TIF)

Figure S4 TNF-α represses AHR target genes via HIF-α subunit levels. (A) HeLa cells were treated with 20 ng/mL TNF-α for the indicated periods of time prior to RNA extraction. qPCR was performed for the levels of HIF-1α, HIF-1β, and the AHR targets CYP1A1 and CYP1B1. (B) HeLa cells were transfected with control, HIF-1α or HIF-2α siRNA oligonucleotides for a total of 48 hours. Where indicated cells were treated with 20 ng/mL TNF-α prior to total RNA extraction. qPCR was performed for the levels of the indicated genes.
Found at: doi:10.1371/journal.pgen.1001285.s004 (0.22 MB TIF)

Figure S5 Sima, Tango, and Dorsal mRNA analysis in development and adult tissues. qPCR analysis using Drosophila tissue array. mRNA levels were normalised to RPL32 gene.
Found at: doi:10.1371/journal.pgen.1001285.s005 (0.14 MB TIF)

Text S1 Supplementary methods.
Found at: doi:10.1371/journal.pgen.1001285.s006 (0.02 MB DOC)

Author Contributions

Conceived and designed the experiments: HAM SR. Performed the experiments: PvU NSK RW SR. Analyzed the data: PvU NSK HAM SR. Contributed reagents/materials/analysis tools: SM. Wrote the paper: SR.

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