

Casein kinase 2 inhibition decreases hypoxia-inducible factor-1 activity under hypoxia through elevated p53 protein level

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Summary

HIF-1 (hypoxia-inducible factor-1) is the main transcription factor involved in the adaptation of cells to hypoxia. In addition to regulation of HIF-1 α protein level, HIF-1 activity is also enhanced by several pathways involving asparagine hydroxylation and phosphorylation. Here, we investigated the relationship between casein kinase 2 (CK2), p53 and HIF-1. An increase in p53 protein level and transcriptional activity was observed when CK2 was inhibited by different inhibitors under normoxia and hypoxia. This increase was in parallel with a decrease in HIF-1 activity without changes in HIF-1 α protein level, indicating a regulation of its transcriptional activity. Similar results were obtained using CK2 α siRNA. Ectopic overexpression of p53 also led to an inhibition of HIF-1

activity. Conversely, CK2 inhibition had no effect in p53-null cells indicating that the inhibitory effect of CK2 inhibitors requires the presence of p53. p53 activity was not required because overexpression of a p53 mutated in its DNA-binding domain exerted the same effect as wild-type p53 and because the effect of CK2 inhibitors was still observed when p53 activity was inhibited by pifithrin- α . Since CK2 activity is increased in hypoxic conditions, this process provides one more mechanism to ensure enhanced HIF-1 activity under such conditions.

Key words: Hypoxia, HIF-1, p53, CK2, Transcription regulation, MDM2

Introduction

Resistance of tumour cells to chemo- or radiotherapy remains an important obstacle to the successful treatment of human cancer. Several processes are involved in the acquisition of this resistance. One of them is hypoxia. Reduced drug delivery to hypoxic cells for chemotherapy as well as intrinsic radioresistance are contributing factors. Hypoxia develops in most solid tumours as a result of inefficient vascular development and/or of abnormal vascular architecture (Jain, 2003). It elicits cellular responses designed to improve cell survival mainly through the activation of one hypoxia-specific transcription factor HIF-1 (hypoxia-inducible factor-1) (Hopfl et al., 2004; Semenza, 2003). HIF-1 leads to neoangiogenesis promotion, enhanced glycolytic flux for oxygen-independent energy production and upregulation of molecules related to cell survival. HIF-1 is a heterodimer consisting of two subunits: HIF-1 α , which is tightly regulated by oxygen levels and ARNT (aryl hydrocarbon receptor nuclear translocator), which is constitutively expressed. Under normoxia, HIF-1 α protein is kept at low level, usually undetectable, by continuous degradation via the 26S proteasome. A class of prolyl hydroxylases acts as oxygen sensors and hydroxylates two proline residues (Pro402 and Pro564) on HIF-1 α at normal levels of oxygen (Ivan et al., 2001; Jaakkola et al., 2001). Hydroxylated HIF-1 α is then recognized by the Von Hippel Lindau-E3 complex, ubiquitinated and hence targeted for proteasome degradation. A third site of hydroxylation, Asn803, has recently been discovered, marked by an asparagyl

hydroxylase first described as FIH-1 (factor inhibiting HIF) (Mahon et al., 2001). The hydroxylation of this residue leads to a steric inhibition of the interaction between HIF-1 α and the coactivators CBP/p300 interfering with its recruitment (Lando et al., 2002). This process is thought to ensure that HIF-1 α escaping degradation remains inactive. In the absence of oxygen, degradation of HIF-1 α and the hydroxylation of Asn83 no longer occur. HIF-1 α dimerizes with ARNT and HIF-1 binds to HRE (hypoxia-responsive element) located in the promoter or enhancer of target genes thereby activating their expression (Wenger, 2002; Semenza, 2000).

A second mechanism responsible for tumour cell resistance to cell death is the loss of p53. Indeed, p53 is the most commonly mutated tumour suppressor in human cancer (Hollstein et al., 1991). p53 has multiple functions including cell-cycle control in response to DNA damage, induction of apoptosis and DNA repair (Harris, 1996; Levine, 1997). p53 acts as a sequence-specific DNA-binding protein, which activates the transcription of target genes. The cyclin-dependent kinase inhibitor, p21^{waf-1}, appears to be the major effector of p53-mediated cell-cycle arrest (Brugarolas et al., 1995; Waldman et al., 1995) whereas the transcriptional induction of Bax largely contributes to p53-mediated cell death (Miyashita et al., 1994). The activity of p53 is maintained at a low level in normal cells through MDM2-mediated ubiquitylation and proteasome degradation. When DNA damage occurs, p53 becomes phosphorylated resulting in its stabilisation and activation. In addition to other stimuli of p53

activation, it has been proposed that hypoxia stabilises p53 (Giaccia and Kastan, 1998; Hammond et al., 2002; Koumenis et al., 2001). Hypoxic induction of p53 requires concomitant induction of HIF-1 α , whereby HIF-1 α can then bind to and stabilise p53 (An et al., 1998; Blagosklonny et al., 1998). Recent work by Chen et al. (Chen et al., 2003) suggests that MDM2 acts as a bridge between both proteins with parallel protection of MDM2-mediated p53 degradation but enhanced HIF-1 α destruction. Direct interaction between the two proteins has been also demonstrated (Hansson et al., 2002). In addition to provoking HIF-1 α degradation, p53 also represses the transcriptional activity of HIF-1 (Ravi et al., 2000) probably through competition for the shared co-activator p300 (Blagosklonny et al., 1998). As well as to the well-known benefit regarding the inhibition of apoptosis, loss of p53 would thus lead to enhanced HIF-1 activity, hence promoting tumour growth and neoangiogenesis (Ravi et al., 2000).

Finally, CK2 has also been observed to contribute to enhanced tumour cell viability. CK2 protein kinase, formerly known as casein kinase 2, is a constitutive serine/threonine kinase. It is found in tetrameric complexes comprising two catalytic (α and/or α') subunits and two regulatory β subunits. Accumulated evidence indicates that CK2 has a vast array of candidate physiological substrates and participates in a variety of cellular functions including the maintenance of cell viability (for reviews, see Ahmed et al., 2002; Litchfield, 2003). The regulation of its activity is poorly understood. Regulation of the subcellular localisation of the different subunits seems to be important but the actual physiological relevance remains to be determined. CK2 was thought to be messenger independent but this knowledge is now challenged by recent data. In most human cancers that have been examined, as well as experimental tumours, CK2 activity is consistently enhanced (Faust et al., 1999; Landesman-Bollag et al., 2001; Munstermann et al., 1990). This upregulation of CK2 in tumours suggests a role for CK2 in cell growth promotion and/or as an anti-apoptotic protein.

Interestingly, CK2 has been shown to phosphorylate p53, regulating several of its functions, either positively (McKendrick et al., 1999) or negatively (Yap et al., 2004). On the other hand, CK2 is also part of the COP9 signalosome (CSN). CSN is a conserved protein complex that is similar to the 19S proteasome lid (for reviews, see Wei and Deng, 2003; Wolf et al., 2003). CSN is associated with de-ubiquitylation and protein kinase activities, among which IP5/6 kinase and CK2 seem to be important. CSN can phosphorylate p53 (Ser149, Thr150, Thr155, Ser392) *in vitro*, with Thr155 and Ser392 probably being the ones involved *in vivo*. CSN-mediated phosphorylation targets p53 for degradation by the ubiquitin-26S proteasome system.

Recent data from our laboratory shows that CK2 positively regulates HIF-1 transcriptional activity without affecting HIF-1 α protein level (Mottet et al., 2005). Moreover, CK2 activity is enhanced under hypoxic conditions. However, the mechanism of this interaction remains unclear. Since CK2 is known to affect p53 activity and p53 to decrease HIF-1 activity, we sought to determine whether p53 would be a putative necessary intermediate in the CK2-dependent regulation of HIF-1 activity. To this end, we investigated the effects of CK2 inhibitors on p53 and HIF-1 α protein level and activity. Apigenin (Ford et al., 2000; Hanyaloglu et al., 2001; Shen et

al., 2001; Zhu et al., 2002), DRB (5,6-Dichloro-1- β -D-ribofuranosyl-benzimidazole) (Blaydes and Hupp, 1998; Critchfield et al., 1997; Farah et al., 2003) and TBB (3,4,5,6-tetrabromo-triazole) (Ruzzene et al., 2002; Zien et al., 2003) were used as CK2-specific inhibitors. An inverse relationship was observed with decreased HIF-1 activity in parallel with increased p53 protein level and activity in the presence of these inhibitors. Overexpression of p53 and the use of p53-null cells suggest that it is indeed through an increase in p53 that CK2 inhibition diminishes HIF-1 activity. However, MDM2 inactivation did not reproduce the effect of CK2 inhibitors indicating different ways of p53 regulation by both proteins. Finally, an interaction between p53 and HIF-1 was shown by co-immunoprecipitation, which was enhanced when CK2 was inhibited.

Results

Effect of CK2 inhibitors on p53 and HIF-1 activity

HIF-1 and p53 transcriptional activity can be regulated at several levels including regulation of their protein concentration, regulation via phosphorylation and of their actual activity. We first investigated the effects of three inhibitors of CK2, DRB, apigenin and TBB, on HIF-1 α and p53 protein level under normoxia and hypoxia, in two different cell lines, HepG2 and HeLa, which both contain wild-type p53. In both cell lines, HIF-1 α was clearly stabilised under hypoxia whereas it was not present under normoxia (Fig. 1A). DRB had no clear effect on HIF-1 α protein level whereas apigenin seemed to slightly decrease it in HepG2 cells. On the other hand, hypoxia induced a marked decrease in p53 protein level in both cell lines. DRB and to a lesser extent, apigenin increased p53 protein level by several fold, in a concentration-dependent manner, both under normoxia and hypoxia. TBB, a more-specific inhibitor of CK2, also led to an increased p53 protein level (Fig. 1B). Similar results were obtained when HIF-1 α and p53 protein level was assessed by immunofluorescence studies. Both proteins were localised in the nucleus and neither hypoxia nor CK2 inhibitors affected this subcellular localisation (data not shown). The phosphorylation status of p53 on one of the most important CK2 site (serine 392) (Blaydes and Hupp, 1998) was followed by western blotting analysis using a specific antibody. Inhibition of CK2 by DRB or apigenin decreased the phosphorylation state of p53 serine 392 (Fig. 1C).

