HIF-2α phosphorylation by CK1δ promotes erythropoietin secretion in liver cancer cells under hypoxia

Evanthia Pangou¹, Christina Befani¹, Ilias Mylonis¹, Martina Samiotaki², George Panayotou², George Simos¹ and Panagiotis Liakos¹.*

INTRODUCTION

Hypoxia inducible factors (HIFs) are transcriptional activators that orchestrate the cellular response to hypoxia. The HIF family contains HIF-1 and HIF-2, both of which function as heterodimers, with an α subunit and a stably expressed β subunit, also known as ARNT. HIF-1α is ubiquitously expressed and regulates many adaptation responses and metabolic enzymes (Majmundar et al., 2010). The second less-studied isoform (HIF-2α) is encoded by the EPAS1 gene, is restricted to specific cell types and is more involved in angiogenesis and metastasis (Keith et al., 2012). HIF-2 is essential in physiological situations such as embryonic development and especially erythropoiesis, a process in which HIF-2 has a predominant role. This has been shown by studies in HIF-2α-knockout mice documenting HIF-2α as the main regulator of hepatic EPO production and being essential for the maintenance of systemic EPO and iron homeostasis (Rankin et al., 2007). HIF-2 also plays substantial roles in pathological situations, such as during the progression of solid tumors (Patel and Simon, 2008; Zhao et al., 2015).

HIF-2α is regulated by oxygen in a similar way to HIF-1α. Under normoxia, HIF-2α is modified by the prolyl-4-hydroxylases (PHDs), which then leads to recruitment of the von Hippel-Lindau (VHL) tumor suppressor E3 ligase complex, poly-ubiquitylation and finally proteasomal degradation (Ohh et al., 2000). In addition, under normoxia, HIF-2α is also hydroxylated at an asparagine residue by factor inhibiting HIF (FIH, also known as HIF1AN), which inhibits HIF-2α interaction with the transcriptional co-activators p300 (also known as EP300) and CBP (also known as CREBBP) (Kaelin and Ratcliffe, 2008). During hypoxia, hydroxylation of HIF-2α is inhibited because PHDs and FIH, which use oxygen as a substrate, become inactive. This leads to HIF-2α stabilization, transport inside the nucleus, dimerization with ARNT, DNA binding, co-activator recruitment and formation of an active transcriptional complex.

Interestingly, HIF isoforms are also subject to oxygen-independent and differing mechanisms of regulation, both in terms of expression levels and activity. This regulation includes response to various signaling pathways and functional interplay with other transcription factors, which involve a variety of post-translational modifications. Whereas HIF-1α regulation by major signal transduction pathways has been extensively studied (Kietzmann et al., 2016) our knowledge about how HIF-2α is regulated remains quite limited. It has been reported that HIF-2α is acetylated at several lysine residues by CPB (Chen et al., 2012) and de-acetylated by SIRT1 (Dioum et al., 2009; Lim et al., 2010) but there is contradictory evidence concerning the role of these modifications. Moreover, HIF-2α is phosphorylated at Thr324 by PKD1 and, as a result, its interaction with the SP1 transcription factor is inhibited, thus promoting NBS1 (also known as NBN) expression (To et al., 2006). HIF-2α is also phosphorylated by casein kinase 2 (CK2) at Thr844, which results in increased HIF-2 transcriptional activity possibly by lowering the affinity of HIF-2α for FIH (Gradin et al., 2002).

The casein kinases are serine/threonine kinases and comprise the casein kinase 1 (CK1) and CK2 families (Knippschild et al., 2014). In mammals, seven distinct CK1 isoforms have been characterized, all of which are highly conserved and ubiquitously expressed. Up to now, more than 140 in vitro and in vivo substrates of CK1 have been reported, including proteins involved in cell cycle, apoptosis, DNA repair, mitochondrial function and signal transduction. The important role of CK1 family members has been documented by reports linking CK1 isoforms to modulation of key regulatory proteins such as p53, MDM2 and β-catenin, all of which act as signal integration molecules in stress situations and are connected to tumorigenesis (Schitteke and Sinnberg, 2014).

Previous work from our laboratory has addressed the role of CK1δ (also known as CSNK1D1) in the hypoxic response and has shown that phosphorylation of HIF-1α by CK1δ destabilizes the HIF-1α–ARNT complex and reduces HIF-1 transcriptional activity.
In addition, by negatively affecting HIF-1 activity, CK1δ decreases lipin-1 expression and lipid droplet formation and, thus, impairs the metabolic adaptation and proliferation of cancer cells under hypoxia (Kalousi et al., 2010; Kourtì et al., 2015).

In this report, we investigated how CK1δ affects HIF-2α expression and transcriptional activity as well as induction of EPO in hepatic cancer cell lines under hypoxia. Our findings suggest a new regulatory effect of CK1δ on EPO production through direct phosphorylation of HIF-2α.

**RESULTS**

**CK1δ supports HIF-2 transcriptional activity and stimulates EPO secretion**

To investigate the involvement of CK1δ in the regulation of HIF-2α, its expression was suppressed in Huh7 cells by small interfering RNA (siRNA)-mediated silencing. Treatment of cells with CK1δ siRNA under hypoxic conditions substantially reduced the expression of CK1δ, but did not affect the expression levels of endogenous HIF-2α (Fig. 1A). The same treatment drastically reduced the mRNA expression levels of the HIF-2-specific target gene EPO suggesting inhibition of HIF-2 transcriptional activity (Fig. 1B). To explore further the biological function of the CK1δ-mediated regulation of HIF-2α, we measured EPO secretion, and we observed that silencing of CK1δ expression substantially reduced EPO secretion compared to cells treated with control siRNA under hypoxia (Fig. 1C). Similar results were obtained in both Huh7 and HepG2 cells using an alternative CK1δ siRNA sequence (Fig. S1A–E).

