1   **Reprogramming the transcriptional response to hypoxia with a chromosomally**

2   **encoded cyclic peptide HIF-1 inhibitor.**

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10

11   **Abstract**

12   The cellular response to hypoxia is orchestrated by HIF-1, a heterodimeric transcription factor

13   composed of an α and β subunit that enables cell survival under low oxygen conditions by

14   altering the transcription of over 300 genes. There is significant evidence that inhibition of

15   HIF-1 would be beneficial for cancer therapy. We recently reported a cyclic hexapeptide that

16   inhibits the HIF-1α/HIF-1β protein-protein interaction *in vitro* and prevents HIF-1-mediated

17   hypoxia-response signalling in cells. This cyclic peptide was identified from a library of 3.2 x

18   10^6 members generated using SICLOPPS split-intein mediated protein splicing. With a view

19   to demonstrating the potential for encoding the production of a therapeutic agents in response

20   to a disease marker, we have engineered human cells with an additional chromosomal control

21   circuit that conditionally encodes the production of our cyclic peptide HIF-1 inhibitor. We

22   demonstrate the conditional production of our HIF-1 inhibitor in response to hypoxia, and its

23   inhibitory effect on HIF-1 dimerization and downstream hypoxia-response signalling. These

24   engineered cells are used to illustrate the synthetic lethality of inhibiting HIF-1 dimerization

25   and glycolysis in hypoxic cells. Our approach not only eliminates the need for the chemical

26   synthesis and targeted delivery of our HIF-1 inhibitor to cells, it also demonstrates the wider

27   possibility that the production machinery of other bioactive compounds may be incorporated

28   onto the chromosome of human cells. This work demonstrates the potential of sentinel

29   circuits that produce molecular modulators of cellular pathways in response to environmental,

30   or cellular disease stimuli.

31

32   **Introduction**

33   Transcription factors are master regulators of cellular fate and function that orchestrate a

34   coordinated response to a variety physiological stimuli. Exogenous modulation of

35   transcription factor activity therefore holds much therapeutic potential, and is a critical tool

36   for deciphering complex cellular networks. The absolute requirement of assembly for

37 function means that protein-protein interaction (PPI) inhibition is the optimal strategy for

38 intervention, but transcription factors are considered to be one of the most chemically

39 intractable targets in drug discovery.[1] More generally, the challenge of identifying PPI

40 inhibitors means that the majority of tools employed for studying these complexes are nucleic

41 acid-based (e.g. siRNA, or CRISPR) and function to eliminate the targeted protein from the

42 cell. Despite their widespread use, these methods have several drawbacks for studying PPIs;

43 removal of a protein from a system eliminates all of its known and unknown interactions and

44 functions. Thus an observed phenotype may not necessarily be attributed to a given PPI. In

45 cases where validated PPI inhibitors are available, the need for chemical synthesis and

46 intracellular delivery of such compounds places limitations on their adaptation and use. We

47 sought an alternative approach by introducing the components necessary to synthesize a PPI

48 inhibitor onto the genome of a human cell line. Under this scenario, the intracellular

49 production of a non-native molecule is selectively induced by one or more disease specific

50 signals via expression of the machinery needed for its production. This approach would have

51 the advantage that it eliminates the need for chemical synthesis and intracellular delivery of

52 the therapeutic agent. To demonstrate the viability of the proposed approach, we turned to our

53 recently reported cyclic peptide inhibitor of hypoxia inducible factor 1 (HIF-1)

54 heterodimerization.[2]

55 HIF-1 is a heterodimeric transcription factor that drives the cellular response to hypoxia,[3, 4] by

56 altering the transcription of over 300 genes,[5] enabling cell survival and growth in a low

57 oxygen microenvironment. HIF-1 is composed of an oxygen-regulated α-subunit (HIF-1α)

58 and a constitutively expressed β-subunit (HIF-1β). HIF-1α is marked for degradation by

59 prolyl hydroxylases that use oxygen as a substrate.[6, 7] Reduced oxygen levels lead to the

60 stabilization and nuclear translocation of HIF-1α, where it binds HIF-1β to form the active

61 HIF-1 transcription factor. HIF-1α mounts an immediate response to reductions of

62 intracellular oxygen,[8] while two closely related isoforms, HIF-2α (also known as EPAS1) and

63 HIF-3α, are thought to regulate the response to prolonged hypoxia.[9] The intricate interplay

64 between HIF-α isoforms in cancer is complex and yet to be fully deciphered, but the role of

65 HIF-1 activity in angiogenesis, tumour growth and metastasis is well established.[10, 11]

66 Tumours grow rapidly, outstripping the capacity of the local vasculature, which results in a

67 hypoxic microenvironment; HIF-1α is overexpressed in many cancers,[12] and oncogene

68 activation and loss of tumour suppressor function is shown to be associated with HIF-1.[13]

69 We recently reported an inhibitor of the HIF-1α/HIF-1β PPI;[2] this molecule (*cyclo*–CLLFVY,

70 named P1) was identified from a genetically encoded library of 3.2 million cyclic

71 hexapeptides generated using split-intein circular ligation of peptides and proteins

72 (SICLOPPS).[14, 15] P1 selectively binds to the PASB domain of HIF-1α with a K_d of 124 nM,

73   disrupts the HIF-1α/HIF-1β PPI *in vitro* and in cells, and inhibits HIF-1 signalling in hypoxic

74   cells.[2] P1 is isoform-specific and does not bind to, or affect the function of the closely related

75   HIF-2 isoform *in vitro* or in cells.[2] SICLOPPS generated cyclic peptides via *Synechocystis sp*

76   PCC6803 (*Ssp*) DnaE split inteins;[16] the SICLOPPS protein is composed of rearranged N-

77   terminal and C-terminal split inteins flanking a peptide extein sequence in the form of I_C-

78   extein-I_N. The N- and C-terminal split inteins combine to form an active intein that splices to

79   cyclize the extein (Figure S1A). By altering the sequence of the SICLOPPS extein at the

80   DNA level, a variety of cyclic peptides and proteins, including randomized cyclic peptide

81   libraries, may be produced by this approach.[17]

82   Given our goal of incorporating the machinery required for the generation of a molecular PPI

83   modulator into cells, and the significance of HIF-1 in tumour survival and growth, we set out

84   probe the possibility of engineering the conditional production of P1 (via the corresponding

85   SICLOPPS inteins) onto the chromosome of human HEK-293 cells, and to assess the effect

86   of genetically encoded P1 on HIF-1-mediated hypoxia response in these cells.

