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## HIF-1 promotes the expression of its $\alpha$ -subunit via an epigenetically regulated transactivation loop†

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**Hypoxia inducible factor-1 (HIF-1) directs the cellular response to low oxygen and plays a key role in tumour survival and growth. Here we use an inhibitor of the HIF-1 $\alpha$ /HIF-1 $\beta$  protein–protein interaction to show the presence of an epigenetically controlled transactivation loop whereby the HIF-1 transcription factor promotes the expression of its own  $\alpha$ -subunit in hypoxic cancer cells.**

Hypoxia-inducible factor (HIF) is a heterodimeric transcription factor composed of an oxygen-regulated  $\alpha$ -subunit and a ubiquitously expressed  $\beta$ -subunit. HIF-1 $\alpha$  is continuously expressed in normoxia, but is degraded by prolylhydroxylase enzymes that use oxygen as a substrate, and as a result has a half life of less than 5 minutes; reduced oxygen levels in hypoxia lead to the stabilization of HIF-1 $\alpha$  through inactivation of this enzymatic process.<sup>1</sup> As HIF-1 $\alpha$  builds up in hypoxia, this protein translocates to the nucleus,<sup>2</sup> where it binds HIF-1 $\beta$  to form the HIF-1 transcription factor complex. The HIF-1 mediated response to the onset of hypoxia is near instantaneous,<sup>3</sup> with the HIF-1 transcription factor directing the transcription of several hundred genes that enable cell adaptation and survival in hypoxia. The role of HIF-1 activity in angiogenesis, tumour growth and metastasis is well established; HIF-1 $\alpha$  is overexpressed in many cancers, and oncogene activation and loss of tumour suppressor function is shown to be associated with HIF-1 activation.<sup>4,5</sup> There has been significant effort directed towards identification of HIF-1 inhibitors for use as potential anticancer agents.<sup>6–10</sup>

HIF-1 $\alpha$  acts as both the sensor and a key component of the hypoxia response machinery by directing the expression of a wide variety of hypoxia-response genes.<sup>11,12</sup> The HIF-1 transcription factor binds to hypoxia-responsive elements (HRE, 5'-RCGTG-3', where R is either A or G) located in the enhancer and promoter regions of these genes, promoting their expression. HIF-1 $\alpha$  quantity

and activity is tightly controlled at the transcription, translation and post-translation levels.<sup>13,14</sup> The presence of a HRE in the core promoter sequence of the HIF-1 $\alpha$  gene (ACGTG, 156 bases upstream of the transcription start site, referred to as 'HIF-1 $\alpha$  HRE' from here),<sup>15</sup> suggests that the HIF-1 transcription factor may upregulate the transcription its own  $\alpha$ -subunit in hypoxia (Fig. 1A).

More recently, the methylation state of the GC dinucleotide in the HIF-1 $\alpha$  HRE has been demonstrated to directly correlate with an increase in HIF-1 $\alpha$  mRNA in hypoxia; in cell lines with a methylated CG dinucleotide HIF-1 $\alpha$  transcription remained at basal levels in hypoxia (Fig. 1B), whereas a 5–10 fold increase in HIF-1 $\alpha$  mRNA upon induction of hypoxia was observed in cell lines with a demethylated HIF-1 $\alpha$  HRE (Fig. 1A).<sup>16</sup> The binding of HIF-1 to the HRE in the promoter region of the HIF-1 $\alpha$  gene has been demonstrated by chromatin immunoprecipitation.<sup>16</sup> Taken together, these data suggest that the HIF-1 transcription factor upregulates the expression of its  $\alpha$ -subunit in hypoxia (referred to as HIF-1 $\alpha$  transactivation from here). But in the absence of an inhibitor of HIF-1 dimerization, which would disrupt the assembly of this transcription factor without affecting the quantity of HIF-1 $\alpha$  at the onset of hypoxia, the significance of this HRE and its effect on HIF-1 $\alpha$  levels in hypoxia remained unknown. Tools such as siRNA or genetic knockouts cannot be used for this purpose as they themselves affect the cellular quantity of HIF-1 $\alpha$ .