To verify these results, Huh7 and HepG2 cells were treated with the specific CK1δ inhibitor D4476 (10 μM) (Rena et al., 2004) under both normoxia and hypoxia. CK1δ protein was similarly expressed under all conditions, and inhibition of its activity by D4476 did not significantly alter the protein expression levels of endogenous HIF-2α induced by hypoxia (Fig. 1D; Fig. S1F). In line with the results obtained by silencing of CK1δ, treatment of both cell lines with D4476 led to a significant decline in the mRNA levels of the HIF-2-specific target genes EPO and PAI-1 (also known as SERPINE1) under hypoxic conditions (Fig. 1E; Fig. S1G). The requirement of CK1δ for hypoxic stimulation of EPO secretion was verified by

![Fig. 1. Silencing or inhibition of CK1δ impairs HIF-2 transcriptional activity and EPO secretion.](image-url)

(A) Western blot analysis of lysates from Huh7 cells transfected with CK1δ siRNA (10 nM) or scrambled siRNA (10 nM). Cells were incubated for 16 h under hypoxia before collection and lysis. (B) Determination of EPO mRNA levels by quantitative real-time PCR in Huh7 cells incubated and treated as in A. Values are expressed as fold increase in relation to the corresponding normoxic conditions and represent the mean±s.e.m. of three independent experiments performed in duplicate. (C) Determination of EPO secretion by ELISA in Huh7 cells incubated and treated as in A. Values are expressed as mlU/ml protein and represent the mean±s.e.m. of three independent experiments performed in duplicate. (D) Western blot analysis of lysates from Huh7 cells incubated for 16 h under normoxia or hypoxia (1% O2), in the presence or absence of the specific CK1δ inhibitor D4476 (10 μM). (E) Determination of EPO and PAI-1 mRNA levels by quantitative real-time PCR in Huh7 cells incubated and treated as in D. Results are shown as in B. (F) Determination of EPO secretion by ELISA in Huh7 cells incubated and treated as in D. Values are shown as in C. *P<0.05; **P<0.01 (two-tailed unpaired Student’s t-test).
treating cells with D4476 under hypoxia (Fig. 1F; Fig. S1H). Taken together, these data show that inhibition of CK1δ significantly decreases EPO and PAI-1 gene expression, suggesting that CK1δ positively affects HIF-2 activity and HIF-2-dependent EPO production under hypoxia without affecting HIF-2α protein levels in liver cancer cells.

**CK1δ phosphorylates HIF-2α in vitro**

To analyze the observed HIF-2 regulation by CK1δ we examined whether CK1δ directly modifies HIF-2α in vitro. Full-length HIF-2α and its smaller fragments (amino acids 1–820, 1–679, 1–542, 1–497, 1–366, 1–280, 366–870, 366–820, 366–679, 366–542, 542–870 and 542–820), were expressed in bacteria as GST fusion proteins (Fig. 2A), purified and used as substrates in radioactive kinase assays using recombinant CK1δ (Fig. 2B). Very little phosphorylation was detected in the N-terminal (1–280 and 1–366) and C-terminal (542–820 and 542–870) fragments of HIF-2α compared to full-length HIF-2α. All other fragments could be modified by CK1δ suggesting that HIF-2α is directly phosphorylated by CK1δ in vitro predominantly between amino acids 366–542, a region that contains the ODD and the N-TAD domains.

**Fig. 2.** HIF-2α is a phosphorylation target of CK1δ in vitro. (A) Schematic representation of HIF-2α, and its domains and fragments that were cloned, expressed and purified as GST fusion proteins. The parts of HIF-2α that were identified as substrates for recombinant CK1δ based on the results presented in B are marked by +. bHLH, basic helix-loop-helix; IH, inhibitory domain. (B) The indicated GST-tagged HIF-2α fragments were subjected to in vitro phosphorylation by 100 units of recombinant CK1δ and analyzed by SDS-PAGE followed by Coomassie Blue staining (upper panels) and autoradiography (lower panels, 32P). Dots indicate the positions of the recombinant GST-tagged HIF-2α proteins. Numbers on the left indicate the corresponding molecular mass markers.
Identification of candidate CK1δ phosphorylation sites between amino acids 366–542

In order to determine the nature of the modified residues in the 366–542 region of HIF-2α targeted by CK1δ, two-dimensional thin-layer chromatography (TLC) analysis of phosphorylated amino acids was performed on the GST–HIF-2α fragment 366–542 that was previously radioactively labeled by CK1δ and mixed with control phosphorylated serine, threonine and tyrosine amino acids (denoted pSer, pThr and pTyr, respectively). The results of the analysis showed that CK1δ phosphorylated both serine and threonine residues in the region 366–542 of HIF-2α (Fig. 3A). To map more precisely the CK1δ phosphorylation sites, recombinant full-length GST–HIF-2α, as well as the smaller fragments 1–542 and 366–542, were incubated with or without CK1δ and ATP and analyzed by SDS-PAGE. Incubation with CK1δ caused slower migration of the GST–HIF-2α fragments, indicating quantitatively substantial phosphorylation (Fig. 3B). The slower migrating bands were excised, digested by trypsin and subjected to mass spectroscopy analysis. The analysis identified five unique tryptic peptides of HIF-2α (Tables S1 and S2) and peptide GAVSEKSNNLFTK was shown to contain two phosphorylated serine residues (Ser383 and Ser386) (Fig. 3C). Sequence comparison revealed that at least one of the two serine residues was conserved in all known vertebrate HIF-2α homologs as well as in the human HIF-1α isoforms (Fig. 3D). The mass spectroscopy analysis did not produce a candidate threonine residue in the HIF-2α 366–542 region that could be modified by CK1δ, as suggested by the phosphorylated amino acid analysis. However, multiple sequence alignment analysis of this region of HIF-2α showed the presence of a small highly conserved area corresponding to amino acids 518–542 and the presence in this area of a conserved threonine residue (Thr528) that is part of the classical CK1 consensus sequence motif E/DxxS/T (Fig. 3D). This residue, which is not present at the same position in HIF-1α, could be the target of CK1δ detected by our above analysis.