DNA-binding activity of both transcription factors was then measured using an *in vitro* DNA-binding assay. HIF-1 DNA-binding activity greatly increased under hypoxia, DRB had no effect on this activity whereas apigenin decreased HIF-1 DNA binding activity by 35% (Fig. 2A). On the other hand, hypoxia induced a 45% decrease in p53 DNA-binding activity. DRB strongly enhanced p53 DNA binding activity under normoxia whereas both inhibitors had this effect under hypoxia (Fig. 2B). The effects of hypoxia and of CK2 inhibitors on HIF-1 and p53 DNA binding activity were similar to those observed for HIF-1 α and p53 protein levels.

Finally, the actual transcriptional activity of both factors was measured using a reporter system as well as by following the expression of one target gene. To this end, we studied the effects of CK2 inhibitors on activity of the luciferase reporter construct driven by six HREs derived from the EPO enhancer (Michel et al., 2002). Transient transfection experiments

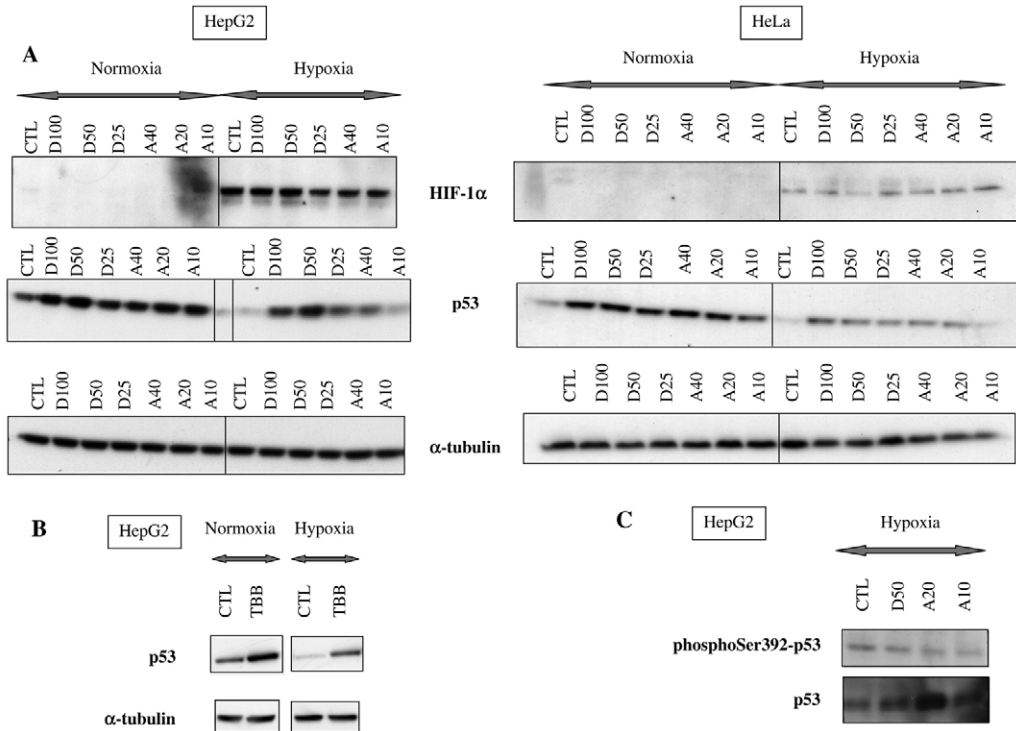


Fig. 1. Effect of CK2 inhibitors on HIF-1 α and p53 protein level in HepG2 and HeLa cells. Cells were incubated for 5 hours under normoxia or hypoxia in the presence or the absence of different concentrations (μ M) of DRB (A,C), apigenin (A,C) or of 10 μ M TBB (B). Total cell lysates were tested for HIF-1 α , p53, phospho-Ser392 p53 and α -tubulin by western blotting.

confirmed induction of the HRE activity by hypoxia. DRB and apigenin strongly inhibited this activity in a concentration-dependent manner (Fig. 3A,B). TBB has a similar effect (Mottet et al., 2005). Very low luciferase activity was detected using pGL3 as a negative control, which was not (or only slightly) affected by CK2 inhibitors. These effects on HIF-1 activity were confirmed by following the expression of one HIF-1 target gene, aldolase (Semenza et al., 1996), by real-time PCR. Results presented in Fig. 3C showed that hypoxia increased the level of aldolase mRNA sevenfold; this increase was inhibited by 50% in the presence of 50 μ M DRB and by 92% in the presence of 20 μ M apigenin. To test the effects of CK2 inhibitors on the activity of p53 more directly, we performed transient transfection assays with a reporter plasmid carrying 13 copies of a p53-responsive promoter driving the expression of the luciferase gene, pG13-Luc (Kern et al., 1992). As already observed for p53 protein level, hypoxia induced a decrease in the activity of this factor (Fig. 4A). Apigenin increased p53 activity under both normoxia and hypoxia. Similarly, hypoxia decreased by 55% the mRNA level of p21, one well-known p53 target gene (el-Deiry et al., 1993) whereas DRB increased the p21 mRNA level sevenfold under normoxia and sixfold under hypoxia (Fig. 4B). A parallel, concentration-dependent increase in p21 protein level was observed in the presence of DRB, apigenin and TBB (Fig. 5C).

In order to verify the specificity of action of the chemical inhibitors, inhibition of CK2 activity was also achieved through diminished expression of the α -catalytic subunit using siRNA. CK2 α siRNA induced ~50% inhibition of CK2 α expression under both normoxia and hypoxia as observed by western blotting (Fig. 5A) or immunofluorescence (data not shown). CK2 α mRNA level was checked by real-time RT-PCR. When normalised to mRNA levels under normoxia (100%), levels were 102% under hypoxia, 95% for cells

exposed to Xtreme alone, 113% for cells exposed to the negative control siRNA and 65% for cells transfected with the CK2 α siRNA. This decrease observed with CK2 α siRNA had no effect on HIF-1 α stabilisation but resulted in an increased p53 level under hypoxia (Fig. 5A). As already observed with the inhibitors, CK2 α expression inhibition induced a complete inhibition of the hypoxia-induced increase in HIF-1 activity (Fig. 5B). This effect was not observed with the negative control siRNA. The level of p53 accumulation with CK2 α siRNA was not as high as that observed when using the chemical inhibitors. This is probably because the inhibition of CK2 α protein expression was not very high whereas the effect of the inhibitors on CK2 activity was much more important.

We therefore conclude that CK2 inhibitors inhibited HIF-1 transcriptional level without significantly affecting HIF-1 α protein level or HIF-1 DNA-binding activity. In parallel, a marked increase in p53 protein level and transcriptional level was observed. It must be noted that p53 is not activated under these conditions of hypoxia but we observed rather decreased levels of p53. The lack of activation of p53 at this degree of hypoxia (mild hypoxia) is consistent with the observations of Kaluzova et al. (Kaluzova et al., 2004) and of Koumenis et al. (Koumenis et al., 2001).

CK2 inhibitors exert their effect on HIF-1 activity through a change in p53 level

Parallel decreases in HIF-1 activity and increases in p53 protein level and activity were observed in the presence of CK2 inhibitors. Since p53 is known to inhibit HIF-1, we wished to establish if it is through p53 that CK2 inhibitors exert their effect on HIF-1 activity. First, we wanted to mirror the increase in p53 protein level and activity induced by CK2 inhibitors, by overexpressing p53 and studying its effect on HIF-1 activity. With the use of the reporter system for p53, we showed that

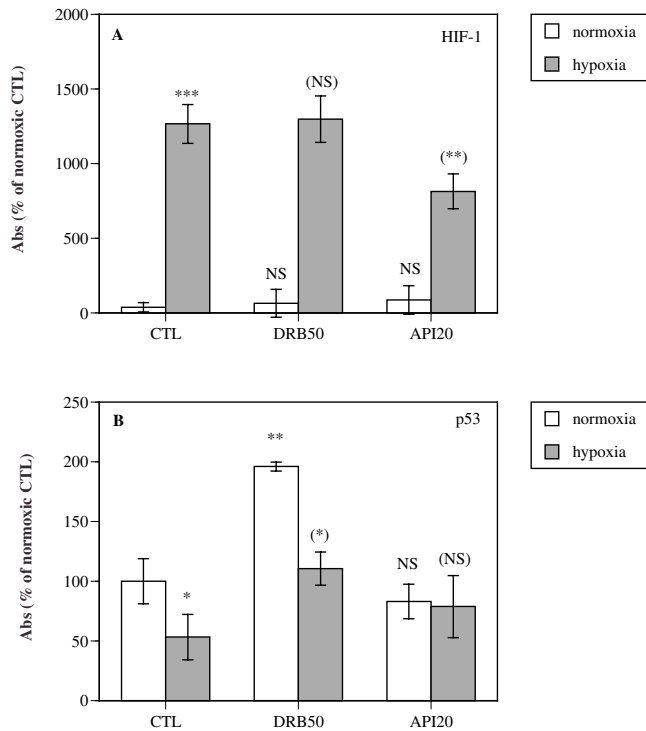


Fig. 2. Effect of CK2 inhibitors on HIF-1 and p53 DNA binding activity in HeLa cells. Cells were incubated for 5 hours under normoxia or hypoxia in the presence or absence of different concentrations (μM) of DRB or apigenin (API). Nuclear extracts were tested for HIF-1 (A) or p53 (B) binding to a specific probe. Results are expressed in absorbance as a percentage of normoxic control (means \pm 1 s.d.; A, $n=3$; B, $n=6$). * $P<0.05$, ** $P<0.01$, *** $P<0.001$, NS, not significant vs. normoxia; (*) $P<0.05$, (**) $P<0.01$, (NS) not significant vs. hypoxia.

p53 overexpression indeed led to a marked increase in p53 activity (Fig. 6B). Under the same conditions, p53 overexpression induced a decrease in HIF-1 activity as measured by the 6HRE reporter system under hypoxia (Fig. 6A). The reason why p53 overexpression led to an increase in the low HIF-1 activity detected under normoxia is not known. These results indicate that an increase in p53 protein level and activity, either due to direct overexpression or to CK2 inhibition, can indeed decrease HIF-1 activity.

