Glycolysis, via NADH-dependent dimerisation of CtBPs, regulates hypoxia-induced expression of CAIX and stem-like breast cancer cell survival

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Abbreviations DMOG: dimethyloxalylglycine, FIH: Factor inhibiting HIF, HIF: hypoxia-inducible factor, MTOB: 2-keto-4-methylthio-2-oxo butyrate, SCLC: stem cell-like cancer.

ABSTRACT

Adaptive responses to hypoxia are mediated by the hypoxia-inducible factor (HIF) family of transcription factors. These responses include the upregulation of glycolysis to maintain ATP production. This also generates acidic metabolites, which require HIF-induced carbonic anhydrase IX (CAIX) for their neutralisation. C-terminal binding proteins (CtBPs) are co-regulators of gene transcription and couple glycolysis with gene transcription due to their regulation by the glycolytic coenzyme NADH. Here we find that experimental manipulation of glycolysis and CtBP function in breast cancer cells through multiple complementary approaches supports a hypothesis whereby the expression of known HIF-inducible genes, and *CAIX* in particular, adapts to available glucose in the microenvironment through a mechanism involving CtBPs. This novel pathway promotes the survival of stem cell-like cancer (SCLC) cells in hypoxia.

INTRODUCTION

Tumour hypoxia is a key element of cancer biology. It correlates with resistance to radiotherapy and chemotherapy and increased metastatic spread. Tumour hypoxia is caused by avascular tumour growth and irregular blood flow, but also by the increased metabolic activity and proliferation of tumour cells or cells present in the tumour microenvironment [1, 2]. It is estimated that more than 40% of breast cancers contain areas of hypoxia or anoxia [3, 4].

Adaptive responses to hypoxia are mediated by the heterodimeric transcription factors hypoxia-inducible factor 1 (HIF-1) and HIF-2. In physiological oxygen conditions, the HIF-α subunit is hydroxylated at conserved proline and asparagine residues by oxygen-dependent prolyl hydroxylases (PHDs) and by factor inhibiting HIF (FIH) [5-7]. This allows for von-Hippel-Lindau protein binding, and subsequent proteasome-mediated degradation [8, 9]. Consequently, under low oxygen pressure, the α subunit accumulates and trans-locates to the nucleus where it binds the β subunit to form an activating complex with the co-activator complex CBP/p300 at the hypoxia-responsive elements of target genes [6, 10-12]. HIF target genes contribute to aggressive tumour phenotypes by promoting increased invasiveness [13], angiogenesis [14], immune evasion [15] and pH regulation [16].

HIF-1 is also an important modulator of tumour cell metabolism as its target genes promote glycolysis by maintaining glycolytic flux required for the synthesis of ATP [17, 18] and metabolites for the synthesis of macromolecules such as nucleic acids [19]. Other HIF-1 targets supress mitochondrial metabolism and the formation of reactive oxygen species [20, 21]. Overall, this response enables tumour cell survival by enabling oxygen-independent energy generation and anabolic metabolism required by highly proliferating cells. Increased HIF-1 activity and the consequent driven changes in metabolism are therefore a major contributor to aggressive tumour growth, and the expression in tumours of increased levels of HIF-1α, or the

products of its key target genes relevant to glycolytic metabolism, such as GLUT1 and CAIX, are known markers of poor patient survival [22, 23].

C-terminal binding proteins (CtBP1 and CtBP2) (CtBPs) are transcription regulators whose activity is directly regulated in response to changing concentrations of a key glycolytic metabolite (reviewed in [24, 25]). CtBPs are highly homologous proteins with a central "dehydrogenase" domain that binds the glycolytic coenzyme NAD(H). This binding results in changes in CtBP dimerisation, conformation and binding to transcription factors, histone modifying enzymes and other co-factors of transcription that contain a PXDLS consensus CtBP-binding motif [26, 27]. Depending on their interaction partners, CtBPs can act as co-repressors [26-32] or co-activators of transcription [33-37]. This, and their higher affinity for NADH over NAD^+, means that they function as regulators of transcription in response to altered rates of glycolytic flux [38]. Glycolysis and CtBP-dependent transcriptional regulation impacts on multiple cellular phenotypes involved in cancer development, including epithelial-to-mesenchymal transition and hypoxia-induced cell migration [39-41], metastasis [42], resistance to cell death [41], suppression of DNA repair [40, 43, 44], maintenance of the fidelity of mitosis [45, 46], resistance to cell cycle inhibition [47], p53-dependent stress responses [48] and the acquisition of stem cell-like phenotypes [35, 40, 49]. Elevated CtBP expression is also associated with poor prognosis in a wide range of solid tumours, including breast cancer [40].

