Regulation of HIF-1 α Activity by Overexpression of Thioredoxin is Independent of Thioredoxin Reductase Status

Salvador Naranjo-Suarez^{1,4}, Bradley A. Carlson¹, Ryuta Tobe¹, Min-Hyuk Yoo¹, Petra A. Tsuji², Vadim N. Gladyshev³, and Dolph L. Hatfield^{1,*}

Under hypoxic conditions, cells activate a transcriptional response mainly driven by hypoxia-inducible factors (HIFs). HIF-1 α stabilization and activity are known to be regulated by thioredoxin 1 (Txn1), but how the thioredoxin system regulates the hypoxic response is unknown. By examining the effects of Txn1 overexpression on HIF-1 α function in HeLa, HT-29, MCF-7 and EMT6 cell lines, we found that this oxidoreductase did not stabilize HIF-1 α , yet could increase its activity. These effects were dependent on the redox function of Txn1. However, Txn1 deficiency did not affect HIF-1 α hypoxic-stabilization and activity, and overexpression of thioredoxin reductase 1 (TR1), the natural Txn1 reductase, had no influence on HIF-1 α activity. Moreover, overexpression of Txn1 in TR1 deficient HeLa and EMT6 cells was still able to increase HIF-1 α hypoxic activity. These results indicate that Txn1 is not essential for HIF-1 α hypoxic stabilization or activity, that its overexpression can increase HIF-1 α hypoxic activity, and that this effect is observed regardless of TR1 status. Thus, regulation of HIF-1 α by the thioredoxin system depends on the specific levels of this system's major components.

INTRODUCTION

Hypoxic conditions occur under physiological (e.g., embryogenesis, high altitude) or pathological conditions (e.g., cancer growth, ischemic diseases) (Wenger et al., 2005). Cells adapt to the decrease in oxygen levels by activating a complex transcriptional response that is mainly driven by the hypoxia inducible factor (HIF) (Semenza et al., 1992). HIF is a heterodimeric complex formed by an α subunit (HIF-1 α or HIF-2 α) and a β subunit (HIF- β , also known as ARNT) (the dimeric complex is designated HIF- α). The genes of both subunits are transcribed under both normoxic and hypoxic conditions. However, under normoxic conditions HIF- α is hydroxylated at two prolines. This

post-translational modification allows the interaction of HIF- α with VHL, which is the substrate recognition subunit of an E3 ubiquitin ligase complex (Ivan et al., 2001; Jaakkola et al., 2001). This interaction induces the ubiquitination of HIF- α , wherein HIF- α becomes targeted for degradation by the proteasome. HIF- α proline hydroxylation is carried out by three enzymes (EGLN1, EGLN2 or EGLN3) that require oxygen, iron and 2-oxoglutarate (Bruick et al., 2001; Epstein et al., 2001) which have been proposed to function as oxygen sensors that directly regulate HIF-1 α stability.

Besides HIF-1 α stabilization, hypoxic conditions also increase HIF-1 α transcriptional activity through the inhibition of another dioxygenase, factor-inhibiting hypoxia-inducible factor (FIH). Under normoxic conditions, FIH induces HIF-1 α hydroxylation at an asparagine residue located in the C-terminal transactivation domain (C-TAD) of HIF-1 α . This hydroxylation in C-TAD of HIF-1 α blocks its interaction with the p300 transcriptional co-activator and weakens HIF-1 α transcriptional activity under normoxic conditions (Lando et al., 2002a; 2002b).

In addition to the hydroxylation reactions on HIF-1 α , other regulatory pathways have been described to be important for the control of HIF-1 α levels or activity, such as reactive oxygen species (ROS) (Chandel et al., 1998), diacylglycerol kinase (Aragones et al., 2001), MAP kinase cascades (Conrad et al., 1999a; 1999b; Richard et al., 1999), Rac1 (Hirota et al., 2001), the PI3K/Akt pathway (Mazure et al., 1997; Zhong et al., 2000; Zundel et al., 2000) and thioredoxin (Trx) redox activity (Carrero et al., 2000; Ema et al., 1999; Huang et al., 1996; Welsh et al., 2002; Zhou et al., 2007). However, the mechanisms by which these pathways affect HIF-1 α stability or function have not been clarified and their implication for HIF-1 α signaling remains controversial (Alvarez-Tejado et al., 2002; Chua et al., 2010; Hoffman et al., 2007; Naranjo-Suarez et al., 2012; Srinivas et al., 2001; Vaux et al., 2001).