Second, if it is really through an increase in p53 protein level that CK2 inhibition led to a decrease in HIF-1 activity, then the effects of CK2 inhibitors should not be observed in p53-null cells. To address this question, we used Hep3B cells which are also human hepatoma cells like the HepG2 cells but are p53^{-/-} cells (Muller et al., 1998). HIF-1 activity was assessed using the pGL3-6HRE reporter system and by following the expression of aldolase. In Hep3B cells, hypoxia also increased the luciferase activity but in contrast to HepG2 cells, no effect of DRB or apigenin was detected on HRE-driven luciferase expression (Fig. 7A). Similar results were obtained when quantifying the aldolase mRNA: there was a 3.3-fold increase under hypoxia, which was only very slightly affected by DRB (Fig. 7B). Therefore, we conclude that because p53 seems to

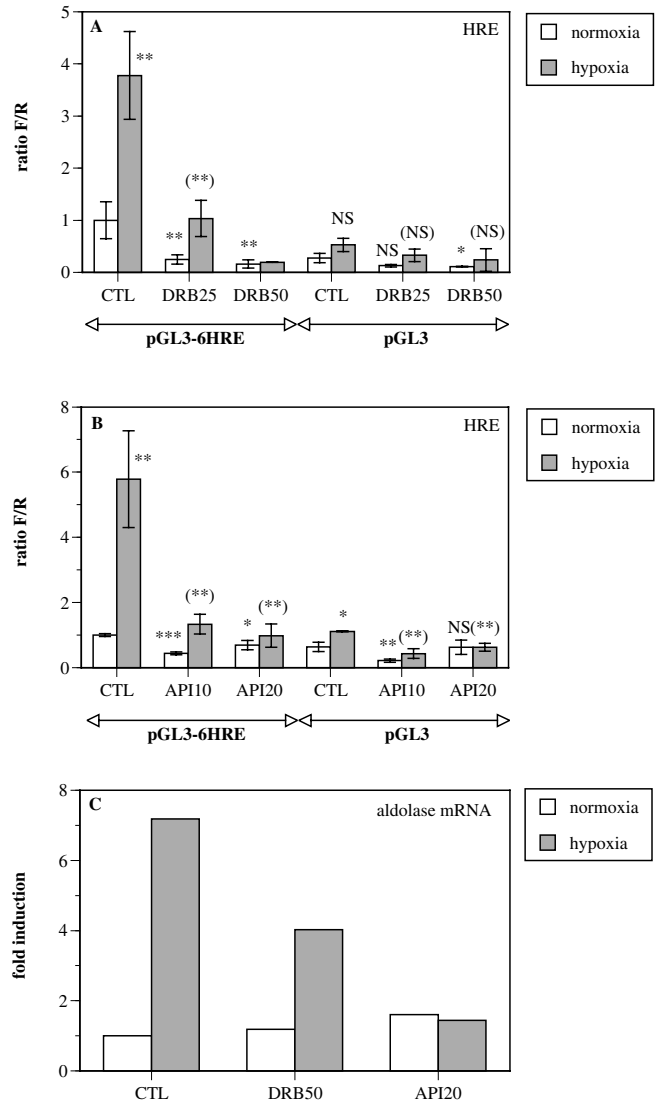


Fig. 3. Effect of CK2 inhibitors on HIF-1 transcriptional activity in HepG2 cells. Cells were incubated for 16 hours under normoxia or hypoxia in the presence of different concentrations (μM) of DRB or apigenin (API). (A,B) Cells were cotransfected with pGL3-6HRE and pRL plasmids. HIF-1 activity is expressed as the ratio of native firefly luciferase to synthetic *Renilla* luciferase activity (means \pm 1 s.d.; $n=3$). (C) Real-time PCR analysis of the aldolase mRNA level. Relative aldolase mRNA levels were calculated as the ratio of aldolase expression in hypoxia-incubated cells to these in control normoxic cells, both normalised to α -tubulin levels. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, NS, not significant vs. normoxia; (*) $P<0.05$, (**) $P<0.01$, (***) $P<0.001$, (NS) not significant vs. hypoxia.

be required for HIF-1 inhibition by CK2 inhibitors, it is through an increase in p53 level that the inhibition of CK2 activity leads to an inhibition of HIF-1 activity.

p53-HIF-1 interaction

Most of the work reporting that p53 is able to inhibit HIF-1 show that this inhibition is due to a decrease in HIF-1 α protein level through an enhanced degradation mediated by MDM2 (Ravi et al., 2000). However, here, DRB and apigenin inhibits HIF-1 activity via p53 but without profound change in HIF-1 α

protein level (Fig. 1A). The contribution of MDM2 has been investigated, using nutlin. Three experiments were performed to follow MDM2, p53 and HIF-1 in the presence of nutlin (Fig. 8). MDM2 protein level decreased under hypoxic conditions, consistent with the decrease observed in p53 protein level and activity. As MDM2 is a p53 target gene, this result suggests that the expression of *MDM2* is directly related to p53 activity and if p53 protein level decreases under hypoxia because CK2 activity increases, this would explain the low p53 activity and the reduced expression of its target genes. On the other hand, when p53 is upregulated, i.e. in the presence of CK2 inhibitors, MDM2 levels also increased (in the presence of TBB after 6 hours incubation) (Fig. 8). These results suggest again that MDM2 levels vary in parallel with (and as a consequence of) p53 activity.

Nutlin at 5 μM was able to increase p53 DNA-binding activity as well as its transcriptional activity measured by a reporter assay. These results were expected knowing the inhibitory effect of nutlin on MDM2 activity. However, despite its marked effect on p53, nutlin had very little or even no effect on HIF-1. Nutlin slightly decreased HIF-1 α protein level but did not affect HIF-1 DNA-binding activity or its transcriptional activity measured by a reporter assay. These results indicate that, although both CK2 and MDM2 inhibition leads to p53

accumulation, inhibition of MDM2 does not reproduce the effect of CK2 inhibition on HIF-1 activity. It has to be noted that MDM2 inhibition markedly increased p53 transcriptional activity, which is not needed for inhibiting HIF-1 in our experimental model (see experiment in the presence of pifithrin- α). Different forms of post-translationally modified p53 accumulating after MDM2 or CK2 inhibition might explain the different effects on HIF-1 activity.

We then sought an interaction between p53 and HIF-1 α . p53 was immunoprecipitated from cells incubated under normoxia or hypoxia, in the presence or the absence of CK2 inhibitors and the amount of HIF-1 α co-precipitated with p53 was assessed by western blot. Results presented in Fig. 9 showed that when there was more p53 present (in the presence of the inhibitors, see lysates), there was more HIF-1 α co-immunoprecipitated whereas the total amount of HIF-1 α was the same (see lysates) (Fig. 9). This was only observed under hypoxic conditions, because no HIF-1 α could be detected by western blot in lysates from normoxic cells. These results indicate that there was more HIF-1 α associated with p53 in the presence of CK2 inhibitors, probably because p53 is more abundant.

p53 activity is not required for downregulation of HIF-1

A transcriptionally inactive p53, mutated in its DNA binding domain retains the ability to inhibit HIF-1 transcriptional activity (Blagosklonny et al., 1998). To investigate whether the HIF-1 inhibitory effect of increased p53 protein level in the presence of CK2 inhibitors requires p53 activity, we coexpressed wild-type or R175H-mutated p53 with the HRE-dependent reporter system in HepG2 cells. The R175H-mutated p53 is mutated in its DNA-binding domain, thus having no transcriptional activity and can act as a dominant-negative mutant (Kern et al., 1992). As can be seen in Fig. 10A, wild-type p53 and the R175H-mutated p53 (DNp53) partially suppressed the HIF-1-dependent reporter activity. On the other hand, wild-type p53 was capable of increasing p53-dependent reporter activity whereas the R175H-mutated p53 completely prevented luciferase expression, as expected from its dominant-negative activity (Fig. 10B). These results suggest that the transcriptional activity of p53 is not required to inhibit HIF-1 activity. Pifithrin- α was used to confirm these results. Pifithrin- α is a chemical

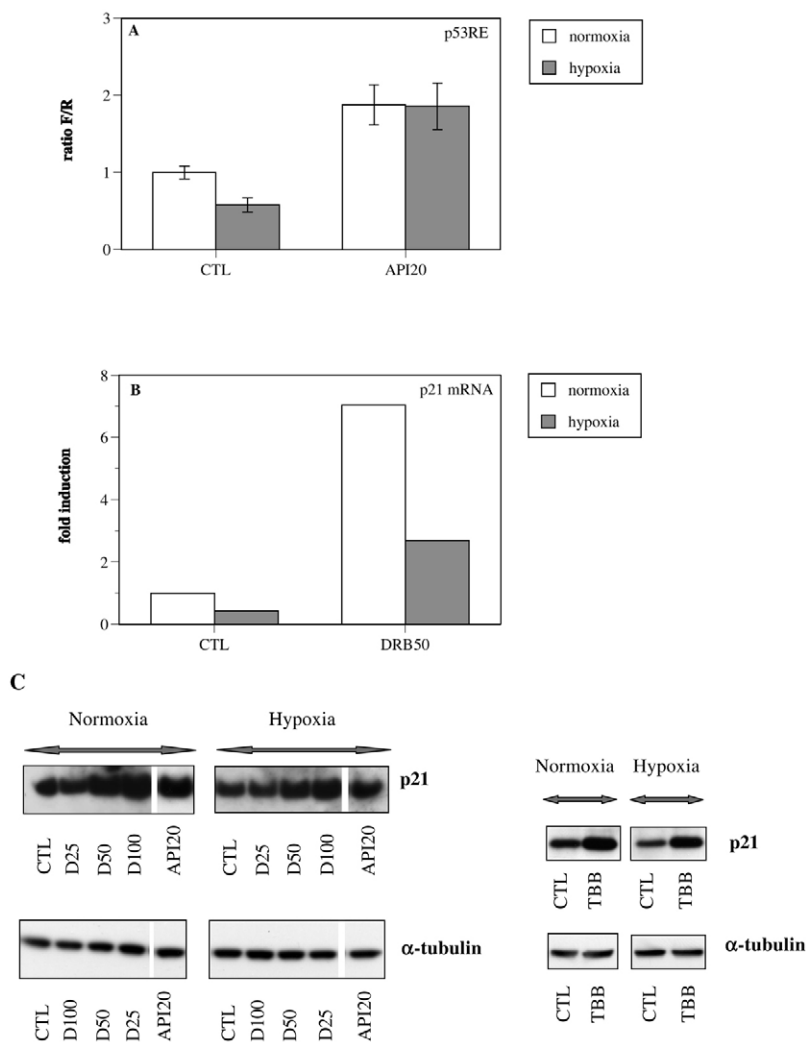


Fig. 4. Effect of CK2 inhibitors on p53 transcriptional activity in HepG2 cells. Cells were incubated for 16 hours under normoxia or hypoxia in the presence or the absence of different concentrations of DRB (μM), 20 μM apigenin (API) or 10 μM TBB. (A) Cells were cotransfected with pG13-Luc and pRL plasmids. p53 activity is expressed as the ratio of native firefly luciferase to synthetic *Renilla* luciferase activity (means \pm 1 s.d.; $n=3$). (B) Real-time PCR analysis of the p21 mRNA level. Relative p21 mRNA levels were calculated as the ratio of p21 expression in hypoxia-incubated cells to these in control normoxic cells, both normalised to α -tubulin levels. (C) Total cell lysates were tested for p21 and α -tubulin protein expression by western blotting.