The magnitude of the oxygen-regulated HIF response is also modulated by metabolites in addition to oxygen, that control the activity of the PHD enzymes [50, 51]. Indeed glycolysis itself can promote HIF-1 activity in an apparent feed-forward mechanism that may help match the magnitude of the HIF response with the availability of glucose and its metabolites in the hypoxic microenvironment [50]. However, less well understood is whether glycolysis may affect the expression of known HIF-inducible genes, to fine-tune the hypoxic response to meet the demands of the metabolic microenvironment. Here, therefore, we have addressed this

question using breast cancer cell models, focussing on CtBPs as potential regulators of such a pathway, and *CAIX* [52] as a known key HIF-response gene that is essential for the maintenance of pH homeostasis in highly glycolytic cells.

MATERIALS AND METHODS

Cell culture, and reagents.

Breast cancer cell lines were cultured at 37 °C with 10% CO_2 in high glucose (25 mM) DMEM media (Gibco #41966-029), 10% foetal calf serum (GE healthcare, Austria), 1% Penicillin/ Streptomycin (Sigma-Aldrich, U.K). Cells were originally obtained from ATCC and re-validated by STR profiling (ECACC) and mycoplasma testing (in house nested PCR of rRNA genes). Adaptation to culture in media containing 10 mM fructose to restrict glycolysis (and matched controls in 25 mM glucose) used DMEM (#D5030 Sigma-Aldrich, U.K) as previously described [53]. Hypoxia was induced using a hypoxia chamber (Billups-Rothenberg, USA) with an atmosphere of 1% O_2, 5% CO_2 and 94% N, or, for experiments using MCF-7 $^{IND}CtBP^{DD}$ cells, 3% O_2, 5% CO_2 and 92% N (O_2/CO_2 incubator MCO-5M-PE, Panasonic Healthcare Co. Ltd, Japan). 2-deoxyglucose (2-DG), Dimethyloxalylglycine (DMOG) and 2-keto-4-methylthio-2-oxo butyrate (MTOB) were from Sigma-Aldrich, UK). siRNA (Ambion, Applied Biosystems, UK) targeting both CtBP1 and CtBP2 (CTBPsi) [54], *HIF1A* (S6541) and silencer negative control #1 (NSsi) were transfected using INTERFERin (PolyplusTransfection, France). his-CtBP2 and his-$CtBP2^{G189A}$ constructs [55] were transfected into cells using FugeneHD (Promega, UK), and single colonies expanded under G418 selection. To generate the $^{IND}CtBP^{DD}$ and $^{IND}CtBP^{DDM}$ vector, $CtBP^{DD}$ (CtBP2[104-361]) and $CtBP^{DDM}$ (CtBP2[104-361]R147L,R169L) [46] were cloned into pENTR4-V5 (Addgene #17425) and then pLentiCMVtighthygroDEST (Addgene #26433) [56], which was co-transfected into HEK-T cells with pCMVdR8.91 and pMD2.G packaging vectors to generate lentiviral particles. The

generated lentiviral particles were transduced into MCF-7, which had previously been transduced with particles generated using pLentiCMVrtTA3 Blast (Addgene #26429) controller vector. Transduced cells were selected with blasticidin (10 μg/mL) and hygromycin (200 μg/mL). Doxycycline was from Clontech, USA.

Analysis of mRNA and protein.

Western blotting, was performed as described in [54]. Blots showing the effects of glycolysis or CtBPs on CAIX protein are representatives of three independent experiments. The following primary antibodies were applied to detect protein expression: mouse anti-human CtBP1 (clone E12) (Santa Cruz Biotechnology, UK), mouse anti-human CtBP2 (#612044) (BD Transduction Laboratories, USA), goat anti-human CtBP2 (E16; required for detection of C-terminally his-tagged CtBP2) (Santa Cruz Biotechnology, USA), goat anti-human CAIX (AF2188) (R&D systems, UK), mouse anti-human V5 tag (clone E10/V4RR) (Thermo Fisher, UK) and rabbit-anti human actin (A5060) (Sigma-Aldrich, U.K). Bands were quantified using ImageJ, background subtracted, and normalised to actin; CAIX abundance following hypoxia or DMOG was set as 100%, and abundance following further manipulation of glycolysis or CtBPs expressed relative to this.

RNA from frozen cell pellets was extracted using the ReliaPrep™ RNA Cell Miniprep System kit (Promega, USA), following the manufacturer's instructions. cDNA was synthesised from RNA concentrations ranging between 250-1000 ng. MMLV reverse transcriptase, oligo dT15 primer and dNTPs were all from Promega, USA. Reverse transcription was performed at 37 °C for 1 h, followed by 5 min at 94 °C. *CAIX* mRNA expression was determined by TaqMan® Reverse Transcription-qPCR, using 5 ng cDNA per reaction (all reagents: Applied Biosystems). Assays were from Applied Biosystems (*CAIX*: Hs00154208_m1, *ACTB*: 4326315E) or Roche Human Universal probe library (Roche, Germany (*PDK1*:

CACCAGACCTCGTGTTGAG/ACGTGATATGGGCAATCCA/#20, *EGLN3*: ATCGACAGGCTGGTCCTCTA/GATAGCAAGCCACCATTGC/#61, *INSIG2*: TTCCTCTATGTTCGTTCTTGGT/TTTCTGCGATAACTTTACATTCG/#67, *FAM162A*: TTCATGGTTATTGAGGGCAAG/GTTCAAGCTTGTTAAAGTCTCGT/#44, *TMEM45A*: GGAGCTATTGCGGTCCAAGTC/GGGGATACAGGACAAATCCA/#24,). Data were analysed using the comparative C_T method relative to *ACTB*. For all experiments, graphs show result in the absence of either hypoxia or DMOG in light orange, and following hypoxia or DMOG in dark blue, with fills of the bars further indicative of the treatments shown on the graph axis.