Thioredoxins (Txns) are a family of small proteins that undergo reversible oxidation through the transfer of reducing equivalent

¹Molecular Biology of Selenium Section, Laboratory of Cancer Prevention, Center for Cancer Research, National Institutes of Health, Bethesda, MD 20892, USA, ²Department of Biological Sciences, Towson University, Towson, MD 21252, USA, ³Division of Genetics, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston MA, 02115, USA, ⁴Present address: Department of Pathology, The Sol Goldman Pancreatic Cancer Research Center, Johns Hopkins Medical Institution, Baltimore, MD 21231, USA

*Correspondence: hatfield@mail.nih.gov

Received April 17, 2013; revised June 15, 2013; accepted June 17, 2013; published online August 1, 2013

Keywords: HIF-1 α stability, HIF transcriptional activity, thioredoxin 1, thioredoxin reductase 1

lents from cysteine residues on their catalytic site to, typically, a protein disulfide substrate. The oxidized Txn is then reduced by selenocysteine-containing NADPH-dependent flavoprotein thioredoxin reductases (TRs). Txns have been implicated in many cellular functions, such as cell growth, chemotaxis, antioxidant defense, redox state of cysteine residues in proteins, and regulation of transcription factors. Expression of Txn1 has been described to increase HIF-1 α DNA binding and its transcriptional activity (Carrero et al., 2000; Ema et al., 1999; Huang et al., 1996; Welsh et al., 2002; Zhou et al., 2007), and a possible mechanism in which Txn reduces HIF-1 α through the nuclear redox regulator, redox factor-1 (Ref-1) has been suggested (Ema et al., 1999). More recent studies have proposed that Txn1 overexpression increases not only HIF-1 α activity, but also HIF-1 α stability, in various mammalian cell lines (Welsh et al., 2002; Zhou et al., 2007).

Herein, we investigated the effect of Txn1 overexpression on HIF-1 α activity and stability in three human cancer cell lines (HeLa, HT-29 and MCF-7) and a mouse breast cancer cell line (EMT6). Under hypoxic conditions, we found an increase in HIF-1 α activity in HeLa, HT-29 and EMT6 cells, but not in MCF-7 cells. However, no effect on HIF-1 α stability was observed in these four cell lines. Overexpression of TR1, which is the only known enzyme to reduce Txn1, did not appear to have an effect on HIF-1 α stability or activity. Interestingly, HIF-1 α stability or activity was not affected after Txn1 or TR1 loss and the effect of Txn1 overexpression on HIF-1 α activity was observed even in the absence of TR1.

MATERIALS AND METHODS

Cell culture and reagents

HeLa, MCF-7 and HT-29 cells were grown in Dulbecco's Minimum Essential Medium (DMEM) (Invitrogen, USA) supplemented with 10% fetal bovine serum (Gibco Invitrogen) and the mouse breast carcinoma cell line, EMT6, was grown in Waymouth's Medium (Invitrogen) supplemented with 15% fetal bovine serum. Antibiotic-antimycotic solution (Invitrogen) was added to DMEM and Waymouth culture media, and cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C. Hypoxia (1% O₂) was induced by culturing cells inside a modular incubator chamber (model MIC-101, Billups-Rothenberg Inc., USA) infused with a mixture of 1% O₂, 5% CO₂, and 94% N₂. For experimental purposes, all cells were cultured under hypoxic or normoxic conditions for 12 h.

Constructs

For Txn1 overexpression, RNA extracted from HeLa cells was retrotranscribed using Imprim-1 reverse transcription system (Promega, USA) following the manufacturer's instructions. The cDNA obtained was used as template for a PCR reaction using primers 1 and 2 (Supplementary Table 1). Amplified Txn1 was cloned into pRV-IRES-GFP (kindly provided by Dr. Antonio Bernad, CNIC, Spain) and into pRV-IRES-Puro using BamHI and XhoI restriction sites. For TR1 overexpression, TR1 cDNA was cloned as described (Naranjo-Suarez et al., 2012). Mutant Txn1 cDNA was amplified by PCR with primers 5 and 6 (Supplementary Table 1) using Txn1 His C32S C35S (Addgene plasmid #21286) as template. The PCR product was then cloned in pRV-IRES-Puro using BamHI and XhoI restriction sites. RNAi target sequences for knocking down mouse TR1 and Txn1 have been reported (Tobe et al., 2012; Yoo et al., 2006). For human TR1 and Txn1 knockdown, RNAi target sequences were designed using siDesign Center (Thermo Scien-

tific) (Supplementary Table 1). Sense-antisense primers for the target genes were annealed and cloned into the *BglIII-HindIII* sites in pSuper-Retro-Puro following manufacturer's instructions (Oligoengine, USA). Retroviral production and cell line infection were carried out as described (Naranjo-Suarez et al., 2012).