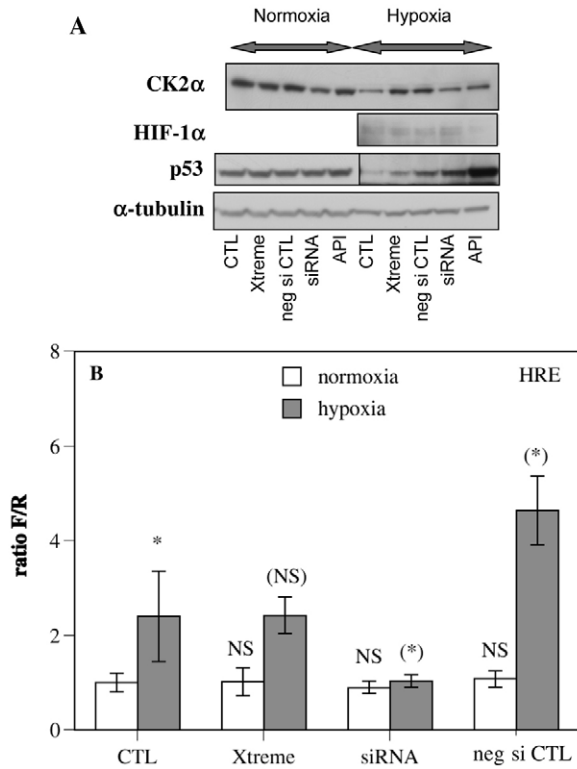


Fig. 5. Effect of CK2 α siRNA on HIF-1 transcriptional activity in HepG2 cells. Cells were transfected with CK2 α siRNA. (A) 42 hours post-transfection, cells were incubated for 6 hours under normoxia or hypoxia in the absence or in the presence of 20 μ M apigenin (API). Total cell lysates were tested for CK2 α , HIF-1 α , p53 and α -tubulin proteins by western blotting. (B) 24 hours post-transfection, cells were cotransfected with pGL3-6HRE and pRL plasmids and then incubated for 16 hours under normoxia or hypoxia. HIF-1 activity is expressed as the ratio of firefly luciferase to *Renilla* luciferase activity (means \pm 1 s.d., $n=3$). Xtreme, cells exposed to the transfection reagent alone; neg si CTL, cells transfected with the negative control siRNA; siRNA, cells transfected with CK2 α siRNA. * $P<0.05$ or NS, not significant vs. normoxia; (*) $P<0.05$ and (NS), not significant vs. hypoxia.

inhibitor of p53, which has been identified and used both in vitro and in vivo (Komarov et al., 1999). It reversibly inhibits p53 transcriptional activity, at least in part through an inhibition of its nuclear import (Bassi et al., 2002; Qin et al., 2002; Toillon et al., 2002). The effect of pifithrin- α was first investigated on HIF-1 α and p53 protein levels. A small decrease in HIF-1 α protein level under hypoxia was observed in the presence of 25 but not 12.5 μ M pifithrin- α (Fig. 11A). Pifithrin- α did not have profound effect on p53 protein level as tested by western blotting (Fig. 11B), although a slight decrease in p53 protein level was observed when immunofluorescence studies were performed (data not shown). In our hands, pifithrin- α did not influence the nuclear localisation of p53 either in normoxia or under hypoxia (data not shown). Next, the effect of this inhibitor was studied on HIF-1 and p53 transcriptional activity. Since it also directly inhibits firefly luciferase activity (Rocha et al., 2003), it was not possible to use this molecule in reporter systems and we

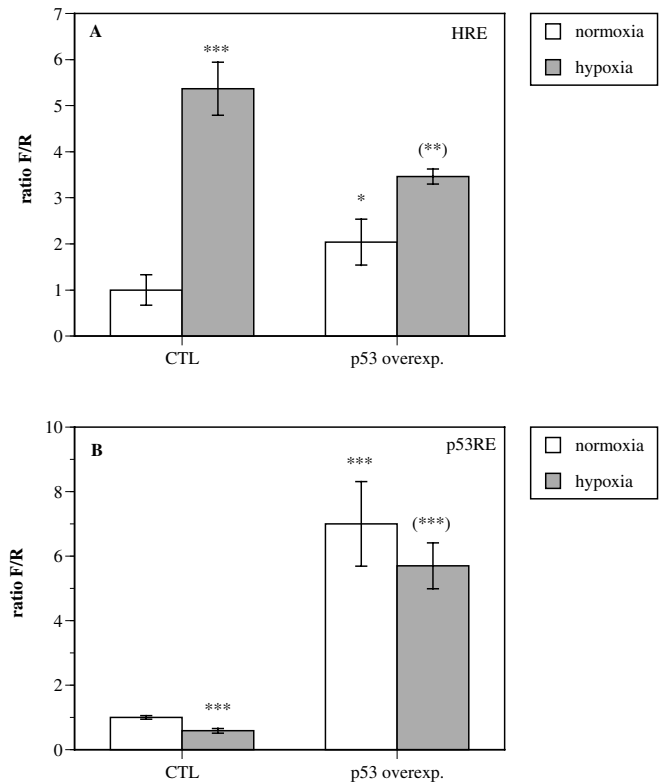


Fig. 6. Effect of p53 overexpression on HIF-1 and p53 transcriptional activity in HepG2 cells. Cells were incubated for 16 hours under normoxia or hypoxia. Cells were cotransfected with pGL3-6HRE (A) or pG13-Luc (B), p53 expression plasmid or pCMV-myc and pRL. p53 activity is expressed as the ratio of firefly luciferase to *Renilla* luciferase activity (means \pm 1 s.d.; $n=3$). * $P<0.05$ and *** $P<0.001$ vs. normoxia; (**) $P<0.01$ and (***) $P<0.001$ vs. hypoxia.

investigated its effect on HIF-1 and p53 activity by following the expression of target genes, respectively aldolase and p21, by real-time PCR. Pifithrin- α alone did not influence aldolase mRNA level under normoxia or under hypoxia. On the other hand, DRB decreased mRNA aldolase level under hypoxia as already observed in Fig. 4. This was further decreased in the presence of pifithrin- α (Fig. 11C), indicating that the inhibitory effect of DRB (i.e. increased p53 protein level) on HIF-1 activity was still observed even if p53 activity was inhibited. That p53 activity was indeed inhibited by pifithrin- α was confirmed by the results of Fig. 11D where a decrease in p21 mRNA level was observed in the presence of this inhibitor. Pifithrin- α also strongly diminished the increase in p21 mRNA level induced by DRB, both under normoxia and hypoxia. Altogether these results indicate that p53 activity is not needed for p53 to inhibit HIF-1 transcriptional activity when its expression is increased by CK2 inhibition.

Discussion

HIF-1 α accumulation and transactivation of HIF-1 constitute the master regulatory system to cope with conditions of reduced oxygen availability. In addition to regulation by hydroxylation, several other mechanisms are involved in positively or negatively regulating HIF-1 activity, including

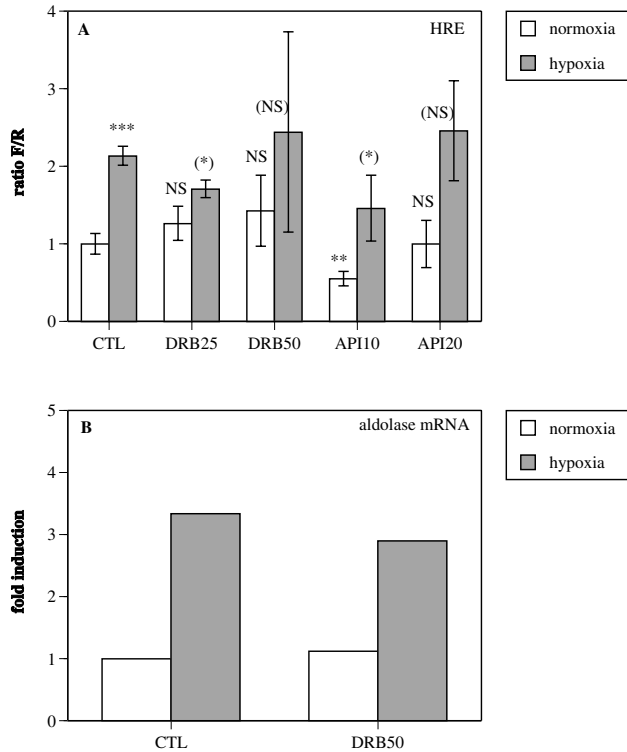


Fig. 7. Effect of CK2 inhibitors on HIF-1 transcriptional activity in Hep3B cells. Cells were incubated for 16 hours under normoxia or hypoxia in the presence or the absence of different concentrations (μM) of DRB or apigenin (API). (A) Cells were cotransfected with pGL3-6HRE and pRL plasmids. HIF-1 activity is expressed as the ratio of firefly luciferase to *Renilla* luciferase activity (means \pm 1 s.d.; $n=3$). (B) Real-time PCR analysis of the aldolase mRNA level. Relative aldolase mRNA levels were calculated as the ratio of aldolase expression in hypoxia-incubated cells to these in control normoxic cells, both normalised to α -tubulin levels. ** $P<0.01$, *** $P<0.001$ or NS, not significant vs. normoxia; (*) $P<0.05$ or (NS), not significant vs. hypoxia.

association with Hsp90 (Isaacs et al., 2002; Minet et al., 1999), phosphorylation (Minet et al., 2000; Mottet et al., 2003; Richard et al., 1999), sequestration in the nucleolus by p14^{ARF} (Fatyol and Szalay, 2001) as well as co-activator recruitment (Sang et al., 2003). Moreover, a complex relationship between HIF-1 and p53 has emerged from various works. Anoxia or severe hypoxia is one of the strongest inducers of p53 (An et al., 1998). This induction of p53 under severe hypoxia requires concomitant induction of HIF-1 α : HIF-1 α binds to and stabilises p53 (Maxwell et al., 1997) leading to an enhanced transcriptional activity of p53 (An et al., 1998). Conversely, HIF-1 activity is inhibited either through enhanced MDM2-mediated degradation of HIF-1 α (Carmeliet et al., 1998; Ravi et al., 2000) or through competition for a common co-activator (Blagosklonny et al., 1998; Schmid et al., 2004). It must however be stressed that p53 accumulation under hypoxia seems to occur at very low oxygen concentrations, near to anoxia (Pan et al., 2004; Piret et al., 2002) and that HIF-1 α upregulation at mild hypoxia (1% O₂) is not sufficient for anoxic p53 induction (Kaluzova et al., 2004; Schmid et al., 2004). However, the