Mammosphere forming assays.

2000 cells were seeded in 100 µl DMEM:F12-based mammosphere media (supplemented with 20 ng/mL recombinant human EGF, 20 ng/mL recombinant human basic FGF, B27 supplement, 0.4% FCS, penicillin-streptomycin, L-glutamine (all from ThermoFisher Scientific, UK) and 5 µg/mL bovine insulin (Sigma-Aldrich, UK)) on polyhema coated low attachment 96-well plates. After 8-10 days mammospheres were measured by alamarBlue. (ThermoFisher Scientific, UK). Mammosphere culture was under normoxic conditions, with no doxycycline added to the media.

Statistical analysis

GraphPad Prism version 6.01 (Graphpad, La Jolla, CA, USA) was used for statistical analysis. For RT-qPCR, statistical significance was calculated using one-way ANOVA and Fisher's LSD test. Unless stated otherwise, all quantitative data are n=3 biological replicates, for qPCR, each of these being derived from technical duplicates, error bars = SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

RESULTS

Regulation of the expression of known hypoxia and HIF-inducible genes by glycolysis

In order establish a model system to examine the effect of glycolysis on the induction of hypoxia-responsive genes, we first performed a time course analysis for the induction of *CAIX* mRNA in three breast cancer cell lines following transfer to a 1% oxygen hypoxic culture environment (Figure 1A). In two of the lines, MCF-7 and MDA-MB-231, reproducible significant induction was observed, and these were selected for further analysis. Induction was maximal after 17 hours and was consistently of substantially greater magnitude in MCF-7 than MDA-MB-231. To establish a role of HIF-1α in this induction of *CAIX*, we transfected MCF-7 cells with *HIF1A* siRNA prior to the hypoxic insult (Figure 1B). The siRNA caused in a significant, albeit incomplete, knockdown of the *HIF1A* transcript, and a significant, 27%, reduction in the abundance on *CAIX* mRNA in hypoxic cells. DMOG is a cell permeable inhibitor of 2-oxoglutarate-dependent diogenases including prolyl-4-hydoxylases, and thus prevents HIF-1α from being targeted for VHL-dependent ubiquitination and degradation in the presence of oxygen [57, 58]. The DMOG- induced expression of *CAIX* was similarly muted by *HIF1A* siRNA. Thus, whilst both hypoxia and DMOG may potentially have effects on gene transcription independently of HIF-1α through effects on other targets, an equivalent proportion of the effects of both interventions on *CAIX* expression is HIF1α-dependent. We cannot determine from these experiments the extent to which HIF-1α-independent pathways may be involved, as the knockdown of HIF1A by the siRNA was incomplete. However prior studies which included the use of *HIF1A* knockout cells have demonstrated a high degree of dependence of *CAIX* expression on HIF-1α, and, unlike most other known HIF-inducible genes, *CAIX* is not effectively induced by HIF-2α [59-61].

We next set out to determine the effect on hypoxia-induced gene expression of partially inhibiting glycolysis at the point of exposure to low oxygen, mimicking an acute and simultaneous reduction loss of blood-derived oxygen and glucose due to restriction of blood supply to a tumour. This was achieved by the addition of 10 mM 2-DG, a competitive inhibitor of hexokinase II [62], to the 25 mM glucose-containing culture medium (Figure 1C). In MCF-7 cells, hypoxia induced a 30.6 fold induction of *CAIX* mRNA compared to untreated controls, whereas in the presence of 2-DG, levels of *CAIX* under hypoxia were significantly 63.7% lower than when glycolysis was not inhibited. This effect of 2-DG was selective for *CAIX*, as hypoxia-induced increases of *PDK1*, *EGLN3*, *FAM162A*, *TMEM45A* or *INSIG2* mRNAs were not inhibited by 2-DG, and indeed *PDK1* induction was significantly increased. In the MDA-MB-231 cell line the magnitude of *CAIX* induction by hypoxia was less than that in MCF-7 (12.3 fold) and its abundance under hypoxia was not decreased by 2-DG. 2-DG did enhance the induction of *PDK1* by hypoxia in MDA-MB-231, and the other mRNAs studied were either not affected, or increased.