Western blotting

Cells were washed with ice-cold Dulbecco's phosphate-buffered saline (Invitrogen) and harvested with Mammalian Protein Extraction Reagent (M-PER) (Thermo Scientific, USA). Protein concentrations in cell extracts were measured using the BCA protein assay reagent (Thermo Scientific). NuPAGE 4-12% Bis-Tris gels (Invitrogen) were used for resolving protein lysates. Nitrocellulose membranes (Invitrogen) were blocked with 5% non-fat dry milk in TBS-T (Santa Cruz, USA) and incubated overnight at 4°C with the indicated antibodies [anti-HIF-1 α , MAB1536 from R&D Systems, (USA); monoclonal anti-TR1 Epitomics, (USA); polyclonal anti- β -actin, sc-1615 from Santa Cruz.; polyclonal anti-Txn1 from Abcam, (USA)]. Immunolabeling was detected by enhanced chemiluminescence (SuperSignal West Dura, Thermo Scientific) and visualized with a digital luminescent image analyzer (Fujifilm LAS-3000, Japan).

Quantitative real-time PCR

Total RNA was extracted from cells using RNeasy mini Kit (Qiagen, USA). cDNA was synthesized from 2 μ g of total RNA using Imprim II Reverse Transcriptase (Promega). For real-time quantitative PCR, 1 μ l of cDNA was used as template in a 25 μ l reaction carried out with the POWER SYBR Master Mix Kit (Applied Biosystems, USA) following the manufacturer's instructions. PCR amplifications were carried out in MyiQ Two-Color Real-Time PCR Detection System (BioRad, USA). Primers for the amplification of vascular endothelial growth factor (VEGF) and adrenomedullin (ADM) were designed as described (Naranjo-Suarez et al., 2012). Primer's sequences for the amplification of HIF-1 are described in Supplementary Table 1. The mRNA levels of target genes were calculated relative to the expression of β -actin.

Flow cytometry

Cells were infected with pRV-IRES-GFP or pRV-Txn1-IRES-GFP. After 48 h, EGFP expression was analyzed by flow cytometry in a FACSCalibur apparatus (BD). Data are presented as green fluorescence intensity versus cell number.

Statistical analyses

Data are presented as the mean $\pm$ S.D. Differences between means were determined by Student's *t*-test using Microsoft Excel and considered statistically significant at $p \leq 0.05$.

RESULTS

Effects of Txn-1 overexpression in HIF-1 α stabilization and activity

Txn1 was overexpressed in HeLa, MCF-7, HT-29 and EMT6 cells to determine whether this oxidoreductase had an effect on HIF-1 α levels or activity. These cells and the corresponding control cells were exposed to normoxic or hypoxic conditions. Hypoxic conditions increased HIF-1 α levels in each cell line examined (Fig. 1A). Overexpression of Txn1 did not have an effect on HIF-1 α protein levels, neither in normoxic nor in hypoxic conditions. In agreement with this observation, we did not find any change in HIF-1 mRNA levels following Txn1 overex-

Fig. 1. HIF-1 α levels and activity in Txn1 overexpressing cells. HeLa, MCF-7, HT-29 and EMT6 cells were infected with retroviruses encoding Txn1 (Txn1) or empty retroviruses (Ctrl) and cultured for 12 h in normoxic (Nx) or hypoxic (Hx) conditions. (A) HIF-1 α , Txn1 and β -actin levels from control or Txn1 overexpressing cells (Txn1) were analyzed by Western blotting. (B) VEGF and ADM mRNA levels were measured by quantitative RT-PCR to determine HIF-1 α activity. Data represent the fold-change relative to normoxic values in control cells and normalized to β -actin. The mean $\pm$ S.D. of three independent experiments is shown (*= $p \leq 0.05$).

pression (Supplementary Fig. 1). To examine the effects of Txn1 overexpression on HIF-1 α activity, we determined the mRNA levels, in normoxic and hypoxic conditions, of two genes, VEGF and ADM, which are angiogenic growth factors induced by HIF-1 α (Comier-Regard et al., 1998; Forsythe et al., 1996; Garayoa et al., 2000). We found that the hypoxic-induction of VEGF and ADM mRNA was significantly increased in HeLa, HT-29 and EMT6 cells overexpressing Txn1, but not in MCF-7 cells (Fig. 1B). Contrary to earlier studies (Welsh et al., 2002; Zhou et al., 2007), these results show that Txn1 overexpression affects hypoxic HIF-1 α activity, but not stability under normoxic or hypoxic conditions, in some cell lines.