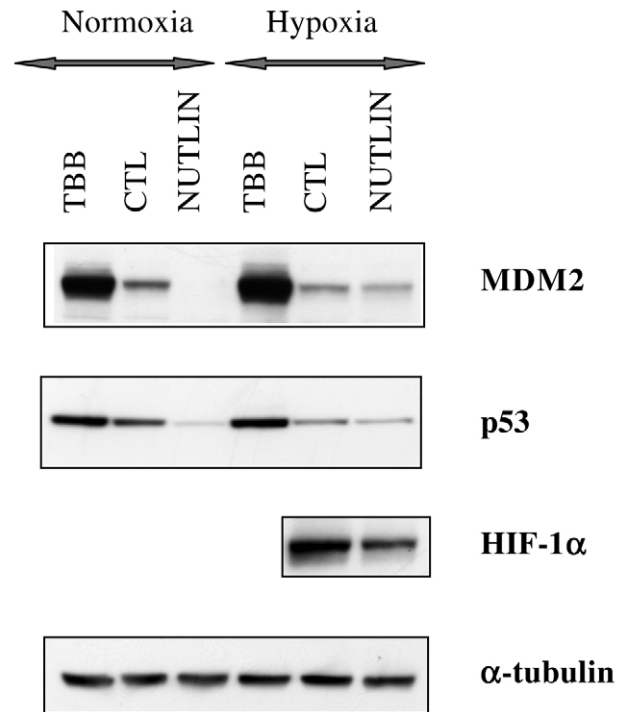


Fig. 8. Effect of nutlin and TBB on MDM2, HIF-1 α and p53 protein levels in HepG2 cells. Cells were incubated for 5 hours under normoxia or hypoxia in the presence or the absence of 10 μM TBB or of 5 μM nutlin. Total cell lysates were tested for HIF-1 α , p53, MDM2 and α -tubulin protein expression by western blotting.

influence of p53 under mild hypoxia on HIF-1 activity remained unclear.

We recently showed that CK2 activity was enhanced under hypoxia and that this kinase positively regulates HIF-1 transcriptional activity without affecting HIF-1 α protein level (Mottet et al., 2005). However, the exact mechanism of this regulation was not known. Here, we showed that CK2 activity maintained p53 protein at a low level, because its inhibition led to an overexpression of p53, both under normoxia and hypoxia. In addition, under our experimental conditions, the p53 protein level was decreased under hypoxia in a time-dependent manner. This decrease in p53 protein level is mirrored by a decrease in its activity as observed using a reporter system and by following the expression of p21, a p53 target gene. Similar results were obtained by Pan et al. (Pan et al., 2004), also with HepG2 cells. We propose that this decrease may be due to a higher CK2-mediated targeting for proteasome degradation, for example via the COP9 signalosome, owing to higher CK2 activity. It must be noted that Achison and Hupp (Achison and Hupp, 2003) reported a decrease in Ser392 phosphorylation of p53, that is a CK2 phosphorylation site, under conditions of mild oxygen as used here. This was observed without a change in p53 protein level and in human colorectal carcinoma HCT116 cells, which may explain the discrepancy with our results. It must also be noted that DRB at concentrations used in this work actually decreased p53 phosphorylation on Ser392. On the other hand, at higher concentrations, DRB may

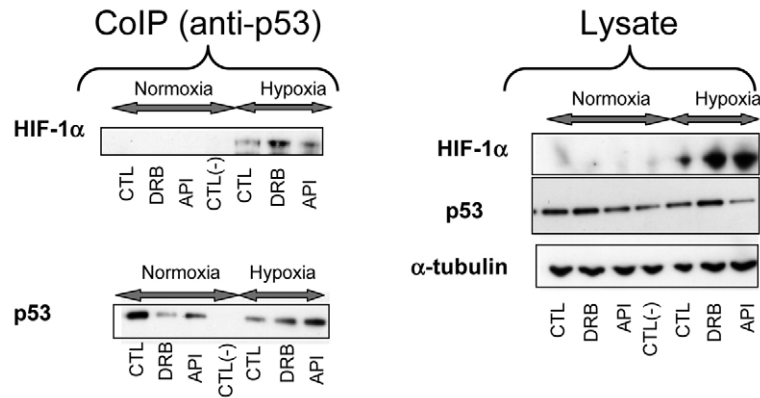


Fig. 9. Effect of CK2 inhibitors on HIF-1 α -p53 interaction in HepG2 cells. Cells were incubated 5 hours under normoxia or hypoxia in the presence or the absence of 50 μ M DRB or 20 μ M apigenin (API). 50 μ l of total cell lysates were tested for HIF-1 α , p53 and α -tubulin by western blotting and the remaining 950 μ l were immunoprecipitated for p53. The amount of p53 and HIF-1 α protein in the immunoprecipitated samples was detected by western blot. CTL(-) are samples immunoprecipitated without anti-p53 antibodies.

lead to a paradoxical increase (Blaydes and Hupp, 1998) in this phosphorylation without increasing p53 degradation.

The pressure exerted on p53 by increased CK2 activity under hypoxic conditions may be the mechanism by which CK2 positively regulates HIF-1 activity. Indeed, we recently showed that CK2 activity is enhanced under hypoxia (Mottet et al., 2005). We have summarised this hypothesis schematically in Fig. 12, knowing that more fine-tuning will probably be involved and that further work is still needed to strengthen it. Two experimental results support this hypothesis. First, ectopic

overexpression of p53 inhibited HIF-1 activity, indicating that in conditions of mild hypoxia, p53 can negatively influence HIF-1 activity with no need of a further signal. Artificial overexpression of p53 has already been reported to inhibit HIF-1 (Blagosklonny et al., 1998; Fontanini et al., 1998). Second, the inhibitory effect of CK2 inhibition on HIF-1 activity was not observed in p53-null cells demonstrating that this protein is a necessary intermediate between CK2 and HIF-1.

Two mechanisms have been proposed to explain HIF-1 inhibition by p53. In the first, large amounts of p53 target HIF-1 α for degradation through MDM2-mediated ubiquitylation (Ravi et al., 2000). This pathway is probably not involved here because no marked change in HIF-1 α protein level was observed. On the other hand, another study demonstrated that p53 overexpression downregulates HIF-1 transcriptional activity by competition for p300 coactivator (Blagosklonny et al., 1998). In this study, it was reported that the transcriptionally inactive mutant p53, mutated in its DNA binding domain, retained the ability to inhibit HIF-1 transcriptional activity, whereas a double mutant in the DNA-binding domain and in the p300-interaction domain lost this inhibitory function. More recently, Schmid et al. (Schmid et al., 2004) showed that low p53 expression represses HIF-1 transactivation without affecting HIF-1 α protein amount whereas high p53 levels lead to HIF-1 α destruction, implying that distinct mechanisms suppress HIF-1 depending on relative p53 expression. The first effect was due to competition between p53 and HIF-1 for limiting amounts of the shared co-activator p300. HIF-1 α and p53 bind to distinct regions of p300. The HIF-1-binding site has been localised to a region encompassing amino acids 326-410 in the CH1-domain (Arany et al., 1996) whereas a fragment comprising amino acids 1514-1922 interacts with p53 (Avantaggiati et al., 1997; Gu et al., 1997). These observations raise the possibility that p53 and HIF-1 may bind to p300 simultaneously, leading to interference by p53 of HIF-1-p300-mediated transactivation. This could lead to co-immunoprecipitation without direct HIF-1 α -p53 interaction. Such a model is compatible with our observations (Fig. 7) showing that, despite similar amounts of HIF-1 α , more of this protein is pulled down when more p53 is present in the cells, i.e. in the presence of CK2 inhibitors. It has to be mentioned that direct interaction between p53 and HIF-1 α has also been demonstrated (Sanchez-Puig et al., 2005).

This hypothesis is reinforced by the fact that our data showed that p53 activity was not needed to exert its HIF-1 inhibitory effect. Indeed, a mutated form of p53 no longer able to bind DNA inhibited HIF-1 activity to the same extent

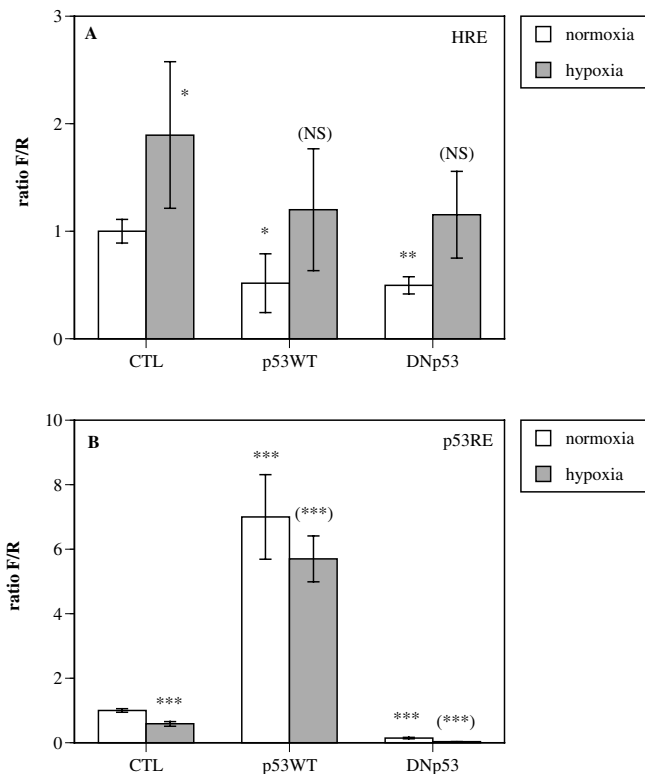


Fig. 10. Effect of wild-type and mutated p53 overexpression on HIF-1 transcriptional activity in HepG2 cells. Cells were incubated for 16 hours under normoxia or hypoxia. Cells were cotransfected with pGL3-6HRE (A) or pG13-Luc (B), p53 expression plasmid or pCMV-myc and pRL. HIF-1 or p53 activity is expressed as the ratio of firefly luciferase to *Renilla* luciferase activity (means \pm 1 s.d., $n=3$). * $P<0.05$, ** $P<0.01$ or *** $P<0.001$ vs. normoxia; (***) $P<0.001$ or (NS), not significant vs. hypoxia.