87

88   **Results**

89   <u>Expression and processing of SICLOPPS constructs in HEK-293 cells</u>

90   We began by constructing a cell line capable of conditional P1 production and assessing the

91   ability of the SICLOPPS construct to correctly function in human cells. To achieve inducible

92   expression of the SICLOPPS construct encoding our HIF-1 inhibitor, we used a cassette

93   containing a CMV promoter, followed by two copies of the tetracycline operator (tetO),

94   enabling regulation of transcription with doxycycline (dox), followed by the gene for

95   SICLOPPS (Figure 1A). Engineered *Nostoc punctiforme* DnaE (*Npu*) inteins that splice

96   significantly faster than the *Ssp* inteins typically used in SICLOPPS[18-20] were utilized for the

97   production of P1, with CLLFVY as the extein to be cyclized. We used flippase-flippase

98   recognition target (Flp-FRT) recombination[21] to stably integrate this cassette onto the

99   chromosome of human HEK-293 cells (T-REx-293) to give T-REx-P1 cells. We first sought

100   to demonstrate the production of functional P1 from the chromosome of human cells. Intein

101   production was probed by immunoblotting with an antibody against the chitin-binding

102   domain (CBD) present on the C-terminus of the N-terminal intein; we only observed the CBD

103   band in the integrated cells, and only when cultured with dox (Figure S2A). The change in

104   transcription of the chromosomal SICLOPPS construct in response to dox was quantified by

105   RT-qPCR as ~37-fold in both normoxia and hypoxia (Figure 1B), which was also reflected at

106   the protein level (Figure 1C). A time course measuring intein protein production over 24 h in

107   hypoxic cells illustrated the steady build-up of SICLOPPS inteins (Figure 1D). The splicing

108   efficiency of the SICLOPPS protein encoding P1 was measured by immunoblot analysis for

109   the CBD. We only observed a single band at ~18 kDa corresponding to the spliced N-intein,

110 suggesting full splicing of the SICLOPPS protein (Figure S2B). We next sought to directly

111 detect the presence of P1 in T-REx-P1 cells. A synthetic sample of P1 was prepared as a

112 standard, and a peak with identical retention time as this sample was observed in the lysate of

113 T-REx-P1 cells treated with dox (Figure 1E). Mass spectrometric analysis of this fraction

114 from the cell lysate revealed peaks corresponding to the mass of P1 (Figure 1F) and the same

115 as observed for the synthetic standard (Figure S3). This data demonstrates that the *Npu*

116 SICLOPPS inteins incorporated into the chromosome of human HEK-293 cells are

117 selectively produced in the presence of dox, splicing post translation to give detectable levels

118 of P1 in the engineered human cell line.
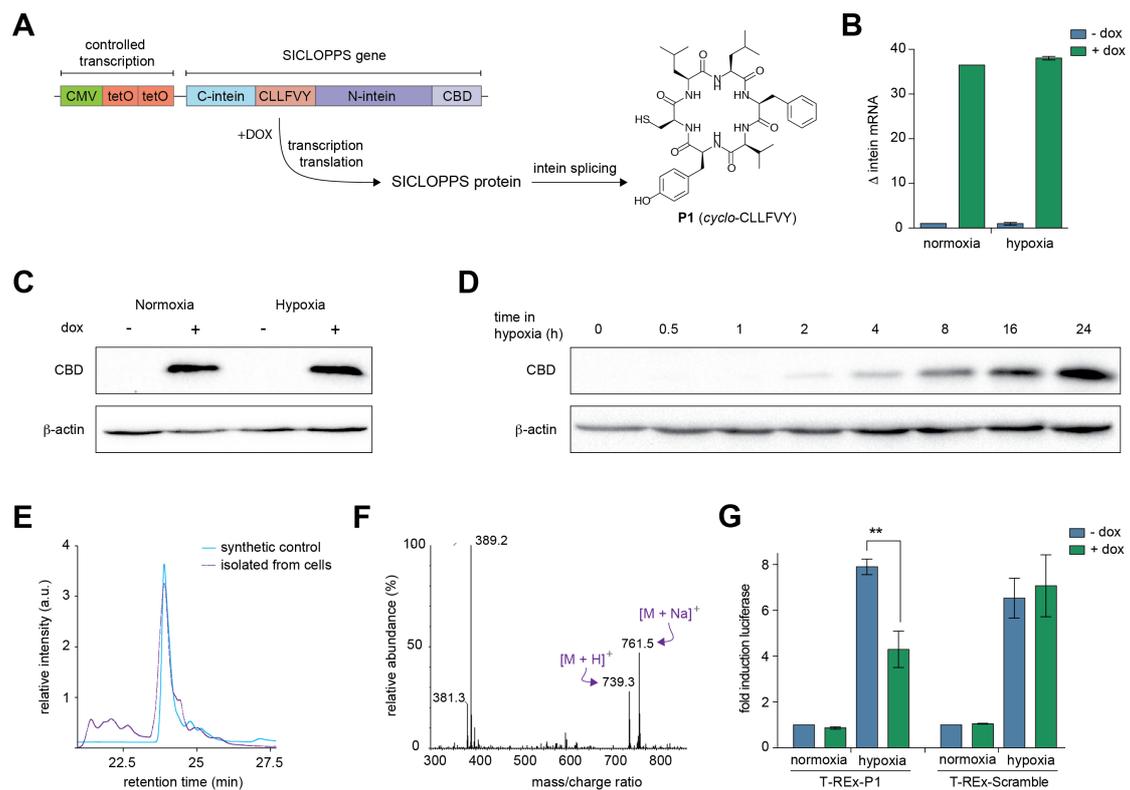
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120 **Figure 1. Production of a functional cyclic peptide HIF-1 inhibitor from the**

121 **chromosome of human HEK-293 cells. (A)** The chromosomally-integrated cassette enables

122 the conditional expression of the SICLOPPS gene, which encodes the *Npu* inteins with

123 CLLFVY as the extein. Splicing of these inteins gives P1. **(B)** RT-qPCR of intein expression

124 in T-REx-P1 cells incubated in normoxia or hypoxia (24 h). **(C)** Immunoblot of T-REx-293

125 and T-REx-P1 cells treated as in B **(D)** Immunoblot of T-REx-HRE cells incubated with dox

126 in hypoxia for 0-24 h **(E)** P1 produced in T-REx-P1 cells has the identical HPLC retention

127 time as the synthetic standard. **(F)** The mass spectrum (ESI+) of fraction shown in F from T-

128 REx-P1 cell lysate shows the presence of P1. **(G)** Firefly luciferase activity in T-REx-P1 and

129 T-REx-Scram cells transfected with a TK-HRE-luciferase and incubated for under normoxia

130 or hypoxia (16 h). Data are means (n=3) ± SEM, **p < 0.01.