Since the largest effects of Txn1 overexpression on VEGF and ADM hypoxic-induction were observed in HeLa and EMT6 cells, we focused on these cell lines in examining Txn1 effects on HIF-1 α . We next generated HeLa and EMT6 cells overexpressing a mutant of Txn1 in which the catalytic cysteines C32 and C35 were changed to serines. The overexpression of this mutant did not have an effect on HIF-1 α stability (Fig. 2A) or activity (Fig. 2B) in normoxic or hypoxic conditions.

Txn1 is not required for HIF-1 α stabilization or activity

HeLa and EMT6 Txn1 deficient cells were generated to examine the significance of this protein on HIF-1 α stabilization and activity. Control and Txn1 deficient cells were cultured in normoxic or hypoxic conditions and HIF-1 α levels analyzed by Western blotting. Txn1 loss had no effect on HIF-1 α levels either in normoxic or hypoxic conditions (Fig. 3A). To elucidate the effect of Txn1 deficiency on HIF-1 α activity, control and Txn1 deficient HeLa and EMT6 cells were cultured under normoxic or hypoxic conditions and HIF-1 α activity assessed by

measuring VEGF and ADM mRNA levels. Txn1 loss had no effect on the normoxic or hypoxic levels of VEGF and ADM mRNAs in either cell line (Fig. 3B).

Overexpression of TR1 does not affect HIF-1 α stability or activity

Since TR1 is the only known reductant of Txn1, we examined whether its overexpression has any effect on HIF-1 α levels and/or activity. HeLa and EMT6 cells overexpressing TR1 were generated, and their analyses revealed that HIF-1 α levels were not affected under normoxic or hypoxic conditions as assessed by Western blotting (Fig. 4A), in agreement with a previous report studying the importance of TR1 on the hypoxia-induced generation of ROS (Naranjo-Suarez et al., 2012). In contrast to what we observed on Txn1 overexpressing cells, TR1 overexpression did not affect HIF-1 α activity (Fig. 4B).

The effect of Txn1 overexpression on HIF-1 α activity is TR1 independent

To assess whether the effect of Txn1 overexpression on HIF-1 α activity was dependent on the presence of TR1, HeLa and EMT6 TR1 deficient cells overexpressing Txn1 were generated. A retrovirus that was bi-cistronic for Txn1 and green fluorescent protein (GFP) was used for Txn1 overexpression. Transduction efficiency was determined by GFP expression as analyzed by flow cytometry (Supplementary Fig. 2). The efficiency of transduction was greater than 90% in both control and Txn1 overexpressing HeLa and EMT6 cell lines.

TR1 expression was down-regulated in HeLa and EMT6 cells expressing shTR1 as determined by Western blotting and its deficiency in these cell lines did not appear to have an effect

Fig. 2. Redox activity of Txn1 is required for its effects on HIF-1 α . HeLa and EMT6 cells were infected with retroviruses encoding a mutant *Txn1* gene (Mut-Txn1) or empty retroviruses (Ctrl) and the cells cultured in normoxic (Nx) or hypoxic (Hx) conditions. (A) HIF-1 α and Txn1 protein levels were analyzed by Western blotting. (B) VEGF and ADM mRNA levels were measured by quantitative RT-PCR to determine HIF-1 α activity. Fold-changes relative to normoxic values in control cells are shown. The data represent the mean value $\pm$ S.D. of three independent experiments.

Fig. 3. Txn1 is not required for HIF-1 α hypoxic stabilization or activity. HeLa or EMT6 cells were infected with retroviruses encoding a scramble sequence (SCR) or a Txn1 shRNA (siTxn1) and cultured in normoxic (Nx) or hypoxic (Hx) conditions. (A) HIF-1 α , Txn1 and β -actin levels were determined by Western blot analysis. (B) VEGF and ADM mRNA levels were determined by quantitative RT-PCR to determine HIF-1 α activity. Data represent the mean value of three independent experiments $\pm$ S.D. and are shown as the fold-change relative to the normoxic values of control cells.

under normoxic or hypoxic conditions on HIF-1 α stability (Fig. 5A) or activity (Fig. 5B). Similarly, the overexpression of Txn1 did not affect HIF-1 α stability either in control or TR1 deficient cells. However, HIF-1 α activity was increased after Txn1 overexpression in control and TR1 deficient cells. Thus, the effects of Txn1 on HIF-1 α activity are TR1 independent.