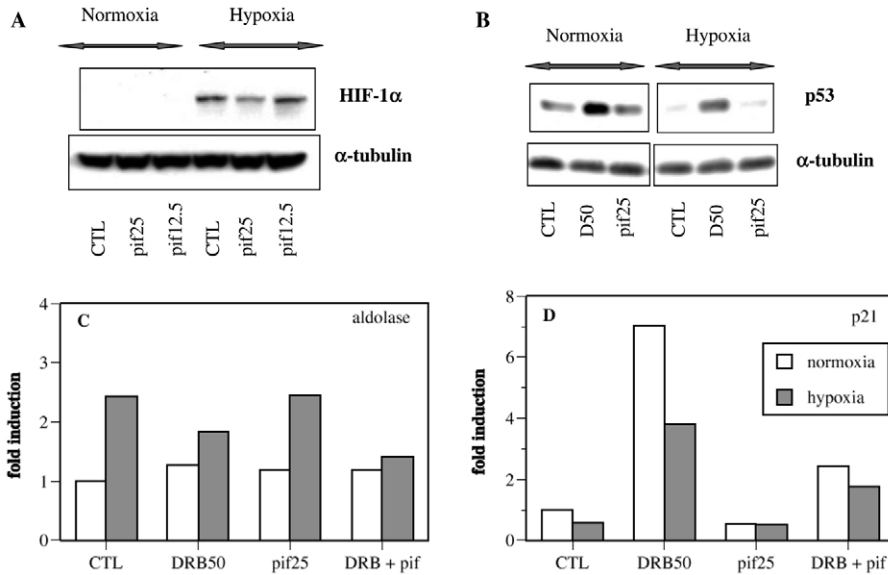


Fig. 11. Effect of pifithrin- α on HIF-1 and p53 protein level and transcriptional activity in HepG2 cells. (A,B) Cells were incubated for 5 hours under normoxia or hypoxia in the presence of different concentrations (μ M) of pifithrin- α or 50 μ M DRB. Total cell lysates were tested for HIF-1 α (A), total p53 (B) and α -tubulin by western blotting. (C,D) Cells were incubated for 6 hours under normoxia or hypoxia in the presence of 25 μ M pifithrin and/or 50 μ M DRB. Real-time PCR analysis of the aldolase (C) and p21 (D) mRNA level was performed. Relative aldolase or p21 mRNA levels were calculated as the ratio of aldolase or p21 mRNA expression in hypoxia-incubated cells to these in control normoxic cells, both normalized to α -tubulin levels.

as wild-type p53. In addition, DRB had a similar effect in the presence than in the absence of pifithrin- α , an inhibitor of p53. This is similar to that described by Blagosklonny et al. (Blagosklonny et al., 1998) but contrary to the results of Kaluzova et al. (Kaluzova et al., 2004), which show that p53 activity is needed for p53 to inhibit HIF-1-mediated overexpression of carbonic anhydrase IX. However, in this case, p53 was chemically activated by DNA damage. Recent work by Fang et al. (Fang et al., 2005) shows that apigenin decreases HIF-1 activity and VEGF expression through a MDM2-p53 pathway. However, the exact mechanism by which apigenin was acting was not determined in this study.

In conclusion, these data unravel a new mechanism of hypoxia-induced activation of HIF-1, through a CK2-dependent downregulation of p53. Since CK2 is often overexpressed in human tumours, this pathway suggests a

further mechanism by which tumour cells adapt to their hypoxic environmental conditions.

Materials and Methods

Cell culture

Human HepG2 hepatoma cells lines containing wild-type p53 and Hep3B (p53 null) were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing respectively 1 or 4.5 g/l glucose, supplemented with 10% fetal calf serum (Invitrogen), 100 U/ml penicillin, 100 μ g/ml streptomycin and 50 ng/ml amphotericin B. HeLa cells (wt p53) were cultured in DHG supplemented with 10% fetal calf serum. Cells were kept at 37°C in humidified atmosphere of 5% CO₂, 95% air. For hypoxia experiments (1% O₂), cells were incubated in serum-free CO₂-independent medium (Invitrogen) supplemented with 1 mM L-glutamine (Sigma) with different concentrations of DRB (Biomol), apigenin (Sigma), TBB (Calbiochem), nutlin (Calbiochem) or pifithrin- α (Alexis Biochemicals).

Western blot analysis

Total cell extracts were prepared from HepG2 or HeLa cells grown to subconfluency in 25 cm² flasks. After incubation, cells were scraped in lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM KCl, 1 mM EDTA, 1% Triton X-100 (all from Merck), protease inhibitors (Complete, Boehringer/Roche) and phosphatase inhibitors (25 mM Na₂VO₄, 10 mM PNPP, 10 mM β -glycerophosphate and 5 mM NaF). The lysate was centrifuged for 5 minutes at 13,000 g at 4°C and the supernatant kept frozen. The protein concentration of each sample was determined by the Bradford method. Samples were applied to 10% NuPAGE Bis-Tris gels (Invitrogen), according to the manufacturer's instructions and then transferred to Hybond-PVDF membrane (Amersham). Membranes were blocked overnight at 4°C in TBS-T solution containing 20 mM Tris, 140 mM NaCl, 0.1% Tween 20, pH 7.6, containing 5% non-fat dry milk. Then, membranes were probed with monoclonal anti-HIF-1 α antibody (BD Bioscience) at a final dilution 1:2000, monoclonal anti-p53 antibody (Upstate) at a final dilution 1:5000, polyclonal anti-phospho-Ser392 p53 (Cell Signaling) at a final dilution 1:5000, polyclonal anti-p21 antibody (Santa Cruz) at a final dilution 1:10,000 or monoclonal anti-MDM2 (BD Bioscience) at a final dilution 1:5000, 2 hours at room temperature. After three 15-minute washes in TBS-T with 5% non-fat dry milk, membranes were incubated for 1 hour at room temperature with HRP-conjugated secondary antibodies at a final dilution 1:50,000 and washed twice for 15 minutes in TBS-T with 5% non-fat dry milk.

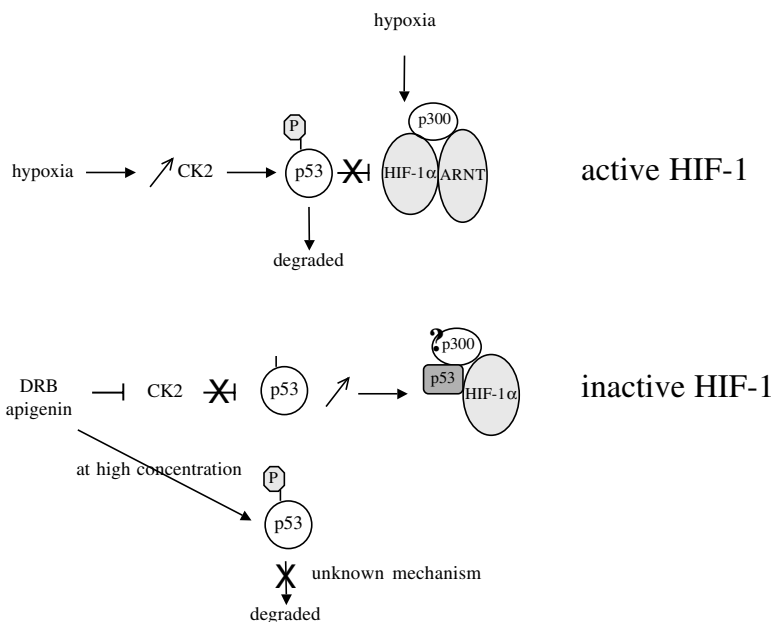


Fig. 12. Schematic representation of the possible role of CK2 in p53-dependent regulation of HIF-1 activity. Under hypoxia, elevated CK2 activity triggers p53 degradation, thus relieving p53 inhibition on HIF-1, leading to active HIF-1. When CK2 is inhibited, p53 is stabilized, competes with HIF-1 α for p300, lowering HIF-1 activity.

and twice for 5 minutes in TBS-T. Proteins were visualised by ECL (Amersham) according to the manufacturer's instructions. Membranes were probed with α -tubulin antibody (Sigma, final dilution 1:50,000) for normalisation.

Co-immunoprecipitation

HepG2 cells were grown to subconfluence in 75 cm² flasks and incubated under normoxia or hypoxia for 5 hours before cell lysis. Cells were lysed in 1 ml co-IP lysis buffer [25 mM Tris-HCl pH 7.8, 100 mM NaCl, 1 mM EDTA, 10% glycerol (Merck), 0.2% NP-40 (Sigma), 1 mM DTT (Sigma), 1 mM PMSF (Sigma), 5 mM NaF (Merck), 1 mM NaMoO₄ (Sigma) and 60 μ M MG132 (Biomol)]. An aliquot of 50 μ l was kept for assessing the protein level of p53 and HIF-1 α in the cell extracts. Lysates were incubated for 2 hours at 4°C with 2.5 μ g polyclonal anti-p53 antibody (Santa Cruz), then 2 hours at 4°C with 50 μ l anti-Myc agarose beads (BD Bioscience). The beads were washed three times with 800 μ l of the co-IP lysis buffer, after which the associated proteins were recovered by incubated the beads in Laemmli buffer 10 minutes at 100°C. Samples were resolved on 10% NuPAGE Bis-Tris gels (Invitrogen) and western blots for p53 and HIF-1 α were performed.

Preparation of nuclear extracts

HeLa cells were grown to subconfluence in 75 cm² flasks and incubated under normoxia or hypoxia for 5 hours before cell lysis. Cells were washed with ice-cold PBS containing 1 mM Na₂MoO₄, and 5 mM NaF. Then, cells were incubated for 3 minutes with ice-cold hypotonic buffer (HB) containing 20 mM HEPES pH 7.9, 5 mM NaF, 1 mM Na₂MoO₄ and 0.1 mM EDTA. Cells were harvested in lysis buffer containing HB, supplemented with 5% NP-40 (Sigma). Lysates were centrifuged for 30 seconds at 13,000 g and supernatants were discarded. Pellets were dissolved in 50 μ l resuspension buffer, composed of HB supplemented with 20% glycerol and protease inhibitors (Complete, Roche) and phosphatase inhibitors (25 mM Na₂VO₄, 10 mM PNPP, 10 mM β -glycerophosphate and 5 mM NaF). Then, 50 μ l salt buffer, composed of RE buffer, supplemented with 400 mM NaCl, were added. Samples were placed at 4°C for 30 minutes under gently rotation for nuclear protein extraction. Then, samples were centrifuged for 10 minutes at 13,000 g at 4°C and supernatants were stored at -70°C.

Colorimetric assay for HIF-1 or p53 DNA binding

HIF-1 or p53 DNA binding activity was measured using a colorimetric assay (TransAM) developed in our laboratory (Renard et al., 2001) and sold by Active Motif. Assays were performed according to the manufacturer's instructions.