CK1δ modifies HIF-2α at Ser383 and Thr528 and enhances its transcriptional activity

To verify the mass spectroscopy data for Ser383 and Ser386, and test the hypothesis that Thr528 is also a target of CK1δ, each of these residues was converted into an alanine residue by site-directed mutagenesis. The three mutant proteins, HIF-2α S383A, HIF-2α S386A and HIF-2α T528A (denoted 383 SA, 386 SA and 538 TA) or the corresponding mutant 366–542 fragments were expressed as GST fusions in bacteria, purified and used as substrates for in vitro phosphorylation assays with recombinant CK1δ or Huh7 total cell extracts. Phosphorylation of HIF-2α S383A and HIF-2α T528A by CK1δ was reduced by ~50% and 23%, respectively, relative to wild-type HIF-2α (Fig. 4A, upper panel), whereas phosphorylation of HIF-2α 366–542 S383A and HIF-2α 366–542 T528A was reduced by 49% and 40%, respectively, showing that CK1δ might preferentially target Ser383 (Fig. 4A, lower panel). When recombinant CK1δ was replaced by total soluble protein extracts of Huh7 cells as a kinase source, the impact on residues Ser383 and Thr528 appeared to be quite similar (Fig. 4B). By contrast, mutation of Ser386 to an alanine residue did not cause any substantial change in the in vitro phosphorylation by CK1δ (Fig. S2A). Taken together, our analysis identified residues Ser383 and Thr528 as the predominant in vitro CK1δ phosphorylation sites of human HIF-2α. The fact that these sites are also subjected to modification by the kinases present in the Huh7 total cell protein extracts indicates that CK1δ represents one of the kinases that interact with HIF-2α in Huh7 cells.

To study the effect of Ser383 and Thr528 phosphorylation on HIF-2α function, we also constructed, by site-directed mutagenesis, the double alanine replacement mutant (denoted DM SA/TA) as well as the phospho-mimetic mutants [HIF-2α S383D (383 SD), HIF-2α T528E (528 TE) and the double mutant HIF-2α (DM SD/TE)], the negative charges of which could potentially mimic the charges of the phosphorylated residues. Wild-type HIF-2α and all its mutant forms were sub-cloned into the pFLAG-CMV2 mammalian expression vector and introduced as Flag-tagged proteins into Huh7 and HepG2 cells by transient transfection. Western blotting analysis showed that the protein expression levels of all mutant forms were similar to those of the wild-type HIF-2α (Fig. 4C). Using a luciferase reporter gene assay, we observed that all the phosphorylation-deficient mutant forms demonstrated an ~50% decrease in their hypoxia-response element (HRE)-dependent transcriptional activity compared with that of the wild-type HIF-2α (Fig. 4D). By contrast, when the HRE-dependent transcriptional activity of the phospho-mimetic mutants was determined, we observed that it was similar to that of the wild-type HIF-2α form, suggesting that wild-type HIF-2α is subject to substantial phosphorylation at the mutated sites.

The data described so far show that both inhibition of CK1δ and the HIF-2α S383A and HIF-2α T528A mutations negatively affect HIF-2 activity, suggesting that Ser383 and Thr528 are true in vivo as well as in vitro targets of CK1δ. To further confirm that the exerted CK1δ effect is specific and linked to the modification of the Ser383 and Thr528 residues, the wild-type, phospho-deficient SA/TA and phospho-mimetic SD/TE mutant forms of HIF-2α were expressed as Flag-tagged proteins in HepG2 cells, and HIF-2 transcriptional activity was determined in the absence or presence of the CK1δ-specific inhibitor D4476 under normoxia (i.e. in the absence of endogenous HIF-2α) (Fig. 4E). As expected, the wild-type form responded to inhibition of CK1δ by exhibiting significantly lower activity, exactly reproducing the behavior of endogenously hypoxically induced HIF-2α. However, neither the low transcriptional activities of the phospho-deficient HIF2α mutant forms nor the normal (compared to wild-type) activities of the phospho-mimetic forms were significantly affected by the CK1δ inhibitor. These results strongly suggest that regulation of HIF-2α by CK1δ in hepatoma cell lines predominantly involves Ser383 and/or Thr528.

Phosphorylation at Ser383 and/or Thr528 is required for HIF-2-dependent stimulation of EPO secretion from liver cancer cells

To explore further the biological effect of Ser383 and Thr528 phosphorylation on HIF-2α function, we tested the mRNA expression of the endogenous HIF-2 specific target genes EPO and PAI-1 in Huh7 and HepG2 cells. We observed that their induction by the phospho-deficient HIF-2α mutant forms was significantly lower relative to the wild-type HIF-2α form, suggesting that both Ser383 and Thr528 are required, most likely as CK1δ phosphorylation targets, for full activation of HIF-2 transcriptional function in both cell lines (Fig. 5A,C). Moreover, in agreement with the in vitro phosphorylation results, which did not support modification of Ser386, mutation of this site did not alter the expression of EPO and PAI-1 in relation to wild-type HIF-2α (Fig. 5B,C). To further confirm that the effect of CK1δ on EPO production is mediated by HIF-2α phosphorylation at Ser383 and/or Thr528, EPO secretion was analyzed in Huh7 and HepG2 cells expressing wild-type Flag-HIF-2α or its phospho-deficient mutant forms under normoxia. As shown in Fig. 5B,D, mutations at the CK1δ phosphorylation sites Ser383 or Thr528 inhibited HIF-2α-mediated induction of EPO secretion. Overall, these data suggest that direct phosphorylation of HIF-2α at Ser383 or Thr528 by CK1δ increases...
Fig. 3. HIF-2α Ser383 and Thr528 are candidate CK1δ phosphorylation sites. (A) Phosphorylated amino acid analysis following in vitro phosphorylation of GST–HIF-2α (366-542) by CK1δ. 32P-labeled HIF-2α (366-542) was subjected to hydrolysis, two-dimensional TLC and autoradiography (right panel). The phosphorylated amino acid standards, pSer (P-S), pThr (P-T), and pTyr (P-Y), were marked after exposure of TLC plate to ninhydrin staining solution (left panel). Circles indicate origin of migration; arrows indicate direction of migration. (B) Western blot analysis of recombinant full-length GST–HIF-2α or its fragments incubated with or without 100 units of recombinant CK1δ in the presence of non-radioactive ATP, using anti-GST antibody. In the presence of CK1δ slower migrating bands are observed, indicating substantial HIF-2α phosphorylation. (C) Fragmentation spectrum of a doubly-charged peak at m/z 794.34735 produced a near-complete b and y ion series (in blue and red) together with prominent neutral loss ions (in green). The corresponding peptide is identified as GAVS(p)EKS(p)NFLFTK confirming that both serine 4 and serine 7 are phosphorylated. Tables S1 and S2 show the different fragments found in the MS2 CID spectrum in detail. (D) Schematic representation of the peptide sequence identified as the phosphopeptide by mass spectrometry and the conserved amino acid sequence from 518 to 542 aligned to corresponding amino acid sequences from vertebrate HIF-2α homologs and from human HIF1α.
HIF-2α transcriptional activity and subsequent EPO production in liver cancer cells.