DISCUSSION

Txn1 has been shown to regulate HIF-1 α . Initially, the effects of Txn1 overexpression on HIF-1 α were reported to be exclusively on its transcriptional activity (Carrero et al., 2000; Ema et al., 1999; Huang et al., 1996), but, more recently, Txn1 overexpres-

Fig. 4. TR1 overexpression has no effect on HIF-1 α hypoxic stabilization or activity. HeLa or EMT6 cells were infected with viruses encoding *TR1* (TR1) or empty retroviruses (Ctrl) and cultured for 12 h in normoxic (Nx) or hypoxic (Hx) conditions. (A) HIF-1 α , TR1 and β -actin levels were analyzed by Western blotting. (B) VEGF and ADM mRNA levels were measured by quantitative RT-PCR to determine HIF-1 α activity. The data are represented as the fold-change relative to the normoxic values of control cells. The mean value $\pm$ S.D. of three independent experiments is shown.

Fig. 5. TR1 is not required for the effects of Txn1 on HIF-1 α . HeLa or EMT6 cells expressing a scramble sequence (SCR) or a TR1 shRNA (siTR1) were infected with empty (Ctrl) or *Txn1* encoding (Txn1) retroviruses, cultured for 12 h in normoxic (Nx) or hypoxic (Hx) conditions. (A) HIF-1 α , TR1, Txn1 and β -actin levels were analyzed by Western blotting. (B) VEGF and ADM mRNA levels were determined by quantitative RT-PCR to determine HIF-1 α activity. The data represent the fold-change relative to normoxic values of control cells (*= $p \leq 0.05$).

sion was reported to induce HIF-1 α stabilization (Welsh et al., 2002; Zhou et al., 2007). In these studies, no changes in the mRNA levels of HIF-1 α were reported. Thus, the effect at the protein levels was suggested to be due to regulation of HIF-1 α stability. HIF-1 α degradation in normoxic conditions is due to the activity of three prolyl hydroxylases, the EGLNs. Their ac-

tivity was reported to be inhibited when any of its co-substrates (oxygen, 2-oxoglutarate and Fe^{2+}) are limiting (Gimenez-Roqueplo et al., 2001; Selak et al., 2005; Zhao et al., 2009), or when the ROS level was increased (Chandel et al., 1998), although the role of ROS in HIF-1 α stabilization is controversial (Naranjo-Suarez et al., 2012 and references therein). Therefore,

the mechanism by which Txn1 overexpression affected HIF-1 α stability is unknown. To study the effects of Txn1 overexpression on HIF-1 α activity and stability, we generated HeLa, EMT6, HT-29 and MCF-7 cells overexpressing Txn1. We found that Txn1 overexpression increased HIF-1 α hypoxic-activity in each cell line, except MCF-7. However, we did not detect any effect on HIF-1 α levels in any of these cell lines in contrast to what two other studies reported (Welsh et al., 2002; Zhou et al., 2007). These results provide evidence that the effect of Txn1 on HIF-1 α is limited to its transcriptional activity.

Previously, the mutation of a cysteine in C-TAD of HIF-1 α abolished its interaction with the transcriptional co-activator p300, and thus, inhibited HIF-1 α transcriptional activity (Ema et al., 1999). A mechanism was proposed, wherein Txn1, through Ref-1, would reduce a cysteine in the transactivation domain of HIF-1 α and allow its interaction with p300. However, these studies were previous to the discovery of the hydroxylation by FIH of an asparagine in the transactivation domain of HIF-1 α that is critical to its interaction with p300. The cysteine that was proposed to be reduced by Ref-1 is part of the sequence that is recognized by FIH (Lando et al., 2002a; 2002b). Thus, the lack of interaction between HIF-1 α and CBP/p300 observed after the mutation of this cysteine was likely due to an inhibition of FIH activity on HIF-1 α and not due to an ability of Ref-1 to reduce this cysteine.