We recently reported a cyclic peptide (named P1, Fig. 1C), that selectively disrupts the HIF-1 $\alpha$ /HIF-1 $\beta$  protein–protein interaction by binding to the PAS-B domain of HIF-1 $\alpha$  with 124 nM affinity.<sup>17</sup> Here, we report the use of this compound as a chemical tool to probe the effect of the epigenetically regulated HIF-1 $\alpha$  transactivation loop on HIF-1 $\alpha$  mRNA and protein levels at the onset of hypoxia in cancer cells.

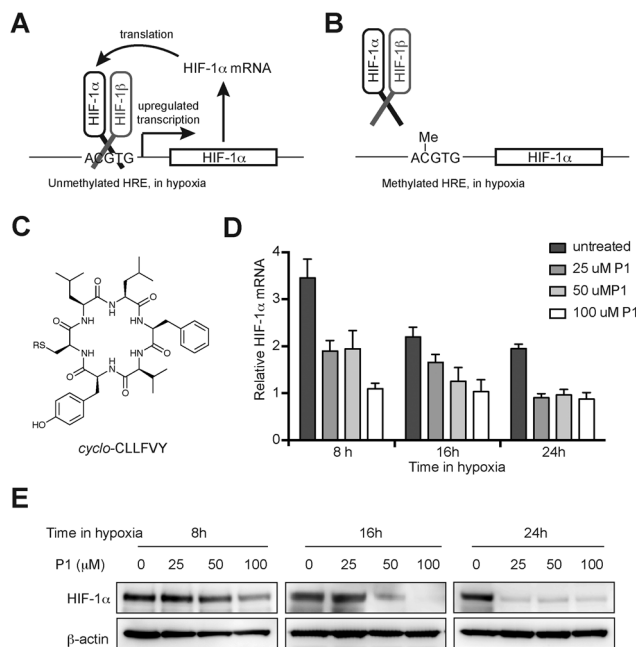
We began by measuring changes in HIF-1 $\alpha$  transcription by qPCR in MCF-7 breast cancer cells. As the CG dinucleotide of the HIF-1 $\alpha$  HRE is unmethylated in this cell lines,<sup>17</sup> an increase in HIF-1 transcription (compared to its basal transcription in normoxia) would be expected upon induction of hypoxia. We observed

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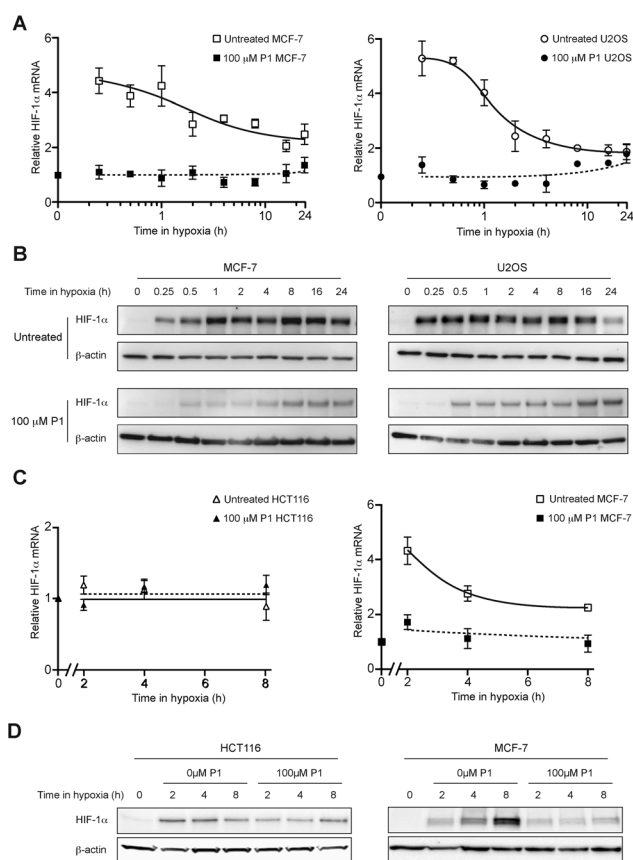