As an alternative approach for the suppression of glycolysis in the breast cancer cell lines we have recently described a model in which cells are adapted to culture in media containing 10 mM fructose, rather than 25 mM glucose. Cells uptake and retain fructose less effectively than they do glucose, and therefore, whilst fructose enters the glycolytic pathway and enables the proliferation of adapted cells at comparable rates to the glucose-cultured counterparts, their rates of glycolysis are markedly reduced [53]. Again, in MCF-7 cells, restriction of glycolysis resulted in a substantial reduction in the hypoxia-induced increase in *CAIX* mRNA (Figure 1D). In this model, the induction of all the other hypoxia-inducible genes was also decreased in the less glycolytic cells; this difference compared to the 2-DG model may either be due to the different methods used to supress glycolysis, or the fact that the 2-DG was only added at the

point when hypoxia was induced, whereas the fructose-cultured cells had adapted to reduced glycolysis over a period of > 30 days.

As hypoxia-induced *CAIX* mRNA expression was attenuated by inhibiting glycolysis in MCF-7 in both models, we also examined the effects of glycolysis on CAIX protein expression under hypoxia. In MCF-7 cells, the changes in CAIX protein essentially mirrored those of the mRNA in both the 2-DG (Figure 1Ei) and fructose (Figure 1Eii) models. In MDA-MB-231 cells, 2-DG did reduce hypoxia-induced CAIX protein induction, despite no effect on the mRNA having been observed. This suggests that CAIX protein abundance in these cells may also be regulated though another mechanism, such as post-transcriptional control.

To determine whether these observed effects of glycolysis on hypoxia-induced expression of *CAIX* and other known HIF-targets in the MCF-7 model was dependent on hypoxia *per se*, we used DMOG as an alternative intervention that induces a response that includes increased cellular HIF activity.. In MCF-7 cells, DMOG caused a 211 fold increase in the abundance of *CAIX* mRNA, whereas in the presence of 2-DG, levels of *CAIX* following DMOG-treatment were significantly 56.7% lower than when glycolysis was not inhibited (Figure 2A). Of the other genes examined, the DMOG-induced upregulation of *EGLN3*, *FAM162A*, and *TMEM45A* mRNAs were also inhibited by 2-DG, and the remainder were unaffected. In the fructose model of glycolytic restriction, DMOG-induced *CAIX* mRNA expression was also repressed in the less glycolytic cells, as was the expression of *PDK1*, *EGLN3*, and *TMEM45A* (Figure 2B). Western blotting confirmed that DMOG induces an increase in CAIX protein expression, and that this increase is supressed by inhibiting glycolysis in both the 2-DG (Figure 2Ci) and fructose (Figure 2Cii) models.

Regulation of the expression of known hypoxia and HIF-inducible genes by CtBPs

Having established that glycolysis is required for effective induction of *CAIX* mRNA and protein in the MCF-7 model system, we set out to determine the role of CtBPs in the response. Firstly, we silenced CtBP1 and CtBP2 expression using an siRNA that targets the mRNA of both genes, and found that, similarly to the effects of glycolytic restriction, this resulted in a significant 47.0% decrease in the abundance of *CAIX* mRNA in hypoxic MCF-7 cells (Figure 3A). The expression of *PDK1* and *INSIG2* mRNAs were also reduced. MTOB is a metabolite in the methionine salvage pathway that is known to bind CtBPs and inhibit their ability to regulate gene expression [63]. Treatment of MCF-7 cells with MTOB resulted in an 87.8% reduction in the abundance of *CAIX* mRNA in hypoxic MCF-7 cells (Figure 3B). Compared to CtBP1/2 siRNA, MTOB was less selective in its effects on *CAIX*, as it reduced the induction of all the known HIF-target genes examined, although the effect was greatest on *CAIX*.

To complement the CtBP inhibition experiments, we also examined the effect of CtBP overexpression on the induction of known HIF-inducible genes. CtBP2 was used for these studies as it contains a nuclear localisation sequence whereas CtBP1 does not [64]. In glycolytic MCF-7 cells cultured in standard 25 mM glucose-containing media and exposed to hypoxia, the expression of *CAIX* mRNA in cells overexpressing CtBP2 was 213% higher than in cells containing the empty vector (Figure 3Ci). A mutated version of CtBP2 ($CtBP2^{G189A}$) that is compromised in its ability to bind NADH, showed an intermediate effect between vector control and wild-type CtBP overexpression. Of the other known HIF-inducible genes studied, there was a trend for CtBP2 overexpression to modestly increase their expression in hypoxic cells, though for wild-type CtBP2 this was only significant for *TMEM45A*. In the cells in which glycolysis was restricted with culture in fructose, the overall expression of known HIF-inducible genes was reduced compared to glycolytic cells (Figure 3Cii) as shown previously, but again there was a clear positive effect of wild-type CtBP2 on the expression of *CAIX* mRNA,

as well as mRNAs for *EGLN3*, *FAM162A*, *TMEM45A* and *INSIG2*. Notably, the effect of the G189A mutation on this activity of CtBP2 was more pronounced in these less glycolytic cells.