In agreement with this hypothesis, Txn1 deficient cells manifested a hypoxic-induction of HIF-1 α target genes similar to control cells. These observations suggested that Txn1 is not required for HIF-1 α transcriptional activity. Therefore, overexpression of Txn1 is likely acting as a synergistic signal for HIF-1 α transcriptional activity. A possible means of how this could occur is through the effect of Txn1 on other transcription factors. Txn1 has been shown to regulate AP-1 (Fos and Jun homodimers) transcriptional activity through Ref-1 (Meyer et al., 1993; Schenk et al., 1994) and AP-1 transcriptional factors have been reported to bind to cis-acting elements present in the promoter of several HIF-1 target genes, such as VEGF, tyrosine hydroxylase, endothelin-1, erythropoietin and lactate dehydrogenase A. Furthermore, c-Jun has been described to cooperate with HIF-1 α in hypoxia-induced gene transcription (Alfranca et al., 2002). This cooperative effect does not require direct binding of c-Jun to DNA. c-Jun and HIF-1 α were shown to be able to bind *in vivo*, but not *in vitro*, suggesting that this interaction involves additional protein(s) and/or posttranslational modifications of these factors (Alfranca et al., 2002). Thus, c-Jun reduction by Ref-1 could be required for its association with HIF-1 α , and the Txn1 effect on HIF-1 α transcriptional activity could be an indirect effect of Txn1 actions on c-Jun. This hypothesis would explain why overexpression of Ref-1 has the same effects on HIF-1 α transcriptional activity as overexpression of Txn1 (Ema et al., 1999). Because hypoxic conditions induce an increase in ROS, it is possible that hypoxic cells use all their endogenous reduced Txn1 to reverse the oxidative stress generated under these conditions. This would explain why the HIF-1 transcriptional activity effect is observed when Txn1 is overexpressed but not when it is downregulated.

In this study, we report that a sulfhydryl-transfer defective mutant of Txn1 had no effect on HIF-1 α transcriptional activity, suggesting that the antioxidant activity of Txn1 is involved. However, overexpression of TR1 did not reproduce the effects observed by Txn1 overexpression alone. This result suggests that the endogenous levels of Txn1 are limited and that an increase in the endogenous levels of the reduced form of Txn1 is not enough to affect HIF-1 α transcriptional activity.

Finally, we found that Txn1 overexpression in TR1 deficient cells was still able to increase HIF-1 α transcriptional activity. This result could be explained by the presence of another, yet undescribed reductase that is capable of reducing Txn1. Another explanation is that Txn1 is synthesized as a reduced protein, and therefore when overexpressed, Txn1 can reduce its substrates even in the absence of TR1. Thus, it is possible that some cells could increase their synthesis of Txn1 to compensate for the decrease in TR1 activity and protein levels induced by hypoxic conditions (Naranjo-Suarez et al., 2012) as has been previously described to occur in hepatocytes from liver-specific selenocysteine tRNA knockout mice (Turanov et al., 2010).

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

This work was supported by the Intramural Research Program of the National Institutes of Health, NCI, Center for Cancer Research to D.L.H., NIH grants to V.N.G. and the National Cancer Institute, National Institutes of Health, under Contract No. HHSN261200800001E.

REFERENCES

- Alfranca, A., Gutierrez, M.D., Vara, A., Aragonés, J., Vidal, F., and Landazuri, M.O. (2002). c-Jun and hypoxia-inducible factor 1 functionally cooperate in hypoxia-induced gene transcription. *Mol. Cell. Biol.* 22, 12-22.
- Alvarez-Tejado, M., Alfranca, A., Aragonés, J., Vara, A., Landazuri, M.O., and del Peso, L. (2002). Lack of evidence for the involvement of the phosphoinositide 3-kinase/Akt pathway in the activation of hypoxia inducible factors by low oxygen tension. *J. Biol. Chem.* 277, 13508-13517.
- Aragonés, J., Jones, D.R., Martin, S., San Juan, M.A., Alfranca, A., Vidal, F., Vara, A., Merida, I., and Landazuri, M.O. (2001). Evidence for the involvement of diacylglycerol kinase in the activation of hypoxia-inducible transcription factor 1 by low oxygen tension. *J. Biol. Chem.* 276, 10548-10555.
- Bruick, R.K., and McKnight, S.L. (2001). A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* 294, 1337-1340.
- Carrero, P., Okamoto, K., Coumault, P., O'Brien, S., Tanaka, H., and Poellinger, L. (2000). Redox-regulated recruitment of the transcriptional coactivators CREB-binding protein and SRC-1 to hypoxia-inducible factor 1 α . *Mol. Cell. Biol.* 20, 402-415.
- Chandel, N.S., Maltepe, E., Goldwasser, E., Mathieu, C.E., Simon, M.C., and Schumacker, P.T. (1998). Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proc. Natl. Acad. Sci. USA* 95, 11715-11720.
- Chua, Y.L., Dufour, E., Dassa, E.P., Rustin, P., Jacobs, H.T., Taylor, C.T., and Hagen, T. (2010). Stabilization of hypoxia-inducible factor-1 α protein in hypoxia occurs independently of mitochondrial reactive oxygen species production. *J. Biol. Chem.* 285, 31277-31284.
- Conrad, P.W., Freeman, T.L., Beitner-Johnson, D., and Millhorn, D.E. (1999a). EPAS1 trans-activation during hypoxia requires p42/p44 MAPK. *J. Biol. Chem.* 274, 33709-33713.
- Conrad, P.W., Rust, R.T., Han, J., Millhorn, D.E., and Beitner-Johnson, D. (1999b). Selective activation of p38 α and p38 γ by hypoxia. Role in regulation of cyclin D1 by hypoxia in pc12 cells. *J. Biol. Chem.* 274, 23570-23576.
- Cormier-Regard, S., Nguyen, S.V., and Claycomb, W.C. (1998). Adrenomedullin gene expression is developmentally regulated and induced by hypoxia in rat ventricular cardiac myocytes. *J. Biol. Chem.* 273, 17787-17792.
- Ema, M., Hirota, K., Mimura, J., Abe, H., Yodoi, J., Sogawa, K., Poellinger, L., and Fujii-Kuriyama, Y. (1999). Molecular mechanisms of transcription activation by HLF and HIF1 α in response to hypoxia: their stabilization and redox signal-induced interaction with CBP/p300. *EMBO J.* 18, 1905-1914.