131

132 <u>Genetically encoded P1 inhibits HIF-1 activity</u>

133 With the engineered cell line in hand and having demonstrated the conditional production of

134 P1, the functionality of the genetically encoded cyclic peptide HIF-1 inhibitor was next

135 probed. T-REx-P1 cells were transfected with a HIF-dependent luciferase reporter plasmid,

136 where activation of HIF results in increased luciferase expression.[22] As expected, there was

137 no change in the luciferase signal with dox in normoxia, while an ~8-fold increase in

138 luciferase activity was observed in hypoxic T-REx-P1 cells without dox (Figure 1G).

139 Induction of P1 production with dox in these cells resulted in a 50% decrease in luciferase

140 activity (Figure 1G), suggesting that chromosomally encoded P1 inhibits HIF-1 function. To

141 demonstrate that our observations are due to P1 and not the SICLOPPS inteins, we generated

142 a negative control cell line (T-REx-Scramble) that chromosomally encoded *cyclo*-CFVLYL

143 (a scrambled variant of P1) as the extein of *Npu* SICLOPPS inteins. Splicing and conditional

144 production of this scrambled peptide was demonstrated by immunoblotting (Figure S4). The

145 above luciferase assay was repeated in this cell line, and a ~7-fold increase in luciferase

146 activity was observed upon induction of hypoxia without dox. There was however, no change

147 in luciferase activity when these cells were incubated with dox in hypoxia (Figure 1G),

148 indicating that the scrambled peptide, or the SICLOPPS inteins do not affect HIF-1

149 dimerization. To validate that the effect from P1 was on HIF-1 rather than on luciferase, we

150 used a control SV40-luciferase plasmid, and did not see any significant change in luciferase

151 signal upon induction of P1 in T-REx-P1 cells with dox (Figure S5).

152 The effect of chromosomally produced P1 on HIF-1 activity was further assessed via its target

153 genes vascular endothelial growth factor (VEGF) and carbonic anhydrase IX (CAIX).

154 Chromosomally produced P1 reduced the VEGF transcription by ~30% (Figure S6A) and

155 CAIX transcription by ~45% (Figure S6B), with no effect of the scrambled peptide observed

156 on either gene (Figure S6). Together, the above data demonstrates that chromosomally

157 encoded P1 is functional, and able to inhibit HIF-1 signalling in hypoxia as expected from our

158 previous studies with the synthetic, tat-tagged variant of the compound.

159

160 <u>Engineering physiological control of peptide expression</u>

161 We next sought to engineer an additional layer of control into the above system by limiting

162 the dox-dependent production of P1 to hypoxic cells. The motivation for constructing this

163 dual-control system was our long-term goal of generating an *in vivo* model that contains such

164 a circuit on its chromosome. Such a system would not only allow expression of the HIF-1

165 inhibitors in hypoxic tissues (as an HRE-only promoter would), but also allow temporal

166 control over initiation of P1 production via addition of dox. Thus the effect of HIF-1

167 inhibition at various stages of the tumour development may be assessed. A hybrid promoter

168 was designed and constructed; three copies of the HRE from the inducible nitric synthase

169 promoter[22] were placed upstream of two copies of TetO, resulting in a dual physiological and

170 chemically controlled conditional promoter that would only function in hypoxia and with dox

171 (Figure 2A). This cassette was incorporated onto the chromosome of HEK-293 cells by Flp-

172 FRT recombination (as above) to give T-REx-HRE cells. Analysis of SICLOPPS cassette

173 transcription by RT-qPCR showed ~9-fold upregulated transcription in hypoxic cells that

174 were incubated with dox (Figure 2B). Immunoblot analysis showed the presence of

175 SICLOPPS protein only in cells cultured in hypoxia and with dox (Figure 2C), further

176 illustrating that the dual conditional promoter was functioning as designed. The quantity of

177 SICLOPPS protein was next compared with T-REx-P1 cells. Interestingly, we observed

178 higher levels of inteins in the CMV-promoted cell line than the HRE-promoted cells

179 incubated in hypoxia and with dox (Figure S7). Analysis of these bands by densitometry

180 indicated that there was ~7-fold more SICLOPPS intein in T-REx-P1 cells than in T-REx-

181 HRE cells after 24 h. There are two reasons for this difference; first, the transgene expression

182 rate from the HRE promoter is known to be lower than that from a CMV promoter,[23, 24] and

183 second, the spliced product of the promoted protein (P1) is an inhibitor of HIF-1 dimerization.

184 Therefore, as P1 builds up, it will also inhibit the transcription factor promoting its own

185 production. To further assess the effect of this feedback loop on P1 production, a time course

186 analysis of intein production was conducted. We observed the steady build-up of SICLOPPS

187 mRNA (Figure 2D) and protein (Figure 2E) in hypoxic T-REx-HRE cells over 24 h, with a

188 noticeable increase in both after 8 hours in hypoxia.

189 The effect of P1 on the interaction of HIF-1α and HIF-1β in hypoxic T-REx-HRE cells was

190 next directly probed using an *in situ* proximity ligation assay (PLA).[2, 25] A PLA signal was

191 observed in hypoxic T-REx-HRE cells incubated without dox (Figure 2F, left hand panel),

192 corresponding to the hypoxia-induced stabilization of HIF-1α and subsequent dimerization of

193 HIF-1α and HIF-1β. The PLA signal was not observed in these cells when incubated with

194 dox (Figure 2F, right hand panel), nor in normoxic cells with or without dox (Figure S8). This

195 data demonstrates the disruption of HIF-1 dimerization by genetically encoded P1 in cells.