Western blotting for CAIX protein confirmed that the effects of CtBPs on *CAIX* mRNA abundance also resulted in concomitant changes in the protein in the siRNA experiments (Figure 3Di) and MTOB experiments (Figure 3Dii). CtBP2 overexpression only resulted in a modest increase in CAIX protein (Figure 3Diii), suggesting that the enhanced increase in *CAIX* mRNA was not fully translated into increased protein synthesis. Immunoblotting for CtBPs confirmed the expected effects of the siRNA and overexpression plasmids on CtBP protein abundance in these experiments.

We then performed a comparable series of experiments using DMOG, as opposed to hypoxia, and again examined the role of CtBPs in the transcriptional response to this intervention. In the presence of DMOG, CtBP1/2 siRNA significantly reduced the abundance of *CAIX* mRNA by 29% (Figure 4A). *PDK1* and *EGLN3* mRNA were also supressed, whereas *TMEM45A* expression was upregulated. Similarly to the hypoxia experiments, MTOB also caused a large 82.6% reduction in *CAIX* mRNA abundance in DMOG-treated cells (Figure 4B), and the expression of all the other known HIF-inducible genes was significantly negatively affected. CtBP2-overexpressing cells cultured in high glucose media and DMOG showed 355% higher expression of *CAIX* mRNA than vector control cells (Figure 4Ci), and again the G189A mutant exhibited intermediate levels of expression. CtBP2 overexpression also enhanced the expression of the majority of the other known HIF-inducible genes analysed, albeit to a lesser extent in most cases. In the less glycolytic cells cultured in fructose-containing medium, the overexpression of CtBP2 has no effect on DMOG-induced *CAIX* mRNA expression, and limited effects on the other known HIF-inducible genes (Figure 4Cii).

Western blotting experiments demonstrated CtBP1/2 siRNA reduced CAIX protein expression in DMOG-treated cells (Figure 4Di), as does MTOB treatment (Figure 4Dii), whereas CtBP2 overexpression increases it (Figure 4Diii).

Effect of CtBP dimerisation on hypoxia-induced expression of CAIX, and the abundance of stem cell-like cancer cells

Sustained hypoxia over several days can increase the occurrence of stem cell-like cancer (SCLC) cells in a HIF-dependent manner [65], and furthermore the induction of CAIX appears to be an important determinant of this effect [66]. We therefore wished to determine the role of CtBPs in SCLC cells under hypoxia. Furthermore, given the evidence using the $CtBP2^{G189A}$ mutant which suggested a role for NADH-dependent CtBP dimerisation in the regulation of CAIX expression, a function that is regulated by glycolysis, we specifically set out to further test this aspect of CtBP regulation in the control of CAIX expression and SCLC cell biology. We have previously developed an approach of microinjection of fragments of CtBP proteins to act as dominant negative inhibitors of specific functions [45, 46], including $CtBP^{DD}$, which encompasses the dehydrogenase/dimerisation domain and thus competes for dimerisation of endogenous CtBP dimers [46]. We therefore constructed an inducible system where the expression of V5-tagged $CtBP^{DD}$ could be switched on by doxycycline in stably transduced MCF-7 cells. A control system, $CtBP^{DDM}$, which carries two point mutations in the dimerisation domain that inhibits dimerisation [46, 67] and thus renders $CtBP^{DD}$ ineffective, was also generated. Figure 5A shows, the effects of inhibition of CtBP dimerisation after 18 hours of mild hypoxia (3% $O_2 \cong 3$ kPa) that are commonly observed in tissues [68]. Induction of $CtBP^{DD}$ by doxycycline resulted in a significant, up to 56.1%, inhibition of *CAIX* expression in hypoxic cells, having a comparable effect to 2-DG. $CtBP^{DDM}$ had no effect, demonstrating that the effect was due to the specific effects of $CtBP^{DD}$ on CtBP dimerisation, and none of the

other known HIF-inducible genes were significantly downregulated by CtBP^{DD}, demonstrating that the effect is selective to *CAIX*. Figure 5B shows induction of the CtBP^{DD} and CtBP^{DDM} proteins, and Figure 5C shows that the effects on *CAIX* mRNA is reflected in the abundance of CAIX protein. We then performed mammosphere-forming assays [69] to assess the prevalence of SCLC cells in the population. These experiments were performed after only 24 h induction of CtBP^{DD} followed by 18 h by hypoxia to limit the possibility of any observed effects being to secondary effects of CtBP inhibition. Compared to the previously published reports using ~72 h hypoxia [65, 66], under these short-term assay conditions there was no significant enrichment of SCLC cells in the population by hypoxia (Figure 5D). The inactive control CtBP^{DDM} had no negative effect on mammosphere-formation when induced by doxycycline under any conditions, and likewise the CtBP dimerisation inhibitor CtBP^{DD} had no effect when induced in normoxic cells. However, in hypoxic cells, after addition of the inducing agent, CtBP^{DD}-expressing cells demonstrated a significant, 29.7%, reduction of mammosphere-forming potential compared to non-induced cells. Thus, within the relatively short time they were exposed to mild hypoxia, at least a proportion of the SCLC cells in the population had acquired a dependency on CtBP dimerisation for their survival. These data are therefore consistent with a role of CtBP dimerisation in controlling the expression of HIF-target genes such as *CAIX*, that are required for the selective survival of SCLC cells in a hypoxic microenvironment.