Transient transfection and luciferase activity measurement

To assay the transcriptional activity of HIF-1, the pGL3-SV40/6HRE reporter vector containing an artificial promoter with TATA box and six copies of the erythropoietin HRE cis-element upstream of the firefly luciferase gene was used (Michel et al., 2002). p53 activity was assessed using a reporter plasmid carrying 13 copies of a p53-responsive promoter driving the expression of the luciferase gene, pG13-Luc (Kern et al., 1992). pGL3-SV40 (Promega) was used as the negative control. HepG2 transfection was performed in a 24-well plate with Superfect transfection reagent (Qiagen). 200 ng pGL3-SV40/6HRE or pG13-Luc were co-transfected with 100 ng control vector pRL-SV40 (Promega) and 1 μ g expression or null vector (pCMV-myc, Clontech BD Bioscience). Thereafter, the transfection mix was replaced by fresh culture medium. 5 hours post-transfection, cells were incubated under normoxia or hypoxia for 16 hours. Luciferase activity measurements were performed in a luminometer using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

siRNA treatment of Cells

HepG2 cells were seeded at 60% confluence onto 24-well plate the day before transfection. CK2 α siRNAs were synthesised (5'-GUCACGGCUUACUGCAAGA-3', Eurogentec). The negative control siRNA oligonucleotide was a unique sequence and did not correspond to any known gene in the human genome database (negative siRNA control, Eurogentec). For transfections, siRNAs were diluted with OptiMEM (Invitrogen), incubated with Xtreme Transfection Reagent (Roche) according to the manufacturer's protocol and diluted to a final concentration of 100 nM. The cells were incubated with siRNA complexes for 5 hours, whereupon the medium was replaced. Analyses were performed 48 hours post-transfection.

Extraction of RNA and cDNA synthesis

Preparation of total RNA was performed using RNAgent kit (Promega) according to the manufacturer's instructions. mRNAs were then retrotranscribed into cDNA using oligodT primers and Superscript II reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions.

Real-time PCR analysis

The levels of p21 or aldolase transcripts were determined by real-time reverse transcriptase (RT)-PCR using SYBR Green (Invitrogen). To normalize for input load of cDNA between samples, human α -tubulin was used as an endogenous standard. Specific primers were used: p21 forward, 5'-CTGGAGACTCTCAGG-

TCGAA-3' and p21 reverse, 5'-CCAGGACTGCAGGCTTCCT-3'; aldolase forward, 5'-GAATTGGATGAAAGATAAAGCCCTTA-3' and aldolase reverse, 5'-TTGCCAGACCATCCGTACTG-3'; α -tubulin forward, 5'-CCAGGAGGCACTACACT-3' and α -tubulin reverse, 5'-CAGGGAGGTGAACCCAGAAC-3'. cDNA was added to SYBR Green Master Mix PCR (300 nM of each specific primer). PCRs were performed in a total volume of 25 μ l in a real-time PCR cycler (ABI PRISM 7700 Sequence Detector, PE Applied Biosystems). Thermal cycling conditions were: initial incubation of 10 minutes at 95°C, followed by 40 cycles of 30 seconds at 95°C, 1 minute at 57°C annealing temperature, and 30 seconds at 72°C. All cDNA samples were tested in duplicate and analysed with ABI Prism Sequence Detection Software v.1.7 (PE Applied Biosystems). Samples were compared using the relative Ct method. The Ct value, which is inversely proportional to the initial template copy number, is the calculated cycle number where the fluorescence signal is significantly above background levels. Fold induction or repression was measured relative to controls and calculated after adjusting for α -tubulin using $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct$ tested gene - Ct α -tubulin and $\Delta\Delta Ct = \Delta Ct$ control - ΔCt treatment (Winer et al., 1999).

Statistical analysis

Statistical analyses were performed using Student's *t* test. For each set of data, real triplicates were performed in one experiment.

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References

- Achison, M. and Hupp, T. R. (2003). Hypoxia attenuates the p53 response to cellular damage. *Oncogene* **22**, 3431-3440.
- Ahmed, K., Gerber, D. A. and Cochet, C. (2002). Joining the cell survival squad: an emerging role for protein kinase CK2. *Trends Cell Biol.* **12**, 226-230.
- An, W. G., Kanekal, M., Simon, M. C., Maltepe, E., Blagosklonny, M. V. and Neckers, L. M. (1998). Stabilization of wild-type p53 by hypoxia-inducible factor 1 α . *Nature* **392**, 405-408.
- Arany, Z., Huang, L. E., Eckner, R., Bhattacharya, S., Jiang, C., Goldberg, M. A., Bunn, H. F. and Livingston, D. M. (1996). An essential role for p300/CBP in the cellular response to hypoxia. *Proc. Natl. Acad. Sci. USA* **93**, 12969-12973.
- Avantaggiati, M. L., Ogryzko, V., Gardner, K., Giordano, A., Levine, A. S. and Kelly, K. (1997). Recruitment of p300/CBP in p53-dependent signal pathways. *Cell* **89**, 1175-1184.
- Bassi, L., Carloni, M., Fonti, E., Palma de la Pena, N., Meschini, R. and Palitti, F. (2002). Pifithrin- α , an inhibitor of p53, enhances the genetic instability induced by etoposide (VP16) in human lymphoblastoid cells treated in vitro. *Mutat. Res.* **499**, 163-176.
- Blagosklonny, M. V., An, W. G., Romanova, L. Y., Trepel, J., Fojo, T. and Neckers, L. (1998). p53 inhibits hypoxia-inducible factor-stimulated transcription. *J. Biol. Chem.* **273**, 11995-11998.
- Blaydes, J. P. and Hupp, T. R. (1998). DNA damage triggers DRB-resistant phosphorylation of human p53 at the CK2 site. *Oncogene* **17**, 1045-1052.
- Brugarolas, J., Chandrasekaran, C., Gordon, J. I., Beach, D., Jacks, T. and Hannon, G. J. (1995). Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature* **377**, 552-557.
- Carmeliet, P., Dor, Y., Herbert, J. M., Fukumura, D., Brusselmans, K., Dewerchin, M., Neeman, M., Bono, F., Abramovitch, R., Maxwell, P. et al. (1998). Role of HIF-1 α in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* **394**, 485-490.
- Chen, D., Li, M., Luo, J. and Gu, W. (2003). Direct interactions between HIF-1 α and Mdm2 modulate p53 function. *J. Biol. Chem.* **278**, 13595-13598.
- Critchfield, J. W., Coligan, J. E., Folks, T. M. and Butera, S. T. (1997). Casein kinase II is a selective target of HIV-1 transcriptional inhibitors. *Proc. Natl. Acad. Sci. USA* **94**, 6110-6115.
- el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W. and Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. *Cell* **75**, 817-825.
- Fang, J., Xia, C., Cao, Z., Zheng, J. Z., Reed, E. and Jiang, B. H. (2005). Apigenin inhibits VEGF and HIF-1 expression via PI3K/AKT/p70S6K1 and HDM2/p53 pathways. *FASEB J.* **19**, 342-353.
- Farah, M., Parhar, K., Moussavi, M., Eivemark, S. and Salh, B. (2003). 5,6-Dichlororibifuranosylbenzimidazole- and apigenin-induced sensitization of colon cancer cells