196 The downstream effect of disrupting HIF-1 dimerization with chromosomally encoded P1

197 was elucidated via analysis of the transcription of HIF-1 target genes VEGF and CAIX. The

198 expression of both genes was measured by RT-qPCR in cells incubated in hypoxia for 24 h

199 with or without dox. Induction of P1 with dox resulted in a ~40% reduction in VEGF mRNA

200 (Figure 2G) and a ~50% reduction in CAIX mRNA in hypoxic T-REx-HRE cells (Figure

201 2H). It should be noted that although lower amounts of the SICLOPPS protein are produced

202 in the T-REx-HRE cells than in T-REx-P1 cells, the extent of the effect of P1 on these HIF-1

203 reporter genes was similar in both cell lines, indicating that P1 concentration is not a limiting

204 factor in the observed inhibition of HIF-1 signalling.
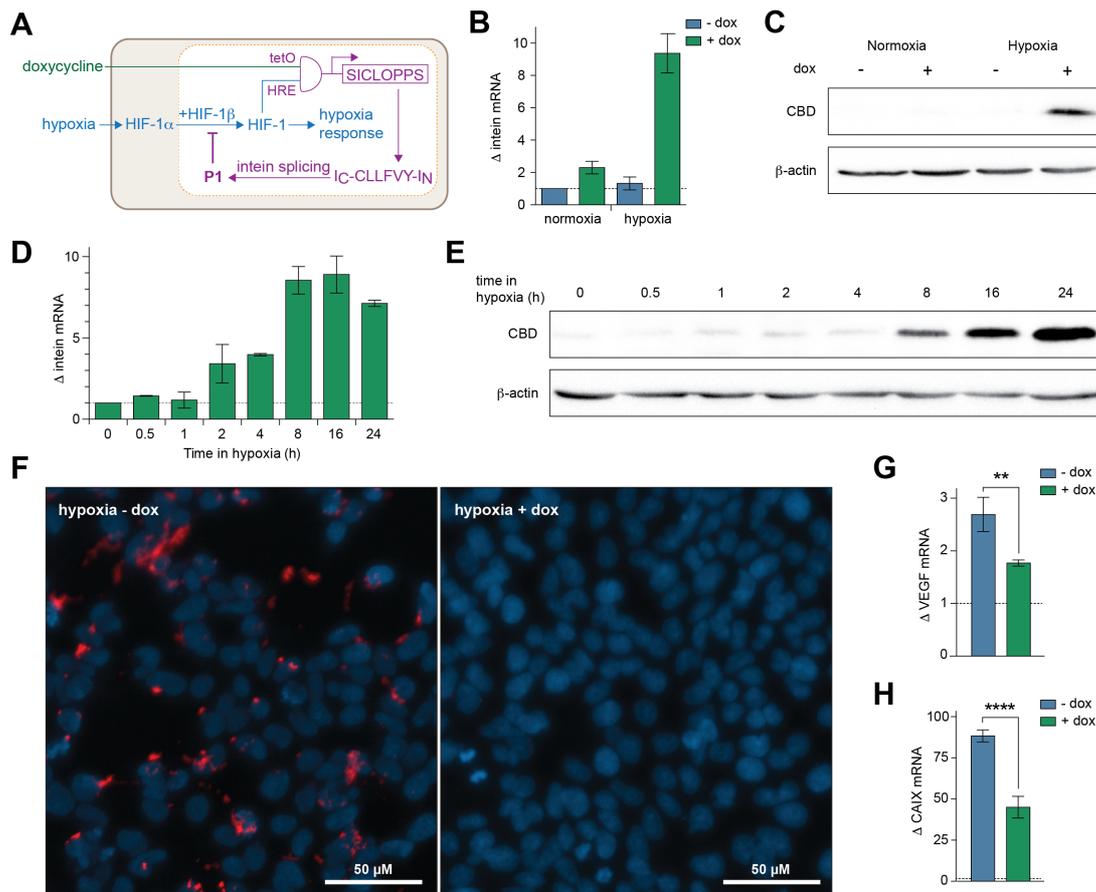
Figure 2: Conditional production of P1 in human cells. (A) The hybrid HRE/TetO promoter requires dual input signals of HIF-1 and dox in an AND process for expression of the SICLOPPS construct. Intein splicing produces P1, which inhibits HIF-1 dimerization. **(B)** RT-qPCR analysis of intein expression in T-REx-HRE cells incubated for 24 h in normoxia or hypoxia, with or without 1 μg/mL dox. **(C)** Immunoblot of T-REx-HRE cells treated as in panel B. **(D)** RT-qPCR analysis of SICLOPPS mRNA levels in T-REx-HRE cells incubated in hypoxia for 0-24 h and treated with 1 μg/mL dox. **(E)** Immunoblot for production of SICLOPPS protein over time. T-REx-HRE cells treated as in panel D. **(F)** PLA of T-REx-HRE cells treated with vehicle (left panel) or dox (right panel) and incubated in hypoxia for 24 h. **(G and H)** RT-qPCR analysis of (G) VEGF and (H) CAIX expression in T-REx-HRE cells incubated in hypoxia for 24 h with or without 1 μg/mL dox. Data are means (n=3) ± SEM, **$p < 0.01$, ****$p < 0.0001$.

Endogenous P1 expression alters transcriptional response to hypoxia

We broadened our analysis of the effect of P1 on HIF-1 signalling by using a focused microarray assessing expression of 43 hypoxia-associated genes (Table S1) in T-REx-HRE cells cultured in normoxia, hypoxia, and hypoxia with dox. The data showed altered expression of these genes in T-REx-HRE cells that were incubated in hypoxia with dox

7

224   (Figure 3A), illustrating the reprogramming of hypoxia response by P1 in these cells. We

225   have previously demonstrated (*in vitro* and in cells) that *cyclo*-CLLFVY is a specific inhibitor

226   of HIF-1 dimerization that does not affect the interaction of HIF-2α and HIF-1β.[2] We

227   therefore aimed to illustrate the potential of using genetically encoded P1 to separate the

228   effect of HIF-1 inhibition on hypoxia signalling from that of the closely related transcription

229   factor HIF-2. Our control experiments showed that treatment of T-REx-HRE cells with dox

230   had no effect on HIF-2α mRNA levels (Figure S9A), and that siRNA knockdown of HIF-2α

231   did not significantly affect the expression of HIF-1α mRNA or protein (Figure S9B and S9C)

232   or SICLOPPS protein (Figure S9D).