- Epstein, A.C., Gleadle, J.M., McNeill, L.A., Hewitson, K.S., O'Rourke, J., Mole, D.R., Mukherji, M., Metzén, E., Wilson, M.I., Dhanda, A., et al. (2001). *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 107, 43-54.
- Forsythe, J.A., Jiang, B.H., Iyer, N.V., Agani, F., Leung, S.W., Koos, R.D., and Semenza, G.L. (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol. Cell. Biol.* 16, 4604-4613.
- Garayoa, M., Martínez, A., Lee, S., Pio, R., An, W.G., Neckers, L., Trepel, J., Montuenga, L.M., Ryan, H., Johnson, R., et al. (2000). Hypoxia-inducible factor-1 (HIF-1) up-regulates adrenomedullin expression in human tumor cell lines during oxygen deprivation: a possible promotion mechanism of carcinogenesis. *Mol. Endocrinol.* 14, 848-862.
- Gimenez-Roqueplo, A.P., Favier, J., Rustin, P., Mourad, J.J., Plouin, P.F., Corvol, P., Rotig, A., and Jeunemaitre, X. (2001). The R22X mutation of the SDHD gene in hereditary paraganglioma abolishes the enzymatic activity of complex II in the mitochondrial respiratory chain and activates the hypoxia pathway. *Am. J. Hum. Genet.* 69, 1186-1197.
- Hirota, K., and Semenza, G.L. (2001). Rac1 activity is required for the activation of hypoxia-inducible factor-1. *J. Biol. Chem.* 276, 21166-21172.
- Hoffman, D.L., Salter, J.D., and Brookes, P.S. (2007). Response of mitochondrial reactive oxygen species generation to steady-state oxygen tension: implications for hypoxic cell signaling. *Am. J. Physiol. Heart Circ. Physiol.* 292, H101-108.
- Huang, L.E., Arany, Z., Livingston, D.M., and Bunn, H.F. (1996). Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its α subunit. *J. Biol. Chem.* 271, 32253-32259.
- Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J.M., Lane, W.S., and Kaelin, W.G., Jr. (2001). HIF α targeted for VHL-mediated destruction by proline hydroxylation: implications for O₂ sensing. *Science* 292, 464-468.
- Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J., et al. (2001). Targeting of HIF- α to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. *Science* 292, 468-472.
- Lando, D., Peet, D.J., Gorman, J.J., Whelan, D.A., White, M.F., and Bruck, R.K. (2002a). FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes Dev.* 16, 1466-1471.
- Lando, D., Peet, D.J., Whelan, D.A., Gorman, J.J., and Whitelaw, M.L. (2002b). Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. *Science* 295, 858-861.
- Mazure, N.M., Chen, E.Y., Laderoute, K.R., and Giaccia, A.J. (1997). Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidylinositol 3-kinase/Akt signaling pathway in Ha-ras- transformed cells through a hypoxia inducible factor-1 transcriptional element. *Blood* 90, 3322-3331.
- Meyer, M., Schreck, R., and Baeuerle, P.A. (1993). H₂O₂ and antioxidants have opposite effects on activation of NF- κ B and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. *EMBO J.* 12, 2005-2015.
- Naranjo-Suarez, S., Carlson, B.A., Tsuji, P.A., Yoo, M.H., Gladyshev, V.N., and Hatfield, D.L. (2012). HIF-independent regulation of thioredoxin reductase 1 contributes to the high levels of reactive oxygen species induced by hypoxia. *PLoS One* 7, e30470.
- Richard, D.E., Berra, E., Gothie, E., Roux, D., and Pouyssegur, J. (1999). p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1 α (HIF-1 α) and enhance the transcriptional activity of HIF-1. *J. Biol. Chem.* 274, 32631-32637.