**CK1δ does not affect protein stability of HIF-2α or its interaction with transcriptional co-activator USF2**

To resolve the mechanism through which lack of phosphorylation by CK1δ negatively affects HIF-2 transcriptional activity, we first investigated the effect of CK1δ inhibition on the stability of HIF-2α by determining its half-life after blocking translation or after termination of hypoxia. Huh7 cells that were incubated under hypoxia with or without D4476 were treated with the translation inhibitor cycloheximide or subjected to re-oxygenation, and HIF-2α expression was monitored for different time periods. As shown in Fig. 6A,B, treatment with D4476 did not alter the protein stability of HIF-2α, which was as rapidly degraded as in the absence of D4476, both after inhibition of protein translation or re-oxygenation. In
addition, comparison at the same time point of 15 min suggests that, as expected, HIF-2α is more stable during hypoxia (without new protein synthesis) than during normoxia (despite ongoing protein translation).

We then tested the effect of D4476 on the interaction of HIF-2α with the upstream stimulatory factor 2 (USF2), as it has been reported that this is necessary for optimal gene expression of EPO and PAI-1 (Pawlus et al., 2012). Huh7 cells overexpressing Flag-tagged USF2 and incubated under hypoxia in the presence or absence of D4476 were subjected to immunoprecipitation with an anti-Flag antibody. As shown in Fig. 6C, HIF-2α co-precipitated with Flag–USF2, as expected by their known association (Befani et al., 2013). However, its interaction with USF2 was not affected by D4476, suggesting that stimulation of their association is not the reason for the observed positive effect of CK1δ on HIF-2-dependent activation of EPO and PAI-1 gene expression.

**Phosphorylation by CK1δ inhibits CRM1-dependent nuclear export of HIF-2α**

Having eliminated an effect of phosphorylation by CK1δ on HIF-2α stability or its interaction with the co-activator USF2, we next analyzed the HIF-2α subcellular distribution. Wild-type Flag-HIF-2α and its mutant forms were expressed in transiently transfected HepG2 or Huh7 cells and their localization was monitored by immunofluorescence microscopy (Fig. 7A; Fig. S3, left panels). Wild-type Flag–HIF-2α was detected exclusively inside the nucleus in ∼80% of the transfected cells, and displayed both nuclear and cytoplasmic localization in only ∼20% of the cells (Fig. 7B) When the same cells were when treated with D4476, wild-type Flag–HIF-2α was distributed both in the nucleus and the cytoplasm in ∼50% of the cell population, suggesting that inhibition of CK1δ impairs its accumulation in the nucleus. Localization of the phospho-deficient mutant HIF-2α forms was both nuclear and cytoplasmic in ∼50% of the cell population, again suggesting that lack of modification by CK1δ impairs HIF-2α nuclear accumulation, whereas localization of all the phospho-mimetic mutant HIF-2α forms indeed mimicked the distribution of the wild-type Flag-HIF-2α and was predominantly nuclear (Fig. S4A, left panel, Fig. S4B).

Increased detection of mutant HIF-2αS383A, HIF-2αT528A and HIF-2αSA/TA proteins or wild-type HIF-2α protein in the presence of D4476 in the cytoplasm of the tested hepatic cancer cells, could be attributed either to reduced nuclear import or enhanced nuclear
export. To distinguish between these two possibilities, cells were treated with leptomycin B (LMB), a potent inhibitor of CRM1 (also known as XPO1)-dependent nuclear export. Exposure to LMB did not influence the localization of the wild-type HIF-2α (Fig. 7A, right panel, 7C) or the phosphomimetic mutant forms, which remained accumulated inside the nucleus (Fig. S4A, right panel, S4C). However, LMB treatment caused exclusive nuclear localization of endogenous HIF-2α (Fig. 7A, right panel, 7C). These data indicate, first, that HIF-2α shuttles between the nucleus and cytoplasm and, second, that lack of phosphorylation by CK1δ sites is the predominant cause of HIF-2α nuclear export by CRM1 explaining the observed partial HIF-2α inactivation.

**Silencing of CK1δ enhances CRM1-dependent nuclear export of HIF-2α under hypoxia.**

To further verify the above results, we tested the impact of CK1δ silencing on the localization of endogenous HIF-2α under hypoxia. CK1δ expression was suppressed by siRNA-mediated silencing in HeLa cells, which were then incubated under hypoxia in the presence or absence of LMB. HIF-2α localization was then monitored by subcellular fractionation (Fig. 8A). As expected, HIF-2α was almost exclusively localized in the nuclear fraction under hypoxia independently of its exposure to LMB. However, in accordance with the results obtained for the overexpressed HIF-2α SA/TA mutant proteins above, a substantial fraction of HIF-2α protein migrated from the nucleus to the cytoplasm when CK1δ was silenced, which then returned to the nucleus upon LMB treatment. Taken together, these data show that loss of CK1δ promotes HIF-2α nuclear export, whereas LMB rescues the accumulation of HIF-2α inside the nucleus, confirming a CRM1-dependent mechanism controlled by phosphorylation.

We next tested whether LMB-dependent re-localization of phospho-deficient HIF-2α mutants to the nucleus could also restore their low transcriptional activity. Indeed, although LMB did not affect the activity of wild-type HIF-2α or its phospho-mimetic mutants, the activity of all the phospho-deficient mutants was increased and reached levels comparable to the wild-type HIF-2α activity (Fig. 8B). These data show that the predominant reason for the inactivation of HIF-2α by mutations in the CK1δ sites is the enhanced exclusion of HIF-2α from the nucleus. We finally
examined whether HIF-2α lacking modification by CK1δ (e.g. the ST/AA mutant) could change gene target specificity and preferentially activate HIF-1 target genes (such as PGK1) while being less active on HIF-2 target genes (such as EPO). However, neither overexpression of wild-type HIF-2α nor any of the mutant forms affected the normoxic expression of the HIF-1-specific PGK1.
gene, irrespective of the presence or not of LMB (Fig. S4D,E), indicating, that HIF-2 cannot replace HIF-1 following inhibition of CK1δ-mediated phosphorylation of HIF-2α.