- to TNF-alpha-mediated apoptosis. *Am. J. Physiol. Gastrointest. Liver Physiol.* **285**, G919-G928.
- Fatyol, K. and Szalay, A. A. (2001). The p14ARF tumor suppressor protein facilitates nucleolar sequestration of hypoxia-inducible factor-1alpha (HIF-1alpha) and inhibits HIF-1-mediated transcription. *J. Biol. Chem.* **276**, 28421-28429.
- Faust, R. A., Niehans, G., Gapany, M., Hoistad, D., Knapp, D., Cherwitz, D., Davis, A., Adams, G. L. and Ahmed, K. (1999). Subcellular immunolocalization of protein kinase CK2 in normal and carcinoma cells. *Int. J. Biochem. Cell Biol.* **31**, 941-949.
- Fontanini, G., Boldrini, L., Vignati, S., Chine, S., Basolo, F., Silvestri, V., Lucchi, M., Mussi, A., Angeletti, C. A. and Bevilacqua, G. (1998). Bcl2 and p53 regulate vascular endothelial growth factor (VEGF)-mediated angiogenesis in non-small cell lung carcinoma. *Eur. J. Cancer* **34**, 718-723.
- Ford, H. L., Landesman-Bollag, E., Dacwag, C. S., Stukenberg, P. T., Pardee, A. B. and Seldin, D. C. (2000). Cell cycle-regulated phosphorylation of the human SIX1 homeodomain protein. *J. Biol. Chem.* **275**, 22245-22254.
- Giacca, A. J. and Kastan, M. B. (1998). The complexity of p53 modulation: emerging patterns from divergent signals. *Genes Dev.* **12**, 2973-2983.
- Gu, W., Shi, X. L. and Roeder, R. G. (1997). Synergistic activation of transcription by CBP and p53. *Nature* **387**, 819-823.
- Hammond, E. M., Denko, N. C., Dorie, M. J., Abraham, R. T. and Giaccia, A. J. (2002). Hypoxia links ATR and p53 through replication arrest. *Mol. Cell. Biol.* **22**, 1834-1843.
- Hansson, L. O., Friedler, A., Freund, S., Rudiger, S. and Fersht, A. R. (2002). Two sequence motifs from HIF-1alpha bind to the DNA-binding site of p53. *Proc. Natl. Acad. Sci. USA* **99**, 10305-10309.
- Hanyaloglu, A. C., Vrecl, M., Kroeger, K. M., Miles, L. E., Qian, H., Thomas, W. G. and Eidne, K. A. (2001). Casein kinase II sites in the intracellular C-terminal domain of the thyrotropin-releasing hormone receptor and chimeric gonadotropin-releasing hormone receptors contribute to beta-arrestin-dependent internalization. *J. Biol. Chem.* **276**, 18066-18074.
- Harris, C. C. (1996). p53 tumor suppressor gene: from the basic research laboratory to the clinic—an abridged historical perspective. *Carcinogenesis* **17**, 1187-1198.
- Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C. C. (1991). p53 mutations in human cancers. *Science* **253**, 49-53.
- Hopfl, G., Ogunshola, O. and Gassmann, M. (2004). HIFs and tumors—causes and consequences. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **286**, R608-R623.
- Isaacs, J. S., Jung, Y. J., Mimnaugh, E. G., Martinez, A., Cuttitta, F. and Neckers, L. M. (2002). Hsp90 regulates a von Hippel Lindau-independent hypoxia-inducible factor-1 alpha-degradative pathway. *J. Biol. Chem.* **277**, 29936-29944.
- Ivan, M., Kondo, K., Yang, H., Kim, W., Valiano, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S. and Kaelin, W. G., Jr (2001). HIF1alpha 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., Krieglshem, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J. et al. (2001). Targeting of HIF-1alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science* **292**, 468-472.
- Jain, R. K. (2003). Molecular regulation of vessel maturation. *Nat. Med.* **9**, 685-693.
- Kaluzova, M., Kaluz, S., Lerman, M. I. and Stanbridge, E. J. (2004). DNA damage is a prerequisite for p53-mediated proteasomal degradation of HIF-1alpha in hypoxic cells and downregulation of the hypoxia marker carbonic anhydrase IX. *Mol. Cell. Biol.* **24**, 5757-5766.
- Kern, S. E., Pietenpol, J. A., Thiagalingam, S., Seymour, A., Kinzler, K. W. and Vogelstein, B. (1992). Oncogenic forms of p53 inhibit p53-regulated gene expression. *Science* **256**, 827-830.
- Komarov, P. G., Komarova, E. A., Kondratov, R. V., Christov-Tselkov, K., Coon, J. S., Chernov, M. V. and Gudkov, A. V. (1999). A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science* **285**, 1733-1737.
- Koumenis, C., Alarcon, R., Hammond, E., Sutphin, P., Hoffman, W., Murphy, M., Derr, J., Taya, Y., Lowe, S. W., Kastan, M. et al. (2001). Regulation of p53 by hypoxia: dissociation of transcriptional repression and apoptosis from p53-dependent transcription. *Mol. Cell. Biol.* **21**, 1297-1310.
- Landesman-Bollag, E., Romieu-Mourez, R., Song, D. H., Sonenshein, G. E., Cardiff, R. D. and Seldin, D. C. (2001). Protein kinase CK2 in mammary gland tumorigenesis. *Oncogene* **20**, 3247-3257.
- Lando, D., Peet, D. J., Whelan, D. A., Gorman, J. J. and Whitelaw, M. L. (2002). Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. *Science* **295**, 858-861.
- Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. *Cell* **88**, 323-331.
- Litchfield, D. W. (2003). Protein kinase CK2: structure, regulation and role in cellular decisions of life and death. *Biochem. J.* **369**, 1-15.
- Mahon, P. C., Hirota, K. and Semenza, G. L. (2001). FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev.* **15**, 2675-2686.
- Maxwell, P. H., Dachs, G. U., Gleadow, J. M., Nicholls, L. G., Harris, A. L., Stratford, I. J., Hankinson, O., Pugh, C. W. and Ratcliffe, P. J. (1997). Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. *Proc. Natl. Acad. Sci. USA* **94**, 8104-8109.
- McKendrick, L., Milne, D. and Meek, D. (1999). Protein kinase CK2-dependent regulation of p53 function: evidence that the phosphorylation status of the serine 386 (CK2) site of p53 is constitutive and stable. *Mol. Cell. Biochem.* **191**, 187-199.
- Michel, G., Minet, E., Mottet, D., Remacle, J. and Michiels, C. (2002). Site-directed mutagenesis studies of the hypoxia-inducible factor-1alpha DNA-binding domain. *Biochim. Biophys. Acta* **1578**, 73-83.
- Minet, E., Mottet, D., Michel, G., Roland, I., Raes, M., Remacle, J. and Michiels, C. (1999). Hypoxia-induced activation of HIF-1: role of HIF-1alpha-Hsp90 interaction. *FEBS Lett.* **460**, 251-256.
- Minet, E., Arnould, T., Michel, G., Roland, I., Mottet, D., Raes, M., Remacle, J. and Michiels, C. (2000). ERK activation upon hypoxia: involvement in HIF-1 activation. *FEBS Lett.* **468**, 53-58.
- Miyashita, T., Krajewski, S., Krajewska, M., Wang, H. G., Lin, H. K., Liebermann, D. A., Hoffman, B. and Reed, J. C. (1994). Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. *Oncogene* **9**, 1799-1805.
- Mottet, D., Michel, G., Renard, P., Ninane, N., Raes, M. and Michiels, C. (2003). Role of ERK and calcium in the hypoxia-induced activation of HIF-1. *J. Cell Physiol.* **194**, 30-44.
- Mottet, D., Pyr Dits Rys, S., Demazy, C., Raes, M. and Michiels, C. (2005). A role for casein kinase 2 in the regulation of HIF-1 activity. *Int. J. Cancer* **117**, 764-774.
- Muller, M., Wilder, S., Bannasch, D., Israeli, D., Lehlbach, K., Li-Weber, M., Friedmann, S. L., Galle, P. R., Stremmel, W., Oren, M. et al. (1998). p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs. *J. Exp. Med.* **188**, 2033-2045.
- Munstermann, U., Fritz, G., Seitz, G., Lu, Y. P., Schneider, H. R. and Issinger, O. G. (1990). Casein kinase II is elevated in solid human tumours and rapidly proliferating non-neoplastic tissue. *Eur. J. Biochem.* **189**, 251-257.
- Pan, Y., Oprysko, P. R., Asham, A. M., Koch, C. J. and Simon, M. C. (2004). p53 cannot be induced by hypoxia alone but responds to the hypoxic microenvironment. *Oncogene* **23**, 4975-4983.
- Piret, J. P., Mottet, D., Raes, M. and Michiels, C. (2002). Is HIF-1alpha a pro- or an anti-apoptotic protein? *Biochem. Pharmacol.* **64**, 889-892.
- Qin, J. Z., Chaturvedi, V., Denning, M. F., Bacon, P., Panella, J., Choubey, D. and Nickoloff, B. J. (2002). Regulation of apoptosis by p53 in UV-irradiated human epidermis, psoriatic plaques and senescent keratinocytes. *Oncogene* **21**, 2991-3002.
- Ravi, R., Mookerjee, B., Bhujwala, Z. M., Sutter, C. H., Artemov, D., Zeng, Q., Dillehay, L. E., Madan, A., Semenza, G. L. and Bedi, A. (2000). Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. *Genes Dev.* **14**, 34-44.
- Renard, P., Ernest, I., Houbion, A., Art, M., Le Calvez, H., Raes, M. and Remacle, J. (2001). Development of a sensitive multi-well colorimetric assay for active NFkappaB. *Nucleic Acids Res.* **29**, E21.
- Richard, D. E., Berra, E., Gothie, E., Roux, D. and Pouyssegur, J. (1999). p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1alpha (HIF-1alpha) and enhance the transcriptional activity of HIF-1. *J. Biol. Chem.* **274**, 32631-32637.
- Rocha, S., Campbell, K. J., Roche, K. C. and Perkins, N. D. (2003). The p53-inhibitor pifithrin-alpha inhibits firefly luciferase activity in vivo and in vitro. *BMC Mol. Biol.* **4**, 9.
- Ruzzene, M., Penzo, D. and Pinna, L. A. (2002). Protein kinase CK2 inhibitor 4,5,6,7-tetramobenzotriazole (TBB) induces apoptosis and caspase-dependent degradation of haematopoietic lineage cell-specific protein 1 (HS1) in Jurkat cells. *Biochem. J.* **364**, 41-47.
- Sanchez-Puig, N., Veprintsev, D. B. and Fersht, A. R. (2005). Binding of natively unfolded HIF-1alpha ODD domain to p53. *Mol. Cell* **17**, 11-21.
- Sang, N., Stiehl, D. P., Bohensky, J., Leshchinsky, I., Srinivas, V. and Caro, J. (2003). MAPK signaling up-regulates the activity of hypoxia-inducible factors by its effects on p300. *J. Biol. Chem.* **278**, 14013-14019.
- Schmid, T., Zhou, J., Kohl, R. and Brune, B. (2004). p300 relieves p53-evoked transcriptional repression of hypoxia-inducible factor-1 (HIF-1). *Biochem. J.* **380**, 289-295.
- Semenza, G. L. (2000). HIF-1 and human disease: one highly involved factor. *Genes Dev.* **14**, 1983-1991.
- Semenza, G. L. (2003). Targeting HIF-1 for cancer therapy. *Nat. Rev. Cancer* **3**, 721-732.
- Semenza, G. L., Jiang, B. H., Leung, S. W., Passantino, R., Concordet, J. P., Maire, P. and Giallongo, A. (1996). Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *J. Biol. Chem.* **271**, 32529-32537.
- Shen, J., Channavajhala, P., Seldin, D. C. and Sonenshein, G. E. (2001). Phosphorylation by the protein kinase CK2 promotes calpain-mediated degradation of IkappaBalpha. *J. Immunol.* **167**, 4919-4925.
- Toillon, R. A., Chopin, V., Jouy, N., Fauquette, W., Boilly, B. and Le Bourhis, X. (2002). Normal breast epithelial cells induce p53-dependent apoptosis and p53-independent cell cycle arrest of breast cancer cells. *Breast Cancer Res. Treat.* **71**, 269-280.
- Waldman, T., Kinzler, K. W. and Vogelstein, B. (1995). p21 is necessary for the p53-mediated G1 arrest in human cancer cells. *Cancer Res.* **55**, 5187-5190.
- Wei, N. and Deng, X. W. (2003). The COP9 signalosome. *Annu. Rev. Cell Dev. Biol.* **19**, 261-286.
- Wenger, R. H. (2002). Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression. *FASEB J.* **16**, 1151-1162.
- Winer, J., Jung, C. K., Shackel, I. and Williams, P. M. (1999). Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. *Anal. Biochem.* **270**, 41-49.

Wolf, D. A., Zhou, C. and Wee, S. (2003). The COP9 signalosome: an assembly and maintenance platform for cullin ubiquitin ligases? *Nat. Cell Biol.* **5**, 1029-1033.

Yap, D. B., Hsieh, J. K., Zhong, S., Heath, V., Gusterson, B., Crook, T. and Lu, X. (2004). Ser392 phosphorylation regulates the oncogenic function of mutant p53. *Cancer Res.* **64**, 4749-4754.

Zhu, C. C., Cook, L. B. and Hinkle, P. M. (2002). Dimerization and phosphorylation of thyrotropin-releasing hormone receptors are modulated by agonist stimulation. *J. Biol. Chem.* **277**, 28228-28237.

Zien, P., Abramczyk, O., Domanska, K., Bretner, M. and Szyszka, R. (2003). TBBz but not TBBt discriminates between two molecular forms of CK2 in vivo and its implications. *Biochem. Biophys. Res. Commun.* **312**, 623-628.