DISCUSSION

Here we have focused on the effects of glycolysis, and the CtBP family of glycolysis-sensing transcriptional regulators, on the hypoxia-induced expression of *CAIX* and other known HIF-inducible genes. Through HIFs, hypoxia induces a broad-ranging transcriptional response to adapt cells to their altered microenvironment, including the upregulation of genes associated with glycolysis to facilitate continued cell growth in the absence of oxygen [17-19]. However,

in a microenvironment such as a tumour, which may have both an underdeveloped blood supply, and contain large numbers of highly metabolic cells, glucose availability can also be reduced [70, 71], potentially limiting the cells ability to increase rates of glycolytic flux. Under these circumstances a modified response may be required, for example this increased expression of proteins such as *CAIX*, which are required in highly glycolytic cells [52], would be an unnecessary waste of metabolic resource. Our experiments using 2-DG to inhibit glycolysis in breast cancer cell lines support this hypothesis, with the data from MCF-7 demonstrating that this is, at least in part, controlled through expression of the mRNA. This response was selective for *CAIX*, demonstrating it is not through global regulation of HIF activity. These findings agree with the prior work of Natsuizaka *et al* who studied the effect of short term low glucose on the hypoxia response in a panel of cancer cell lines and found it to enhance the induction of some genes, whilst the induction of others, notably *CAIX*, was supressed [72]. In a separate study, glucose deprivation also abolished the induction of CAIX protein by hypoxia in fibrosarcoma and pharyngeal carcinoma models [73]. Our model of chronic adaptation to reduced glycolysis differs from acute inhibition of glycolysis in both our 2-DG experiments and the above published studies in that the cells extensively reprogram their gene expression [53] prior to the hypoxic insult. In this model the hypoxic response of all the genes was reduced somewhat, but the effect on *CAIX* was markedly the most substantial. Both acute and chronic inhibition of glycolysis also supresses the induction of *CAIX* mRNA and protein when cells were exposed to DMOG, demonstrating that the mechanism involved is not explicitly dependent upon the cells being hypoxic.

None of the studies cited above provide a mechanism for the effects of glycolysis and *CAIX* expression. In order to test whether CtBPs are required for the glycolysis-dependent regulation of *CAIX*, we applied different approaches to alter CtBP expression and function. We utilised siRNAs and CtBP2 overexpression to alter CtBP expression levels. Furthermore, NADH-

dependent CtBP function was more selectively inhibited by the pharmacological CtBP inhibitor MTOB and an inducible CtBP dimerisation inhibitor mini-protein. We found that in hypoxic MCF-7 as well as in DMOG treated MCF-7, both downregulated total CtBP protein expression as well as impaired NADH-dependent CtBP function caused a reduction in hypoxia-induced *CAIX* expression, whereas CtBP2 overexpression increased *CAIX* gene and protein expression in a manner dependent upon its ability to bind NADH. These results strongly support a glycolysis-dependent regulation of *CAIX* gene and protein expression that is dependent on CtBPs.

Induction of *CAIX* expression is strictly regulated by HIFs and SP1 but can be modulated by MAPK and PI3K signalling in a cell type-specific manner [59, 74, 75]. The impact of pathways such as this which modulate the HIF response are clearly demonstrated by immunohistochemical studies of protein expression in tumour samples, where the expression of CAIX, whilst clinically significant in terms of predicting patient outcome, may only weakly correlate with the expression of HIF proteins, and does not correlate with the products of other HIF-responsive genes [76]. Our demonstration of a glycolysis-NADH-CtBP dependent pathway in the regulation of the hypoxia-induced*CAIX* expression thus advances the understanding of the mechanisms underlying important clinical observations such as these. As CtBPs are recruited to promoters by DNA-binding transcriptional regulators, our data support a model whereby HIFs cooperate on gene promoters with transcription factors that are interaction partners of CtBPs to render some HIF target genes responsive to glycolysis. However validation of this model will require further in-depth mechanistic analysis and the exclusion of other possible explanations, which could include an indirect effect of CtBPs that replicates the effect of glycolysis, rather than transducing it, or an effect on HIF subunits themselves that result in a promoter-selective effect. Supporting a direct effect of CtBPs on hypoxia-responsive promoters, a recent study [77] reanalysed the ChIP-seq and gene

expression data of Di *et al* [40] in CtBP-manipulated MCF-7 cells to identify genes whose expression were both regulated by CtBPs and had CtBPs associated with their promoters, and, using gene ontogeny analysis found the strongest enriched biological pathway to be “response to hypoxia”. Overall therefore, our study may support a shift of paradigm away from CtBPs solely acting as co-repressors of transcription towards a context-dependent co-regulator of transcription, as there is a growing body of literature highlighting the activating role of CtBPs in the co-regulation of their target genes [33-37].