233   The isoform-specificity of a number of HIF-target genes was next assessed using the above

234   approach. While there was no effect on HIF-1α mRNA from either HIF-1 or HIF-2 (Figure

235   3B), HIF-3α expression seemed to be primarily under the control of HIF-2 (Figure 3C). We

236   next assessed the role of HIF isoforms on genes involved in angiogenesis (ANGPLT4 and

237   VEGF) and erythropoiesis (EPO). Following dox treatment, expression of EPO was

238   decreased to normoxic levels, whereas HIF-2α siRNA treatment caused no significant

239   change, indicating EPO is a HIF-1 specific target (Figure 3D). In contrast, although inhibition

240   of HIF-1 significantly reduced the expression of ANGPLT4 (Figure 3E) and VEGF (Figure

241   S9E), a combination of both dox and HIF-2α siRNA treatments was required to reduce gene

242   expression to normoxic levels. This data suggests that expression of ANGPLT4 and VEGF is

243   transactivated by both HIF-1 and HIF-2. Together, the inhibitory effect of P1 expression on

244   genes involved in the promotion of angiogenesis and erythropoiesis supports previous

245   assertions for the potential of targeting HIF-1 dimerization as a therapeutic strategy to prevent

246   tumour vascularisation.

247   Inhibition of HIF-1 dimerization and HIF-2α siRNA treatment also differentially impacted

248   upstream effectors of oxygen-dependent regulation of HIF-1. Induction of P1 expression

249   resulted in a decrease in PHD2 and PHD3 mRNA whereas HIF-2α siRNA has no significant

250   effect (Figure 3F and 3G). These observations are in line with the theory that HIF-1 mediates

251   the acute response to hypoxia whereas HIF-2 is the prominent driver of adaptation to

252   prolonged periods of hypoxic conditions.[26, 27] Another gene primarily controlled by HIF-1

253   was CAIX, with no significant effect from HIF-2α siRNA alone (Figure S9F). This data was

254   in agreement with reports of CAIX as a HIF-1 specific target.[28] Interestingly, although the

255   stress response gene DDIT4 was upregulated in hypoxia, and inhibition of HIF-1 and HIF-2

256   significantly reduced this induction, neither treatment was sufficient to reduce DDIT4

257   expression to normoxic levels (Figure 3H). DDIT4 is also induced in response to endoplasmic

258   reticulum stress and DNA damage related to the regulation of reactive oxygen species,[29, 30]

259   which may be a result of hypoxic exposure, particularly when HIF response pathways are

260   disrupted.[30] Another gene of note was NOTCH-1, whose expression was halved in hypoxic

261   cells treated with dox, but doubled in hypoxic cells treated with HIF-2α siRNA (Figure 3I),

262   illustrating the opposing regulatory roles of HIF-1 and HIF-2 on this gene. The above data not

263   only demonstrates reprogramming of hypoxia response in our engineered human cell line, but

264   also illustrates the potential utility of using genetically encoded P1, a HIF-1 specific inhibitor,

265   to decipher the role of HIF isoforms in hypoxia signalling in a variety of cell lines.

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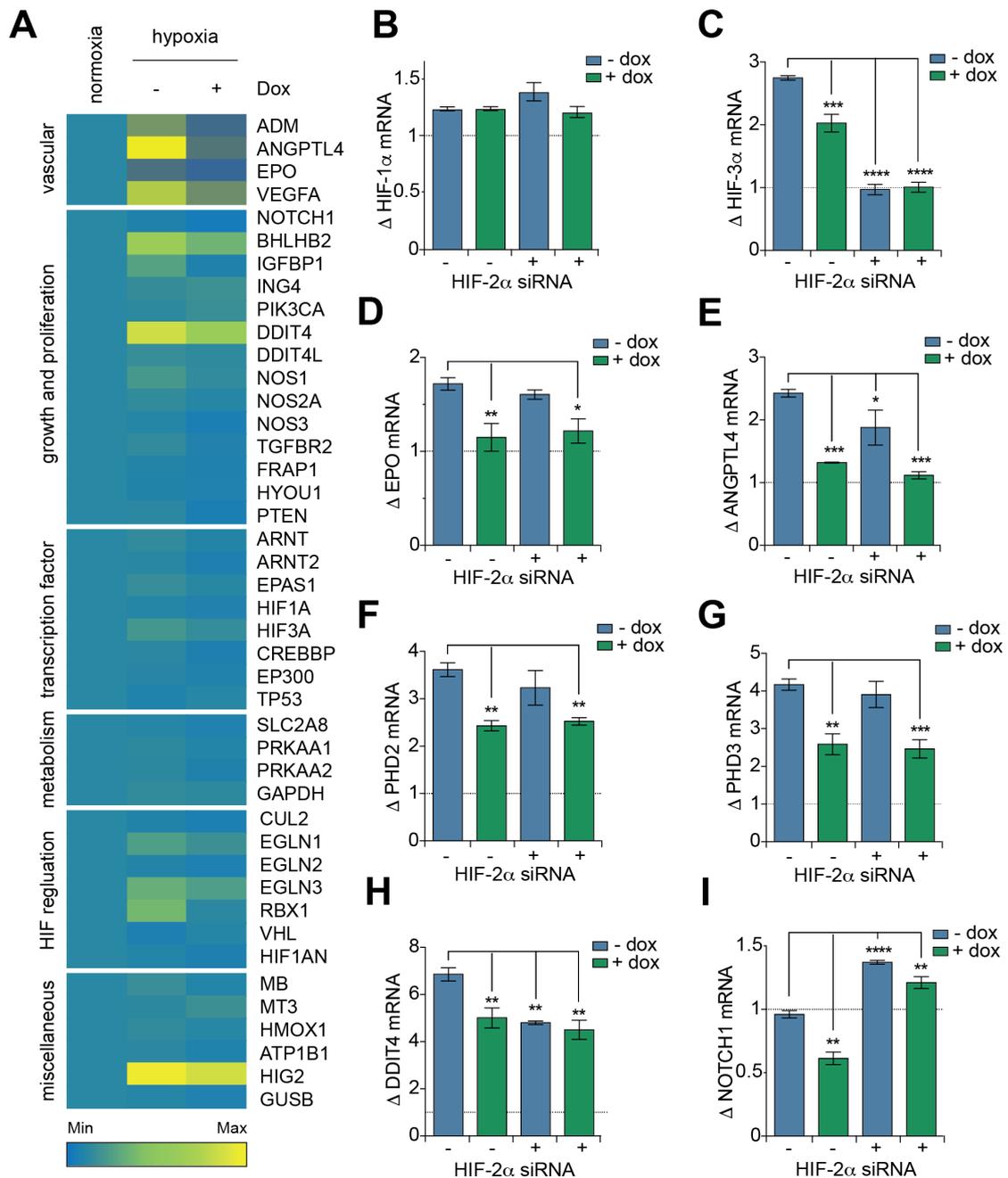
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268   **Figure 3. Expression of genetically encoded P1 alters the transcriptional profile of**

269   **hypoxic HEK-293 cells. (A)** Heat map of relative gene expression in T-REx-HRE cells

270   transfected incubated in normoxia, or 24 h in hypoxia with or without 1 μg/mL dox. Details

271 of genes are given in Table S1. **(B-I)** RT-qPCR analysis of T-REx-HRE cells incubated in

272 normoxia, or 24 h in hypoxia with or without HIF-2α siRNA, and with 1 µg/mL dox with or

273 without HIF-2α siRNA. Fold change in gene expression is shown relative to normoxic

274 expression (dotted line). Data are means (n=3) ± SEM, **p < 0.01, ***p < 0.001, ****p <

275 0.0001.