**DISCUSSION**

In this study, we have identified and characterized a new regulatory mechanism of HIF-2α activity involving CK1δ-mediated phosphorylation of two distinct residues of HIF-2α, Ser383 and Thr528. Our conclusion, that phosphorylation of HIF-2α by CK1δ increases EPO production by blocking CRM1-dependent export of HIF-2α to the cytoplasm (Fig. 8C) is based on the following observations.

First, CK1δ silencing or inhibition, decreases hypoxic induction of two HIF-2-specific target genes, EPO and PAI-1, without affecting HIF-2α protein expression. Second, CK1δ modifies HIF-2α residues Ser383 and Thr528 in vitro. Third, blocking Ser383 or Thr528 phosphorylation (by converting them into Ala) inhibits HIF-2 transcriptional activity whereas phospho-mimetic mutations of the same residues preserve HIF-2 activity. Fourth, CK1δ does not affect HIF-2α protein stability or its interaction with the USF2 co-activator but controls CRM1-dependent nuclear export of HIF-2α. Finally, both suppression of CK1δ activity and mutation of the CK1δ phosphorylation sites on HIF-2α strongly reduce EPO secretion in two liver cancer cell lines.

The first identified site of CK1δ modification, Ser383, is between the PAS-B and ODD domains of HIF-2α. The crystal structure of human HIF-2α, which contains the PAS-B domain, has been determined only until the 350th residue (Erbel et al., 2003; Wu...
Taking all this into account, we suggest that phosphorylation of HIF-2α at Ser-383 or Thr-528 by CK1δ prevents its CRM1-mediated nuclear export and is therefore required for its efficient accumulation inside the nucleus and full HIF-2α activity. How exactly these modifications interfere with the nuclear export of HIF-2α is certainly an interesting issue that merits further future investigation. Nuclear export of HIF-1α is also regulated by phosphorylation, but in this case modification is catalyzed by ERK1/2, which target two serine residues (Ser641 and Ser643) adjacent to a non-conventional CRM1-dependent nuclear export signal (NES; Mylonis et al., 2008, 2006). However, neither these ERK sites nor the NES of HIF-1α are conserved in HIF-2α.

Regulation of HIF-2α and HIF-1α by CK1δ represents a new example of distinct opposite regulation between the two isoforms. Here, we have shown that HIF-2α modification by CK1δ at Ser383 or Thr528 enhances its transcriptional activity by promoting its nuclear accumulation. By contrast, our laboratory has previously reported that HIF-1α is modified by CK1α at Ser247 and this inhibits HIF-1α activity by blocking HIF-1α–ARNT interaction (Kalousova et al., 2010; Kourt et al., 2015). Comparative sequence analysis reveals that the two isoforms display an absolute conservation in the PAS-B region where the phosphorylation of HIF-1α occurs. However, our mapping experiments excluded phosphorylation of the corresponding HIF-2α domain by CK1δ in vitro, and instead led to the identification of Ser383 or Thr528, which lies C-terminally to the PAS-B domain. Ser383 is conserved in the HIF-1α isoform but Thr528 is not. So despite the overall sequence similarities between HIF-1α and HIF-2α, they are targeted by CK1δ at distinct sites and with opposite functional outcome. It is not without precedent that a single factor mediates a distinct regulation between the two HIF-α isoforms. HAF-associated factor (HAF, also known as SART1) has been reported to destabilize HIF-1α in proteasome-dependent manner by binding to its C-terminal domain (Koh et al., 2008) but, by contrast, HAF promotes HIF-2 transcriptional activity by binding to its C-terminal domain (Koh et al., 2011). It is probably important for cells that express both HIF-α isoforms to promote the activity of the one isoform when the other one is inactivated under certain conditions and in a tissue-specific manner, and CK1δ might be involved in regulating this balance. Hence, high CK1δ expression and/or activity might mediate preferential HIF-2α activation in cells expressing both HIF-α isoforms.

CK1 family members are evolutionarily conserved serine/threonine kinases members involved in many cellular processes including proliferation, cell cycle progression, DNA repair, chromosome segregation and have been reported to target several transcription factors (such as TP53, FoxO1 and HIF-1α) (Bischof et al., 2011; Cheong and Virshup, 2011; Greer and Rubin, 2011; Gross and Anderson, 1998; Kalousova et al., 2010; Knippschild et al., 2005, 2014; Schütte and Sinnberg, 2014). HIF-2α is added to this list for the first time. HIF-2α is expressed in hepatocytes and is the main regulator of hepatic EPO production (Rankin et al., 2007), and we have now shown that this function can be controlled by CK1δ. During fetal development, EPO is mainly produced in the liver. Its primary site of production changes to the kidney during late gestation, but EPO continues to be produced in the liver to a lesser extent. Therefore, hepatocytes are the primary source of extra-renal EPO in the adult (Koury et al., 1991), and under conditions of severe hypoxia, liver EPO production increases and might account for more than 33% of total EPO production (Eckardt and Kurtz, 2005). Dysregulated EPO expression results in the development of anemia, when serum EPO levels are inadequate, or polycythemia, as a result of EPO overproduction (Rankin et al., 2007). Therefore, the
identification of CK1δ as a kinase regulating HIF-2α-dependent EPO secretion from liver cancer cells has strong physiological significance and might have important therapeutic applications in these conditions. Moreover, Ribatti et al. have demonstrated that EPO and its receptor are involved in highly vascularized human hepatocellular carcinoma (Ribatti et al., 2007). In this case, EPO is secreted by hepatic tumor cells and it acts on vascular endothelial cells through their EPOR receptors, promoting angiogenesis. It has also been observed that some cases of hepatocellular carcinoma (HCC) are associated with abnormally high levels of erythrocytes. This HCC-associated erythrocytosis is thought to result from increased EPO production by the HCC cells, and many HCC patients have high plasma EPO levels (Sakisaka et al., 2003).

Taking into account the important role of HIF-2 in HCC, many compounds that directly interfere with HIF-2 function or target downstream hypoxia-related pathways have been tested in experimental trials as anti-cancer agents but have proven ineffective (Zhao et al., 2015). The new regulatory communication between CK1δ and HIF-2 described in this work suggests an alternative means to control HIF-2 activity in disease and needs to be further investigated.