**Fig. 1** Epigenetic regulation of HIF-1 $\alpha$  transcription in hypoxia. (A) The unmethylated HRE upstream of the HIF-1 $\alpha$  gene is bound by HIF-1, upregulating the transcription of HIF-1 $\alpha$  in hypoxia. (B) The HIF-1 transcription factor cannot bind a methylated HRE, therefore in these cells, transcription of HIF-1 $\alpha$  will remain at basal levels. (C) The structure of P1, R = CGRKKRRQRRRPPQ. (D) The effect of disrupting HIF-1 by P1 on relative HIF-1 $\alpha$  mRNA (1 = normoxic levels) in MCF-7 cells at 8, 16 and 24 hours in hypoxia. (E) The effect of disrupting HIF-1 by P1 on HIF-1 $\alpha$  protein levels in MCF-7 cells incubated for 8, 16 and 24 hours in hypoxia.

a  $3.5 \pm 0.4$  fold increase in HIF-1 $\alpha$  mRNA in MCF-7 cells incubated in hypoxia for 8 hours (Fig. 1D). We next measured HIF-1 $\alpha$  transcription in cells incubated in hypoxia for 16 and 24 hours; we observed a reduction in the upregulated transcription of HIF-1 $\alpha$  with increased incubation of cells in hypoxia ( $2.2 \pm 0.2$  fold higher than normoxia after 16 hours, and  $1.95 \pm 0.1$  fold higher than normoxia after 24 hours). To probe whether the observed increase in HIF-1 $\alpha$  mRNA was due to transactivation by HIF-1, the experiment was repeated in cells treated with 0, 25, 50 and 100  $\mu\text{M}$  of P1 prior to the induction of hypoxia. The HIF-1 dimerization inhibitor would be expected to eliminate the observed upregulation of HIF-1 $\alpha$  transcription in hypoxia. We observed a dose-dependent drop in the hypoxia-induced upregulation of HIF-1 $\alpha$  mRNA, with transcription of HIF-1 $\alpha$  occurring at similar rates to that observed in normoxic cells for cells pretreated with 100  $\mu\text{M}$  P1 (Fig. 1D). The effect of P1 on the hypoxia-induced increase in HIF-1 $\alpha$  mRNA was also measured in cells incubated in hypoxia for 16 and 24 hours, and was again found to be prevented by P1 (Fig. 1D). These results indicate that the increase in HIF-1 $\alpha$  transcription observed in hypoxic MCF-7 cells is due to upregulation of HIF-1 $\alpha$  transcription by HIF-1. We next assessed the effect of disrupting HIF-1 with P1 on HIF-1 $\alpha$  protein levels by western blot. A dose-dependent and time-in-hypoxia-dependent reduction of HIF-1 $\alpha$  protein was observed in hypoxic MCF-7 cells that were pretreated with P1, mirroring the changes in HIF-1 $\alpha$  mRNA (Fig. 1E). It should be noted that the

observed effects are not due to toxicity of P1, as transcription of housekeeper genes and HIF-1 $\alpha$  in P1-treated cells, did not drop below those observed in normoxia. We have also previously demonstrated (by toxicity assays) that the compound is not toxic to cells.<sup>17</sup>

As a reduction of HIF-1 $\alpha$  mRNA levels was observed with increasing incubation time in hypoxia, we next measured changes in HIF-1 $\alpha$  mRNA as a function of time in hypoxia by qPCR. In untreated MCF-7 cells, HIF-1 $\alpha$  mRNA was observed to rapidly increase by  $4.4 \pm 0.5$  fold within 15 minutes of hypoxia induction (solid line, Fig. 2A, left hand panel); the experiment was repeated in U2OS cells (an osteosarcoma cell line which also contains an unmethylated HIF-1 $\alpha$  HRE),<sup>17</sup> and a  $5.3 \pm 0.6$  fold increase in HIF-1 $\alpha$  mRNA was observed at the same time point (solid line, Fig. 2A, right hand panel). In MCF-7 cells, HIF-1 $\alpha$  mRNA was maintained at this upregulated level for an hour,