276

277 <u>Inhibition of HIF-1 dimerization confers synthetic lethality to glycolysis inhibitor</u>

278 The effect on cell viability from the disruption of hypoxia-response signalling via inhibition

279 of the HIF-1α/HIF-1β PPI by P1 was next probed. The viability of T-REx-HRE cells cultured

280 in normoxia or hypoxia, with or without dox was assessed over 72 hours using 3-(4,5-

281 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). No difference was observed

282 in the viability of cells cultured with dox versus those that were not (Figure 4A and Figure

283 S10), indicating that inhibition of the HIF-1α/HIF-1β PPI in hypoxic HEK-293 cells does not

284 affect viability. Nonetheless, previous studies have shown that knockdown of HIF-1α by

285 siRNA sensitizes cells to the glycolysis inhibitor 2-deoxyglucose (2DG), leading to

286 cytotoxicity.[31] We questioned whether inhibition of HIF-1α/HIF-1β PPI with P1 would

287 render HEK-293 cells susceptible to 2DG. This was initially assessed using MTT assays as

288 above. We observed a 37% reduction in viability after 72 hours in hypoxic T-REx-HRE cells

289 incubated with dox (Figure 4B). This observation was further probed with a triplex assay that

290 uses protease biomarkers to assess viability, cytotoxicity and apoptosis.[32] We observed a 45%

291 decrease in the ratio of viable to cytotoxic T-REx-HRE cells after 72 h (Figure 4C), in line

292 with our observations from the MTT assay. Interestingly, no significant increase in caspase-

293 3/7 activity was observed in these cells (Figure 4D), suggesting that the observed synthetic

294 lethality of 2DG combined with P1 is not driven by apoptosis.[33]
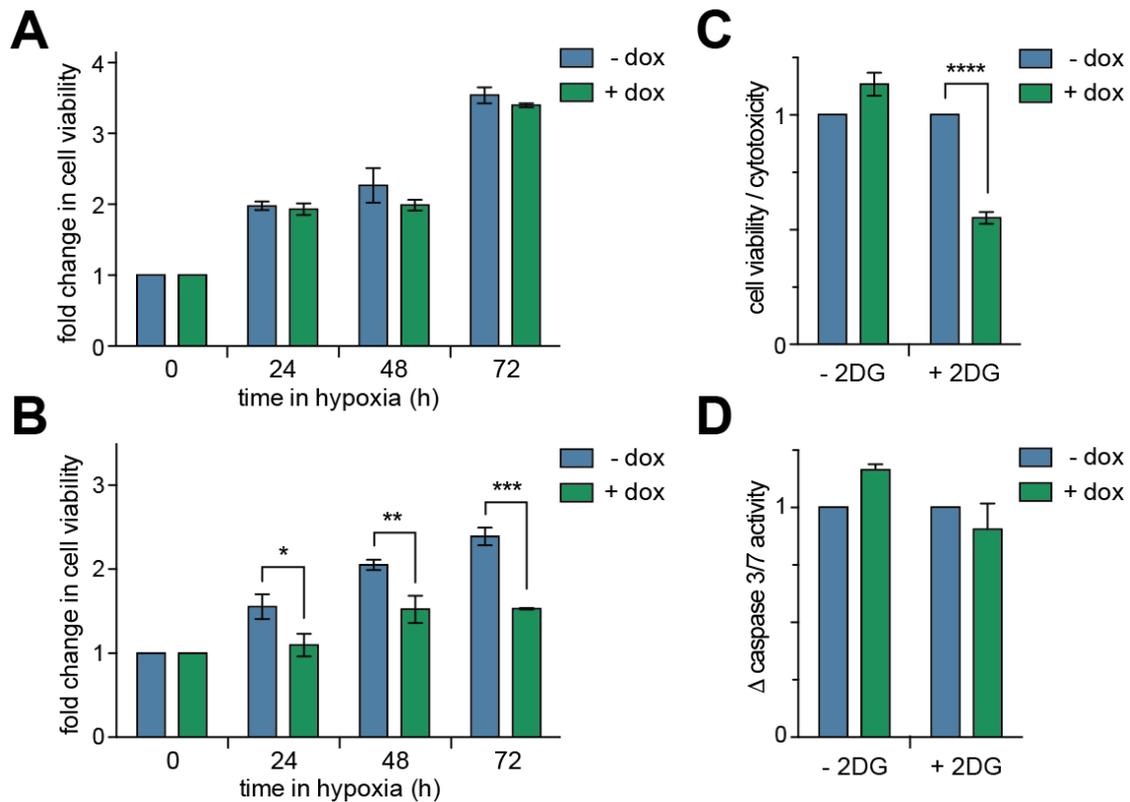
Figure 4. Assessing the effect of HIF-1 inhibition on T-REx-HRE cell viability. (A) Cells were incubated in hypoxia for up to 72 hours with or without 1 µg/mL dox. Cell viability was assessed with an MTT assay. **(B)** Cells were treated as in panel A with or without the addition of 3 mg/mL 2DG to the culture media. **(C)** Cells were treated as in panel B and incubated in hypoxia for 72 h. The ratio of cell viability to cytotoxicity was determined using a triplex reporter assay. **(D)** Cells were treated as in panel C and apoptotic cell death was assessed via a caspase 3/7 activity assay. Data are means (n=3) ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Discussion

The conditional production of P1, an inhibitor of the HIF-1α/HIF-1β PPI, has been encoded onto the chromosome of a human cell line, and demonstrated to be a viable approach for the inhibition of HIF-1 signalling. Two versions of this sentinel circuit are reported, one that initiates P1 production in response to a chemical trigger (doxycycline), and a second that requires an environmental signal (hypoxia) in addition to doxycycline. In addition, the synthetic lethality of HIF-1 dimerization inhibition (by P1) and inhibition of glycolysis (with 2DG) is demonstrated. There is a growing body of evidence that inhibition of HIF-1, or re-direction of cellular pathways away from HIF-1 controlled mechanisms, may improve the anticancer effects of current chemotherapeutic agents.[34-37] Using the T-REx-HRE cell line