- Schenk, H., Klein, M., Erdbrugger, W., Droge, W., and Schulze-Osthoff, K. (1994). Distinct effects of thioredoxin and antioxidants on the activation of transcription factors NF- κ B and AP-1. *Proc. Natl. Acad. Sci. USA* 91, 1672-1676.
- Selak, M.A., Armour, S.M., MacKenzie, E.D., Boulahbel, H., Watson, D.G., Mansfield, K.D., Pan, Y., Simon, M.C., Thompson, C.B., and Gottlieb, E. (2005). Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF- α prolyl hydroxylase. *Cancer Cell* 7, 77-85.
- Semenza, G.L., and Wang, G.L. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol. Cell. Biol.* 12, 5447-5454.
- Srinivas, V., Leshchinsky, I., Sang, N., King, M.P., Minchenko, A., and Caro, J. (2001). Oxygen sensing and HIF-1 activation does not require an active mitochondria respiratory chain electron-transfer pathway. *J. Biol. Chem.* 276, 21995-21998.
- Tobe, R., Yoo, M.H., Fradejas, N., Carlson, B.A., Calvo, S., Gladyshev, V.N., and Hatfield, D.L. (2012). Thioredoxin reductase 1 deficiency enhances selenite toxicity in cancer cells via a thioredoxin-independent mechanism. *Biochem. J.* 445, 423-430.
- Turanov, A.A., Kehr, S., Marino, S.M., Yoo, M.H., Carlson, B.A., Hatfield, D.L., and Gladyshev, V.N. (2010). Mammalian thioredoxin reductase 1: roles in redox homeostasis and characterization of cellular targets. *Biochem. J.* 430, 285-293.
- Vaux, E.C., Metzén, E., Yeates, K.M., and Ratcliffe, P.J. (2001). Regulation of hypoxia-inducible factor is preserved in the absence of a functioning mitochondrial respiratory chain. *Blood* 98, 296-302.
- Welsh, S.J., Bellamy, W.T., Briehl, M.M., and Powis, G. (2002). The redox protein thioredoxin-1 (Trx-1) increases hypoxia-inducible factor 1 α protein expression: Trx-1 overexpression results in increased vascular endothelial growth factor production and enhanced tumor angiogenesis. *Cancer Res.* 62, 5089-5095.
- Wenger, R.H., Stiehl, D.P., and Camenisch, G. (2005). Integration of oxygen signaling at the consensus HRE. *Sci. STKE* 2005, re12.
- Yoo, M.H., Xu, X.M., Carlson, B.A., Gladyshev, V.N., and Hatfield, D.L. (2006). Thioredoxin reductase 1 deficiency reverses tumor phenotype and tumorigenicity of lung carcinoma cells. *J. Biol. Chem.* 281, 13005-13008.
- Zhao, S., Lin, Y., Xu, W., Jiang, W., Zha, Z., Wang, P., Yu, W., Li, Z., Gong, L., Peng, Y., et al. (2009). Glioma-derived mutations in IDH1 dominantly inhibit IDH1 catalytic activity and induce HIF-1 α . *Science* 324, 261-265.
- Zhong, H., Chiles, K., Feldser, D., Laughner, E., Hanrahan, C., Georgescu, M.M., Simons, J.W., and Semenza, G.L. (2000). Modulation of hypoxia-inducible factor 1 α expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res.* 60, 1541-1545.
- Zhou, J., Damdimopoulos, A.E., Spyrou, G., and Brune, B. (2007). Thioredoxin 1 and thioredoxin 2 have opposed regulatory functions on hypoxia-inducible factor-1 α . *J. Biol. Chem.* 282, 7482-7490.
- Zundel, W., Schindler, C., Hass-Kogan, D., Koong, A., Kaper, F., Chen, E., Gottschalk, A.R., Ryan, H.E., Johnson, R.S., Jefferson, A.B., et al. (2000). Loss of PTEN facilitates HIF-1 mediated gene expression. *Genes Dev.* 14, 391-396.