**Fig. 2** P1 inhibits the upregulation of HIF-1 $\alpha$  mRNA and protein levels in hypoxia. (A) The effect of P1 on HIF-1 $\alpha$  mRNA as measured by qPCR in hypoxic MCF-7 and U2OS cells. The data shows that HIF-1 $\alpha$  mRNA levels remain at normoxic levels in P1-treated cells, despite the induction of hypoxia. (B) Representative blots showing pretreatment of MCF-7 and U2OS cells with P1 results in a significant decrease in HIF-1 $\alpha$  protein. (C) The effect of P1 on HIF-1 $\alpha$  mRNA as measured by qPCR in hypoxic HCT116 and MCF-7 cells. The data shows that HIF-1 $\alpha$  mRNA levels remain at normoxic levels in hypoxic HCT116 cells, with no effect from P1. Analogous experiment in MCF-7 cells were conducted and are shown for comparison. (D) Representative blots showing pretreatment of HCT116 cells with P1 does not result in a significant decrease in HIF-1 $\alpha$  protein. Analogous experiments with MCF-7 cells were conducted and are shown for comparison.

before steadily declining over the next 16 hours (solid line, Fig. 2A, left hand panel). In U2OS cells however, HIF-1 $\alpha$  mRNA levels began to decline after 30 minutes (solid line, Fig. 2A, left hand panel). In both cell lines, HIF-1 $\alpha$  transcription steadily declined to around 2-fold the normoxic level after 16 hours in hypoxia.

Transcription of HIF-1 $\alpha$  remained at normoxic levels at all examined time points in hypoxic MCF-7 and U2OS cells that had been treated with 100  $\mu$ M of our HIF-1 inhibitor prior to the induction of hypoxia (dashed lines, Fig. 2A). This data further supports the hypothesis that the upregulated transcription of HIF-1 $\alpha$  observed upon induction of hypoxia in these cells is driven by the HIF-1 transcription factor. As a control, the effect of P1 on the transcription of HIF-1 $\beta$ , which is constitutively expressed and not regulated by hypoxia, was also measured in hypoxic MCF-7 cells. As expected from a HIF-1 dimerization inhibitor, P1 did not significantly alter HIF-1 $\beta$  mRNA levels in hypoxic MCF-7 cells (Fig. S1, ESI $^\dagger$ ).

We next sought to probe the effect of eliminating the HIF-1 $\alpha$  transactivation loop (with P1) on the build up of HIF-1 $\alpha$  protein in hypoxic cells. We observed a rapid increase in HIF-1 $\alpha$  protein levels by western blot within 15 minutes of the induction of hypoxia in both MCF-7 and U2OS cells (Fig. 2B). A significant reduction in HIF-1 $\alpha$  protein at the onset of hypoxia was observed in P1-treated cells; HIF-1 $\alpha$  protein levels still increase with time, but at a significantly slower rate, suggesting that the HIF-1-driven increase of HIF-1 $\alpha$  mRNA translates into an increase in HIF-1 $\alpha$  protein levels in hypoxic MCF-7 and U2OS cells.

We next assessed the effect of P1 in HCT116, a colon carcinoma cell line, in which the GC dinucleotide in the HIF-1 $\alpha$  HRE is methylated. Previous work has shown that HIF-1 $\alpha$  transcription is not upregulated upon induction of hypoxia in these cells, and that the HIF-1 transcription factor does not bind the methylated HIF-1 $\alpha$  HRE,<sup>16</sup> in line with that observed for other hypoxia-response genes with a methylated HRE.<sup>18</sup> The hypoxia-mediated transactivation of HIF-1 $\alpha$  and binding of HIF-1 to the HIF-1 $\alpha$  HRE has been reported to be restored upon treatment of HCT116 cells with the DNA demethylating agent 5-aza-2'-deoxycytidine.<sup>16</sup> To further probe our hypothesis, we measured the effect of P1 on HIF-1 $\alpha$  levels in HCT116 cells. We monitored HIF-1 $\alpha$  transcription at 2, 4 and 8 hours in HCT116 cells by qPCR and in line with the published data, transcription of HIF-1 $\alpha$  remained at normoxic levels despite the hypoxic environment at all examined time points.