11

315 reported here, a variety of chemotherapeutic agents may be screened for synthetic lethality, or

316 increased potency when combined with HIF-1 inhibition.[38, 39]

317 This system has the potential to address several key questions about HIF-1 and its necessity

318 for the survival and growth of tumours. Since its discovery, a large body of evidence has

319 suggested that the HIF-1 transcription factor plays a significant and critical role in cancers,

320 enabling survival and adaptation in the hypoxic tumour microenvironment. However, recent

321 studies have indicated that HIF-1α also functions in the cell as a monomer.[40-42] Approaches

322 that enable disruption of the HIF-1 transcription factor, without reducing the cellular level of

323 HIF-1α have the potential to help decipher the role of HIF-1 in cancer. The sentinel circuit

324 reported above may, for example, be incorporated onto the genome of a variety of cancer cell

325 lines that are used in xenograpft models. P1 production may be initiated at various points

326 during the tumour lifecycle, either globally or only within the hypoxic regions of the tumour,

327 illustrating the significance (or not) of HIF-1 as a target for cancer therapy. Another

328 possibility is the generation of *in vivo* models that contain the sentinel P1-encoding circuit on

329 their chromosome, enabling the effect of long-term HIF-1 inhibition on tumour formation and

330 maintenance to be studied; the dual control promoter limits P1 production to hypoxic cells

331 and allows temporal control over P1 production, enabling the study of HIF-1 inhibition at

332 various stages of the tumour lifecycle.

333 The concept of encoding the conditional production of a non-native small molecule from the

334 genome of engineered human cells has applications beyond the HIF-1α/HIF-1β inhibitor

335 described here. There are a multitude of natural products used as therapeutics that may be

336 hard-coded onto cells via their biosynthetic machinery, and produced as required. The use of

337 sentinel circuits to induce apoptosis in cells in response to specific DNA sequences,[43] or

338 encoding an environment-coupled kill-switch in bacteria[44] attests to the possibilities. Other

339 recent examples include the introduction of new biological components into cells for

340 therapeutic purposes.[45-50] As the field of synthetic biology advances, such examples will grow

341 in number and complexity.

342

343 **Methods**

344 Cell culture

345 All cell culture reagents were purchased from Life Technologies unless otherwise stated. All

346 cells were cultured at 37°C in a humidified 5% CO_2 atmosphere. T-REx-293 cells were

347 maintained in DMEM containing 10% fetal bovine serum (FBS), 100 µg / mL zeocin and 15

348 µg / mL blasticidin and integrated T-REx cell lines were cultured in DMEM containing 10%

349 FBS, 100 µg / mL hygromycin B and 15 µg/ mL blasticidin. Unless otherwise stated, T-REx

350 cells were dosed with 1 µg/ mL doxycycline (dox) to induce expression of integrated

351 constructs. Hypoxia treatment was achieved in a Don Whitley Scientific H35 Hypoxystation

352 with a humidified atmosphere containing 1% O_2 and 5% CO_2. Transfection of plasmids was

353 carried out using FuGENE HD (Promega) according to the manufacturer's instructions and

354 experiments were carried out 24 h after transfection.

355

356 Transfection and selection of stable clones

357 Stable mammalian expression cell lines were generated by Flp recombinase-mediated

358 integration. Flp-In T-REx-293 (T-REx-293) cells and plasmid vectors pcDNA5/FRT/TO and

359 pOG44 (the kind gift of Dr Noel Wortham) and are available commercially from Life

360 Technologies. pOG44 and pcDNA5/FRT vectors were transfected, at a ratio of 9:1, into T-

361 REx-293 cells with Fugene HD for the generation of stable cell lines. Polycolonal selection

362 was carried out with 200 µg/ mL hygromycin in high dilution culture. Integration was

363 confirmed by western immunoblotting.

364

365 Quantitative PCR

366 Total RNA was extracted from cells using ReliaPrep RNA Cell Miniprep System (Promega)

367 and quantified using a Nanodrop ND-1000 spectrophotometer. Complementary cDNA was

368 synthesized in a 20 µL reaction using 1 µg of total RNA with GoScript Reverse Transcriptase

369 (Promega) according to the manufacturer's instructions. Quantitative real-time PCR (RT-

370 qPCR) was performed using Universal Taqman PCR master mix (Applied Biosystems) and

371 the TaqMan gene expression assays of interest (Applied Biosystems) on a CFX-connect 96

372 Real-Time PCR system (Bio-Rad). Expression assays used in this study were: 18S

373 (Hs99999901_s1), ActB (Hs99999903_m1), VEGF (Hs00900055_m1), CAIX

374 (Hs00154208_m1), HIF-1α (Hs00153153_m1) and EPAS1 (Hs01026149_m1). Expression

375 values were expressed as $\Delta\Delta C_T$ normalized to expression of 18S and β-Actin and normoxic

376 gene expression. Hypoxia focused microarray was conducted using TaqMan Array Human

377 Hypoxia plates (Applied Biosystems). Expression values were expressed as $\Delta\Delta C_T$ normalized

378 to expression of 18S and normoxic gene expression.

379

380 Western Immunoblotting

381 For visualisation of expression of CBD tagged inteins, cells were lysed in intein extraction

382 buffer (20mM Tris.HCl, 1 mM TCEP, 0.5 mM NaCl, pH7.8) on ice for 15 minutes. For

383 visualisation of HIF-1α protein, cells were lysed by incubation on ice with

384 radioimmunoprecipitation assay buffer (50 mM Tris (pH 7.4), 150 µM NaCl, 1 mM EDTA,

385 1% v/v Triton X-100), and 1x protease inhibitor cocktail (Sigma) for 20 min.