**MATERIALS AND METHODS**

**Plasmid constructions**

cDNAs encoding the various fragments of HIF-2α were produced by PCR and were cloned as BamHI fragments into pGEX-4T-1 bacterial expression vector (Amersham Biosciences), yielding pGEX-HIF-2α full length (1–870) and pGEX-HIF-2α fragments 1–820, 1–679, 1–542, 1–366, 1–280, 366–870, 366–820, 366–679, 366–542, 542–870 and 542–820. Single point HIF-2α mutants S383A, S383D, S386A, T528A and T528E and double point HIF-2α mutants S383A/T528A, S383D/T528A, S383D/T528E were constructed using pBS-SK(+)–HIF-2α containing the full-length cDNA of HIF-2α as a template, with the QuikChange II site-directed mutagenesis kit (Stratagene). The primer sequences used are available upon request. The DNA sequence of the point mutants was confirmed by sequencing performed by VBC Biotech. All mutant forms of HIF-2α were then subcloned as BamHI fragments into the mammalian expression vector pFlag-CMV2 and into the pGEX-4T-1 bacterial expression vector. Using the pFlag-CMV2 HIF-2α point mutant forms as templates, we obtained by PCR the cDNA fragments corresponding to 366–542 S383A, S386A and T528A and S383A/T528A. The derived mutant fragments were subcloned as BamHI fragments into the pGEX-4T-1 bacterial expression vector. The full-length pCDNA-Flag-USF2 plasmid was kindly provided by Cheng-Jun Hu (Department of Craniofacial Biology, University of Colorado, Aurora, CO) (Pawlus et al., 2012).

**Cell culture, transfection and luciferase assays**

Human Huh7 and HepG2 cells were purchased from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Biosera) containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin (Biosera). Transient transfections were carried out in 10-cm and 6-cm dishes or 12-well plates by using TurboFect (Thermo Scientific) transfection reagent. When required, 24 h after transfection, cells were treated for 16 h with the CK1δ inhibitor D4476 (10 μM, Cayman), for 4 h with 10 ng/ml LMB (Sigma-Aldrich) or with DMSO as solvent control. For hypoxia treatment, cells were exposed for 16 h to 1% O2, 94% N2 and 5% CO2 in an IN VIVO 200 hypoxia workstation (Ruskin Life Sciences). For half-life studies, after 4 h of hypoxia treatment, cells were treated with 10 μg/ml cycloheximide (Sigma-Aldrich) or were re-oxygenated in a time-dependent manner. Luciferase assays were performed in cells transiently co-transfected with equal amounts of plasmids expressing different forms of HIF-2α (or the empty parental vector pFlag-CMV2 as control), the firefly luciferase reporter plasmid pGL3–SHRE–VEGF, kindly provided by Amato J. Giaccia (Stanford University, Stanford, CA) and the Renilla luciferase expressing plasmid pCI–Renilla, generously provided by Martina U. Muckenthaler (University of Heidelberg, Germany). At 24 h post transfection, cells were washed with PBS, lysed and luciferase activity was determined using the luciferase assay kit or the Dual Luciferase Reporter Assay System (Promega, Madison, WI).

**siRNA-mediated silencing of CK1δ**

mRNA encoding CK1δ was targeted using two distinct sets of validated siRNA (Qiagen): Hs-CSNK1D5 HP (5′-CCGGTCTAGGATCGAAATG-3′) and Hs-CSNK1D6 HP (5′-CTCCGCTGAACTTTGCA-3′). AllStars siRNA (Qiagen) was used as negative control. Cells were incubated in serum-free DMEM for 4 h with siRNA (10 nM) in the presence of Lipofectamine™ RNAiMAX (Invitrogen, Life Technologies) as described by the manufacturer. At 24 h post transfection, cells were exposed to hypoxia and 16 h later they were harvested, and cell lysates were prepared for real-time quantitative PCR or for western blot analysis.

**RNA extraction, real-time quantitative PCR and quantification of EPO production**

Total RNA from Huh7 and HepG2 cells was isolated using thepeqGOLD TriFast reagent (Peqlab) and cDNA was synthesized with the High Capacity Reverse Transcription kit (Applied Biosystems). Real-time quantitative PCR was performed with SYBRGreen qPCRSuperMix Universal (Invitrogen) in a MiniOpticon instrument (Bio-Rad). The mRNAs encoding EPO, PAI-1 and HPRT1 were amplified using primers available upon request. Each sample was assayed in duplicate for both target and internal control. Relative quantitative gene expression was calculated using the ΔΔCT method and presented as a fold increase or percent activity in relation to the respective controls. For quantification of EPO secretion, medium was collected from 105 cells in 6-cm culture dishes, and EPO in the medium was measured by using the Human Erythropoietin DRG® EPO (Erythropoietin) ELISA (ELIA-3646) kit (DRG International Inc.).

**SDS-PAGE, western blotting and subcellular fractionation**

Proteins were resolved by 10% SDS-PAGE, and analyzed by Coomassie Blue staining or western blotting using: anti-HIF-2α polyclonal antibody (pAb) (1:750, Novus Biologicals, NB100-122), anti-GST pAb (1:10,000, Novus Biologicals, ab19256), anti-Flag monoclonal antibody (mAb) (1:10,000, Sigma, F4042), anti-tubulin mAb (1:10,000, Cell Signaling #3873), anti-CK1δ pAb (1:250, Santa Cruz Biotechnology, sc-55553) and anti-USF2 pAb (1:250, Santa Cruz Biotechnology, sc-862). Analysis by immunoblotting was carried out as previously described (Lyberopoulos et al., 2007; Mylonis et al., 2008). Subcellular fractions were performed in HeLa cells (because of technical problems encountered with the fractionation of hepatoma cells) as previously described (Andrews and Faller, 1991). Western blot images were taken using an Uvitec Cambridge Chemiluminescence Imaging System with the help of Alliance Software (version. 16.06) and quantified by UviHand software (version. 15.03) provided with the instrument (Uvitec Cambridge).