It is known that CtBPs promote the formation of SCLC cells [35, 40, 49] and CAIX has also been clearly demonstrated to be an important marker of cells with a stem cell-like phenotype [66, 78]. In this light, we investigated the role of glycolysis-CtBP-NADH axis in the formation of SCLC cells in MCF-7 breast cancer cells. We found that inhibition of CtBP dimerisation using the inducible mini-protein reduced the mammosphere forming potential in hypoxia but not in normoxia. These data are consistent with our recent work that metformin, a pharmacological inhibitor of complex I of the mitochondrial electron transport chain, sensitised breast SCLC cells to CtBP inhibition [49]. The data we have shown here, combined with the findings from Ledakie *et al* whose work also focussed on the MCF-7 cell system [66], support a model whereby the regulation of *CAIX* expression by CtBPs may be a key determinant of this effect in hypoxic cells.

In conclusion, we have shown for the first time that CtBPs are a functional link between hypoxia-induced glycolysis and hypoxia-induced expression of the HIF target gene *CAIX*. It will be of great interest to dissect the mechanisms underlying this link, such as any potential functional or physical interactions between HIF-1, CtBPs and other transcriptional regulators at the *CAIX* promoter. Additionally, this work strengthens the understanding of CtBPs as an important oncogene in epithelial cancers and supports the rationale for the further development of CtBPs as potential drug targets for cancer therapy [46, 79, 80].

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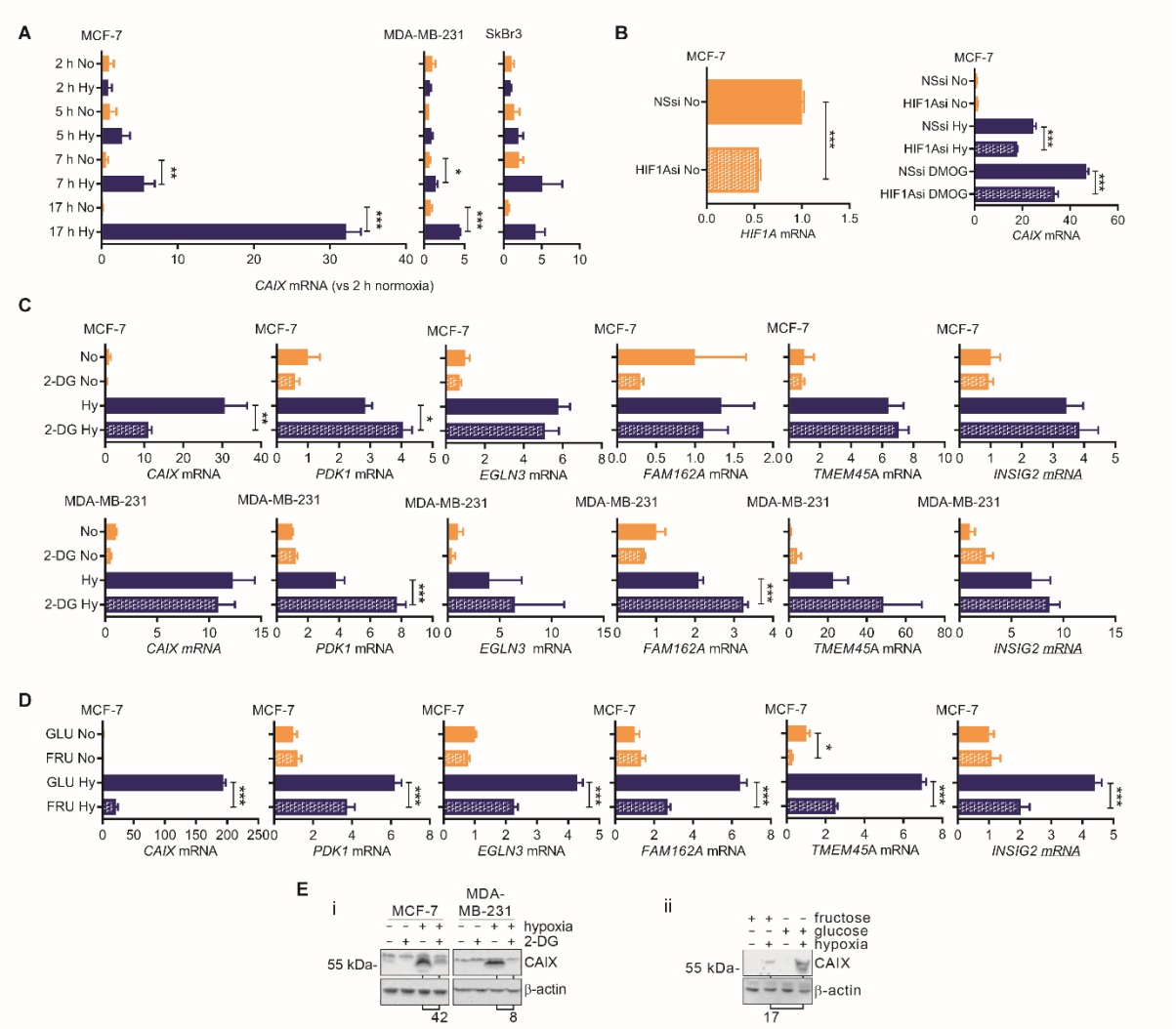