386 Cell lysates were sonicated in an ice water bath then centrifuged at 10,000 rpm for 20 min at

387 4°C, and the protein concentration in the supernatant quantified by Bradford assay. Proteins

388 were separated on an SDS-PAGE gels (15% for CBD, 10 % for HIF-1α), transferred to

389 PVDF membranes (Invitrogen) and subjected to immunoblot analysis. Mouse monoclonal

390 anti-CBD (E8034S, 1:250, New England Biolabs) anti-HIF-1α (610958, 1:250 BD

391 Biosciences) were diluted in PBS containing 5% non-fat powdered milk and 0.1% Tween-20

392 and incubated with the membrane overnight at 4°C overnight. Horseradish peroxidase

393 conjugated anti-mouse antibody was used as the secondary antibody, and monoclonal anti-

394 βactin-peroxidase antibody (A3854, 1:100,000, Sigma) served as a loading control. Bound

395 immunocomplexes were detected using ECL primes western blot detection reagent

396 (RON2232, GE Healthcare) and analyzed using a ChemiDoc Imaging System (Bio-Rad) and

397 Image Lab 4.0 software (Bio-Rad).

398

399 Detection of peptide in cell lysates by HPLC and MS

400 T-REx-P1 cells were scraped in ice cold PBS and the cell pellet froze in N_2 (l). The pellet was

401 thawed and lysed in PMSF lysis buffer (5 mM EDTA, 2 mM EGTA, 0.4 mM PMSF in PBS)

402 containing protease inhibitor cocktail (sigma) by three freeze thaw cycles. 10 µL of TFA was

403 added to precipitate proteins and the lysate centrifuged (8000 rpm, 30 min, 4°C). The

404 supernatant was passed through a 10 kDa cut off filter and the flow through collected and

405 analysed by reverse phase HPLC on a Waters HPLC system equipped with a Waters Atlantis

406 T3, Amide capped C18 5µm, 6 x 100 mm column. Samples were manually injected into a

407 Waters flex inject system into the HPLC system containing a Waters 1525 binary pump. One-

408 minute fractions were collected in the 5 minute window around the elution time of the

409 synthetic peptide and analysed by LC-MS.

410

411 HRE luciferase reporter assay

412 T-REx-P1, T-REx-Scramble, or T-REx-HRE cells were transiently transfected with a HIF

413 dependent firefly luciferase reporter construct (pGL2-TK-HRE) or a HIF independent firefly

414 luciferase reporter construct (pGL3-SV40) as a control. Transfected cells were incubated in

415 the presence or absence of 1 µg/mL dox. After 24 h, cells were recovered and plated at

416 25,000 cells/ well in 96 well plates and incubated for 5 h before either hypoxic or aerobic

417 incubation for 16 h. Firefly luciferase activity was determined using Bright-Glo Reagent

418 (Promega) according to the manufacture's instructions. Luciferase signal was normalized

419 using the corresponding no-transfection controls for each plate.

420

421 Duolink proximity ligation assay

422   Duolink proximity ligation assay (PLA) was conducted using the in situ PLA Kit (O-Link

423   Bioscience, Uppsala, Sweden) according to the manufacturer's instructions. The antibodies

424   used were rabbit monoclonal anti-HIF-1α (NB100-449, Novus Biologicals) and mouse

425   monoclonal anti-HIF-1β (H00000405- B01P, Abnova). Cells were treated with 1 µg/mL dox

426   in normoxia or hypoxia for 24 after which they were fixed with 2% formaldehyde in PBS for

427   10 min and permeabilized with 0.5% Triton (diluted in PBS) for 10 min. After pre-incubation

428   with the Duolink Blocking Reagent for 1 h, samples were incubated overnight with the

429   primary antibodies to HIF-1α (1:500) and HIF-1β (1:500). Duolink PLA probes and reagents

430   were added as recommended by the manufacture's instructions. Cells were imaged with a

431   fluorescent microscope (Zeiss Axio Vert.A1).

432

433   <u>HIF-2α knockdown</u>

434   T-REx-HRE cells were seeded at 200,00 cells/well on 6-well plates and incubated for 24 h

435   such that cell density reached 50-70% confluence just prior to transfection with siRNA. Cells

436   were transfected with siRNA using Lipofectamine RNAiMAX transfection reagent (Life

437   Technologies) according to the manufacturer's instructions for 'forward transfection'. Briefly,

438   cell culture media was removed from cells and replaced with serum-free OptiMEM cell

439   culture medium (Life Technologies). siRNA and Lipofectamine were separately, diluted in a

440   volume of OptiMEM equivalent to 10% of the final volume of cells, and incubated at RT for

441   5 min. The diluted oligonucleotides and transfection reagent were then combined, mixed

442   gently, and incubated at RT for 10 min. The siRNA-Lipofectamine complexes were added

443   drop wise to cells which were then incubated at 37°C for 24 h. The final concentration of

444   siRNA was 5 nM and the final amount of Lipofectamine was 0.2% v/v for all experiments.

445   Cells were either transfected with EPAS1 (HIF-2α) siRNA (Silencer Select pre-designed

446   annealed human oligonucleotide duplex, s4700, Life Technologies), scrambled siRNA

447   (Silencer Select negative control number 2, Life Technologies) or vehicle alone. Following

448   transfection, cells were incubated in hypoxic or aerobic conditions for 24 h in the presence or

449   absence of 1 µg/mL for 24 h, then harvested for total RNA extraction.

450

451   <u>Cell viability assays</u>

452   T-REx-HRE cells were seeded in triplicate at 5,000 cells per well on 96 well plates 24 h prior

453   to dosing with 1 µg/mL dox in 100 uL fresh DMEM, DMEM without glucose or DMEM

454   containing 3 mg/mL 2-deoxy-glucose. MTT-based cell proliferation assays were performed

455   on untreated cells on the day of treatment or treated cells 24, 48 h or 72 h after treatment, as

456   follows: MTT (Sigma) was prepared in sterile PBS added to cells at a final concentration of 1

457   mM (10% v/v). Cells were then incubated at 37°C for 4 h until intracellular punctate purple

458   precipitated were clearly visible under the microscope. 75 µL of the culture medium was the

459   removed from each well and 100 µL DMSO added. The cells were incubated for 10 min in

460   the dark, with agitation to dissolve the insoluble formazan particles. Absorbance was

461   measured at 570 nm on a microplate reader (Tecan Infinite M200 Pro). ApoToxGlo assays

462   (Promega) were performed on cells 48 or 72 h after treatment with 1 µg/mL dox according to

463   the manufacturer's instructions.

464

465   <u>Statistical Analysis</u>

466   Data analysis was performed with Prism 6 (Graphpad Software). Statistical significance was

467   evaluated with an unpaired *t*-test for comparison between two means and analysis of variance

468   followed by Bonferroni method for multiple comparisons. A value of $P < 0.05$ was considered

469   to denote statistical significance.

470

471   **Supporting information**

472   Supplementary figures 1-10 (Figures S1-S10), and supplementary table 1(Table S1). The data

473   underlying this publication are available from the University of Southampton data repository,

474   or by emailing the corresponding author of this study.

475

479

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631 **TOC graphic**

632