**Immunoprecipitation and immunofluorescence**

For immunoprecipitation experiments, cells were transfected with pCDNA-Flag-USF2 and at 24 h post transfection were incubated for 16 h under hypoxia in the presence or absence of D4476. Immunoprecipitation analysis was carried out as previously described (Mylonis et al., 2006). For immunofluorescence experiments, cells were grown on coverslips and transiently transfected with pCMV2–FLAG vector expressing wild-type HIF-2α and its mutant forms. In some cases, 4 h post-transfection cells were treated with D4476 for 16 h, or treated with LMB 4 h prior to observation. Coverslips were incubated for 1 h at room temperature with anti-Flag mAb (Sigma), washed twice with PBS, and incubated for 1 h at room temperature with Cy3-conjugated anti-mouse-IgG secondary antibody (Jackson Immunoresearch). Analysis by immunofluorescence was carried out as previously described (Mylonis et al., 2008). Images were taken on a Zeiss Axioscope fluorescence microscope using an AxioCam MRm CCD sensor and 40× objective with filters for DAPI and Cy3.

**Clinical practice implications**

The findings presented in this study suggest that CK1δ could be a novel target for the treatment of HCC, with potential therapeutic implications for the treatment of hypoxia-related diseases.
Protein purification and phosphorylation assays

Wild-type and mutant forms of GST–HIF-2α and its GST fragments 1–820, 1–679, 1–542, 1–366, 1–280, 366–870, 366–820, 366–679, 366–542, 542–870 and 542–820 were expressed in Escherichia coli and purified as previously reported for GST–HIF-1α (Chachami et al., 2005). Phosphorylation reactions were carried out as previously described (Mylonis et al., 2006). The reaction was stopped by adding SDS sample buffer and heating at 95°C for 5 min. Samples were analyzed by SDS-PAGE followed by Coomassie Blue staining and autoradiography. Relative phosphorylation levels were determined by cutting out protein bands from gels and measuring their radioactivity by scintillation counting.

Phosphoamino acid analysis

32P-labeled GST–HIF-2α (366–542) was analyzed by SDS-PAGE, blotted onto immobilon membrane and the relevant radioactive band was excised. The excised strip was treated with 5.7 M HCl at 110°C for 90 min, and the supernatant was dried in a Speed-Vac concentrator. The lyophilized powder was reconstituted in a solution containing pSer, pThr and pTyr (10 nmol each; Sigma) and then subjected to by two-dimensional chromatographic separation (Grangeasse et al., 1999). Briefly, migration in the first dimension was performed by chromatography in a solvent containing propanol:water at a ratio of 70:30 for 90–120 min on a thin layer silica plate (Merk), and in the second dimension in a solvent containing isobutyl alcohol, formic acid and water at a ratio of 80:30:40 for 90–120 min. After separation and drying, phosphorylated amino acids were visualized by ninhydrin staining (0.5% in acetonitrile) followed by autoradiography.

In-gel tryptic digestion

The stained gel band corresponding to the HIF-2α construct (366–542) was excised and cut into small pieces in order to proceed with in-gel tryptic protein digestion according to standard procedures (Shevchenko et al., 2006). Briefly, the gel pieces were destained with 100 mM ammonium bicarbonate and acetonitrile (1:1), dehydrated with acetonitrile (ACN) and then rehydrated with 25 mM ammonium bicarbonate (NH₄HCO₃) buffer. The dried gel pieces were subsequently rehydrated with 12.5 g/ml trypsin (trypsin gold, Promega) in 25 mM NH₄HCO₃ buffer and incubated on ice for 45 min. The samples were finally transferred to a thermomixer (Eppendorf) and incubated overnight at 37°C with mild mixing (300 rpm). Next day, peptides were extracted from the gel pieces by addition of 150 μl acetonitrile containing 5% formic acid (v/v) with shaking for 1 h. The extracted peptide solution was dried down in a vacuum centrifuge (Savant). The samples were reconstituted with 30 μl solution of 2% (v/v) acetonitrile and 0.1% (v/v) formic acid, sonicated in a water bath for 3 min and analyzed by performing liquid chromatography tandem mass spectrometry (LC-MS/MS).

LC-MS/MS analysis

The purified peptides were analyzed by high pressure (HPLC-MS/MS) using coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a nanospray source. 10 μl of the peptide mixtures were pre-concentrated at a flow-rate of 5 μl/min for 10 min using a C18 trap column (Acclaim PepMap) and then loaded onto a 50 cm C18 column (75 μm ID, particle size 2 μm, 100 Å, Acclaim PepMap RSLC, Thermo Scientific). The binary pumps of the HPLC (RSLCnano, Thermo Scientific) contained solution A [2% (v/v) ACN in 0.1% (v/v) formic acid] and solution B (80% ACN in 0.1% formic acid). The peptides were separated using a linear gradient of 4%–40% B in 55 min at a flow rate of 300 nl/min. The column was placed in an oven operating at 35°C. Full scan mass spectrometry spectra were acquired in the orbitrap (m/z 300–1600) in profile mode and data-dependent acquisition with the resolution set to 60,000 at m/z 400 and an automatic gain control target at 10⁵. The six most intense ions were sequentially isolated and multistage activation was used in order to generate more sequence-informative fragments for detection in the linear ion trap. Dynamic exclusion was set to 60 s. Ions with single charge states were excluded. A lockmass of m/z 445,120025 was used for internal calibration. The software Xcalibur (Thermo Scientific) was used to control the system and acquire the raw files.

Data analysis and database search

Protein identification was performed using Proteome Discoverer 1.3 software (Thermo Fischer Scientific, Waltham, MA, USA) equipped with SEQUEST HT search engine. Peak lists were searched against the Uniprot database with an initial mass deviation of 10 ppm, fragmentation mass deviation of 0.8 Da and trypsin digestion specificity allowing up to two missed cleavages. Oxidation of methionine, phosphorylation of threonine and serine residues and acetylation of the protein N-terminus were used as variable modifications. The peptides were filtered according to their XCorr score versus charge state (XCorr: 2≥1.8 and +3≥2.5) and high-confidence peptides were evaluated for their phosphorylation status. The algorithm phosphoRS was used in order to assign and score the phosphosite probabilities.

Statistical analysis

The Graph Pad Instat Statistical package for Windows was used. Data are expressed as means±s.e.m. Differences were examined by Student’s t-test (two-tailed) between two groups or by one-way analysis of variance (ANOVA) within multiple groups. P<0.05 was considered statistically significant.