Treatment with 100  $\mu$ M P1 prior to the induction of hypoxia in HCT116 had no effect on HIF-1 $\alpha$  mRNA levels at the time-points examined (Fig. 2C, left hand panel); in the absence of the HIF-1 $\alpha$  transactivation loop, disruption of HIF-1 dimerization has no effect on HIF-1 $\alpha$  transcription in hypoxia. Concurrent experiments with MCF-7 cells again showed an increase in HIF-1 $\alpha$  mRNA in hypoxia, which was reduced to normoxic level by pretreatment with 100  $\mu$ M P1 (Fig. 2C, right hand panel). As a control we assessed the effect of P1 on the transcription of vascular endothelial growth factor (VEGF), a HIF-1 regulated gene that stimulates vasculogenesis and angiogenesis,<sup>19</sup> in HCT116. We observed a  $5.1 \pm 0.1$  fold increase in VEGF mRNA after hypoxic incubation for 2 hours, remaining upregulated by

$5.5 \pm 0.1$  fold above its normoxic transcription after 8 hours in hypoxia. The hypoxia-mediated upregulation of VEGF transcription was reduced to normoxic levels in HCT116 cells treated with 100  $\mu$ M P1 (Fig. S2, left hand panel, ESI $^\dagger$ ). Repeating this experiment in MCF-7 cells also showed inhibition of the hypoxia-mediated  $4.4 \pm 0.8$  fold increase in VEGF mRNA in P1-treated cells (Fig. S2, right hand panel, ESI $^\dagger$ ).

We next sought to compare the effect of HIF-1 $\alpha$  transactivation on the accumulation of HIF-1 $\alpha$  in hypoxic cells by monitoring changes in HIF-1 $\alpha$  protein in hypoxic MCF-7 and HCT116 cells, in the presence and absence of our HIF-1 inhibitor. In HCT116 cells, HIF-1 $\alpha$  protein is readily observed by western blot in cells incubated in hypoxia for 2 hours, with no significant increase in HIF-1 $\alpha$  protein observed in cells incubated in hypoxia for 4 and 8 hours (Fig. 2D, left hand panel). Treatment with 100  $\mu$ M P1 did not significantly affect accumulation of HIF-1 $\alpha$  in these cells after 2, 4 or 8 hours in hypoxia (Fig. 2D, left hand panel). The stabilization of HIF-1 $\alpha$  protein was also readily observed in MCF-7 cells incubated in hypoxia for 2 hours, but unlike HCT116 cells, HIF-1 $\alpha$  protein continued to rise with increasing incubation time in hypoxia (Fig. 2D right hand panel). HIF-1 protein stabilization after 2 hours in hypoxia was visible by western blot in P1-treated MCF-7 cells, but at significantly lower levels than observed in untreated cells, and these levels did not rise with longer incubation in hypoxia. Taken together, our results show that in cells with an unmethylated HIF-1 $\alpha$  HRE (such as MCF-7), HIF-1 $\alpha$  transactivation and the resulting rise in HIF-1 $\alpha$  mRNA significantly contributes to the build up of HIF-1 $\alpha$  protein in hypoxia. This study demonstrates the potential for using the increasing numbers of protein-protein interaction inhibitors<sup>17,20,21</sup> as chemical tools that enable new insight into biological processes.

## Conclusions

The default epigenetic status of the HIF-1 $\alpha$  HRE in normal tissue is currently unknown, it is nonetheless clear from the above data that demethylation of this HRE serves to significantly accelerate the build up of HIF-1 $\alpha$  protein in hypoxia. Interestingly, a recent study found the HIF-1 $\alpha$  HRE to be methylated in samples of normal colon cells, but demethylated in several colon cancer cell lines and primary colon cancer specimens.<sup>16</sup> This suggests that in addition to the multiple mechanisms of HIF-1 regulation,<sup>22</sup> cellular response to hypoxia is also regulated *via* methylation of the HIF-1 $\alpha$  HRE. While the mechanism that would cause variations in the epigenetic status of HRE is currently unclear,<sup>23,24</sup> analogous epigenetic control of several HIF-1-regulated genes have also been reported.<sup>18,25,26</sup> As the HIF-1 transcription factor has a different binding affinity for each HRE,<sup>12</sup> it may be reasoned that in addition to accelerating the build up of HIF-1 $\alpha$ , demethylation of the GC dinucleotide in the HIF-1 $\alpha$  HRE would result in an altered transcriptional response to hypoxia in these cells. Further experiments to assess this hypothesis are currently underway in our laboratory.

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