Acknowledgements

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Competing interests

The authors declare no competing or financial interests.

Author contributions

E.P. performed experiments and wrote the paper, C.B. performed experiments and assisted with data analysis, I.M. assisted with the in vitro phosphorylation and subcellular fractionation assays and data processing, M.S. and G.P. performed experiments with LC-MS/MS and data analysis, G.S. supervised experiments and wrote the paper, and P.L. designed and supervised experiments and wrote the paper.

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Supplementary information

Supplementary information available online at http://jcs.biologists.org/lookup/doi/10.1242/jcs.191395.supplemental
Fig. S1. Impaired CK1δ activity decreases HIF-2 target genes mRNA and EPO secretion levels.

(A) Western blot analysis of lysates from HepG2 cells transfected with CK1δ siRNA (10nM) or scrambled siRNA (10nM). Cells were incubated for 16 h under hypoxia before collection and lysis. (B) Determination of PAI-1 mRNA levels by quantitative real-time PCR in HepG2 cells incubated and treated as in A. Results are shown as percent of activity in relation to hypoxia and represent the means of three independent experiments performed in duplicate (± s.e.m; **P<0.01). (C) Determination of EPO secretion by ELISA in HepG2 cells incubated and treated as in A. Results are shown as in B (*P<0.05). (D) Western blot analysis of lysates from Huh7 cells transfected with a different set of CK1δ siRNA (10nM) or scrambled siRNA (10nM). Cells were incubated for 16 h under hypoxia before collection and lysis. (E) Determination of EPO mRNA levels by quantitative real-time PCR in Huh7 cells incubated and treated as in (D). Results are shown as percent of activity in relation to hypoxia induced EPO and represent the means of two independent experiments performed in duplicate (± s.e.m).

(F) Western blot analysis of lysates from HepG2 cells incubated for 16 h under normoxia or hypoxia (1% O₂), in the presence or absence of the specific CK1δ inhibitor D4476 (10 μM) hypoxia before collection and lysis. (G) Determination of EPO and PAI-1 mRNA levels by quantitative real-time PCR in HepG2 cells incubated and treated as in A. Results are shown as fold increase in relation to the corresponding normoxic conditions and represent the mean of three independent experiments performed in duplicate (± s.e.m.; *P<0.05). (H) Determination of EPO secretion by ELISA in HepG2 cells incubated and treated as in A. Values are expressed as mlU/ml protein and represent the mean of three independent experiments performed in duplicate (± s.e.m; *P<0.05). (Statistical differences were assessed by using two-tailed Student’s t-test analysis).
**Fig. S2. Mutation of HIF-2α Ser 386 to Ala does not affect HIF-2α phosphorylation and activity.**

**(A)** GST-HIF-2α 366-542 wild type or its point mutants (1μg) were subjected to in vitro phosphorylation by 100 units of recombinant CK1δ and analyzed by SDS-PAGE followed by Coomassie Blue staining (upper panel) or autoradiography (lower panel). Only the relevant parts of the gels are shown. The numbers under each lane represent relative phosphorylation levels measured as indicated under “Experimental Procedures” and are means of three independent experiments. *(B)* Western blot analysis of lysates from Huh7 cells transfected with flag-CMV2 alone, wild-type flag-HIF-2α or its phosphorylation deficient mutants using anti-HIF-2α and antitubulin antibodies. *(C)* Determination of EPO and PAI-1 mRNA levels by quantitative real-time PCR in Huh7 cells transfected as in B. Results are shown as percent of activity in relation to wild-type HIF-2α and represent the mean of three independent experiments performed in duplicate (± s.e.m). (Statistical differences were assessed by using two-tailed Student’s t-test analysis).
Fig. S3. HIF-2α phosphorylation deficient mutant localization in Huh7 cells.
Transfected Huh7 cells expressing flag-CMV2 alone, wild-type flag-HIF-2α, or its phosphorylation deficient mutants (as indicated) were fixed, stained with DAPI and subjected to immunofluorescence microscopy with anti-Flag mouse monoclonal antibody. Cells were treated with 10 ng/ml LMB for 4 h before fixation (as indicated). In one case, cells were treated with 10 μM D4476 for 16 h before fixation (as indicated). White scale bars equal to 10 μm.
A

FLAG
HIF-2α WT
HIF-2α S383D
HIF-2α T528E
HIF-2α DM SD/TE

Flag-HIF2α DAPI Merge

B

HIF-2α WT
HIF-2α S383D
HIF-2α T528E
HIF-2α DM SD/TE

% transfected cells

C

HIF-2α WT
HIF-2α S383D
HIF-2α T528E
HIF-2α DM SD/TE

% transfected cells

D

EPO mRNA levels

E

PGK1 mRNA levels

LMB

FLAG HIF2 WT DM SA/TA DM SD/TE
Fig. S4. HIF-2α phosphomimetic mutations does not affect its localization.

(A) Transfected HepG2 cells expressing flag-CMV2 alone, wild-type flag-HIF-2α, or its phosphomimetic mutants (as indicated) were fixed, stained with DAPI and subjected to immunofluorescence microscopy with anti-Flag mouse monoclonal antibody. Cells were treated with 10 ng/ml LMB for 4 h before fixation (as indicated). In one case, cells were treated with 10 μM D4476 for 16 h before fixation (as indicated). White scale bars equal to 10 μm. (B & C) Number of cells showing nuclear and cytoplasmic staining under each condition was counted, and the percentage calculated. At least 100–150 transfected cells were examined in each case. Each experiment depicted in this figure was performed three separate times and data plotted are the average of the three independent experiments (± s.e.m). (D & E) Determination of EPO (D) and PGK1 (E) mRNA levels by quantitative real-time PCR in Huh7 cells transfected as indicated. Cells were treated with 10 ng/ml LMB for 16 h before lysis. Results are shown as fold increase in relation to the empty FLAG vector and represent the means of two independent experiments performed in duplicate (± s.e.m).
Table S1. Annotation of the different fragment ions derived from the CID spectrum of the monoisotopic m/z: 794.34735 Da (identified as GAVSpEKSpNFLFTK).

(A) b and y ions (B) Ions with phosphorylation losses and (C) Precursor ions.

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Table S2. Sequences of HIF-2α specific peptides identified by the LC-MS/MS analysis and their corresponding scorings.

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