Figure 1: Hypoxia-induced upregulation of HIF-1 responsive genes is influenced by glycolysis. A: *CAIX* mRNA expression was determined in three breast cancer cell lines, following the indicated time in culture in either normoxia (No) or 1% oxygen (hypoxia, Hy). Data are mean ± SD of n=2 biological replicates. B: MCF-7 cells were transfected with non-silencing or *HIF1A* siRNA and 48 later exposed to either normoxia, hypoxia or 1 mM DMOG for 18 h prior to the analysis of *HIF1A* and *CAIX* mRNA expression. C**:** MCF-7 and MDA-MB-231 cells were cultured in either normoxia or hypoxia without/with 10 mM 2-DG for 18 h prior to the analysis of HIF target gene mRNA abundance. **D:** MCF7 adapted to grow in either 10 mM fructose or 25 mM glucose were cultured in either normoxia or hypoxia for 18 h prior to the analysis of HIF target gene mRNA abundance. **E**: Representative western blots demonstrating the effects of restricting glycolysis on hypoxia-induced CAIX protein expression. (i) Experimental conditions as in (C). (ii) Experimental conditions as in (D).

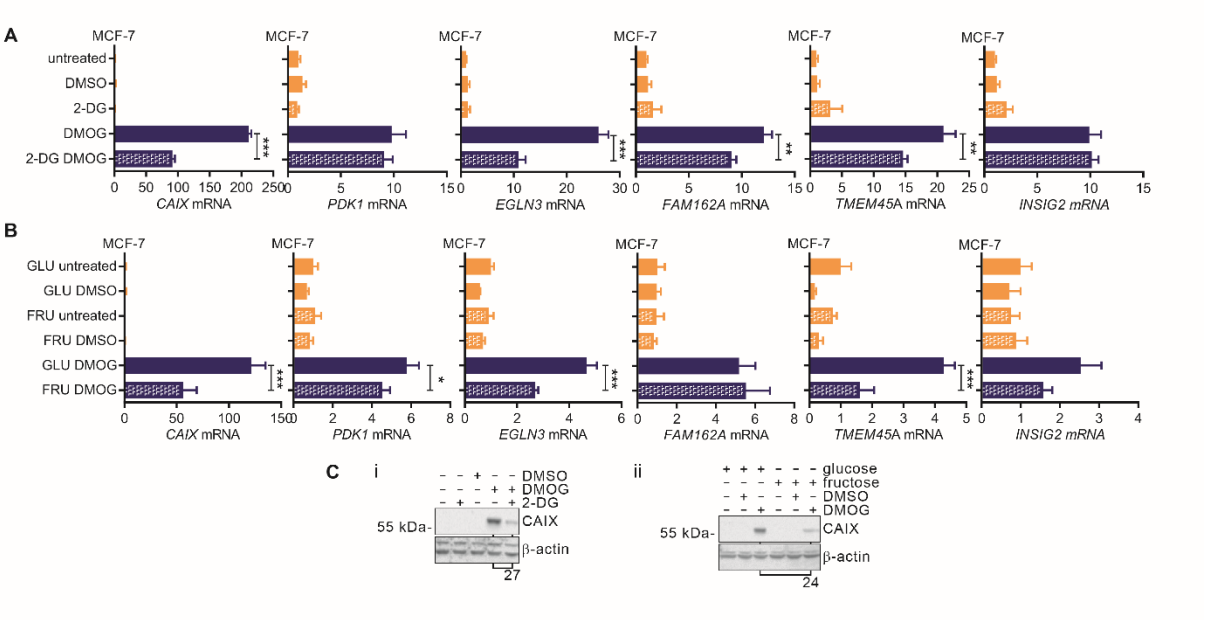

Figure 2: Upregulation of HIF-1 responsive genes by the HIF-activating agent DMOG is influenced by glycolysis. A: MCF-7 cells were cultured in either DMSO carrier or 1 mM DMOG and without/with 10 mM 2-DG for 18 h prior to the analysis of HIF target gene mRNA abundance. **B:** MCF7 adapted to grow in either 25 mM glucose or 10 mM fructose were cultured in either DMSO carrier or 1 mM DMOG and without/with 10 mM 2-DG for 18 h prior to the analysis of HIF target gene mRNA abundance. **C**: Representative western blots demonstrating the effects of restricting glycolysis on DMOG-induced CAIX protein expression. (i) Experimental conditions as in (A), (ii) Experimental conditions as in (B).

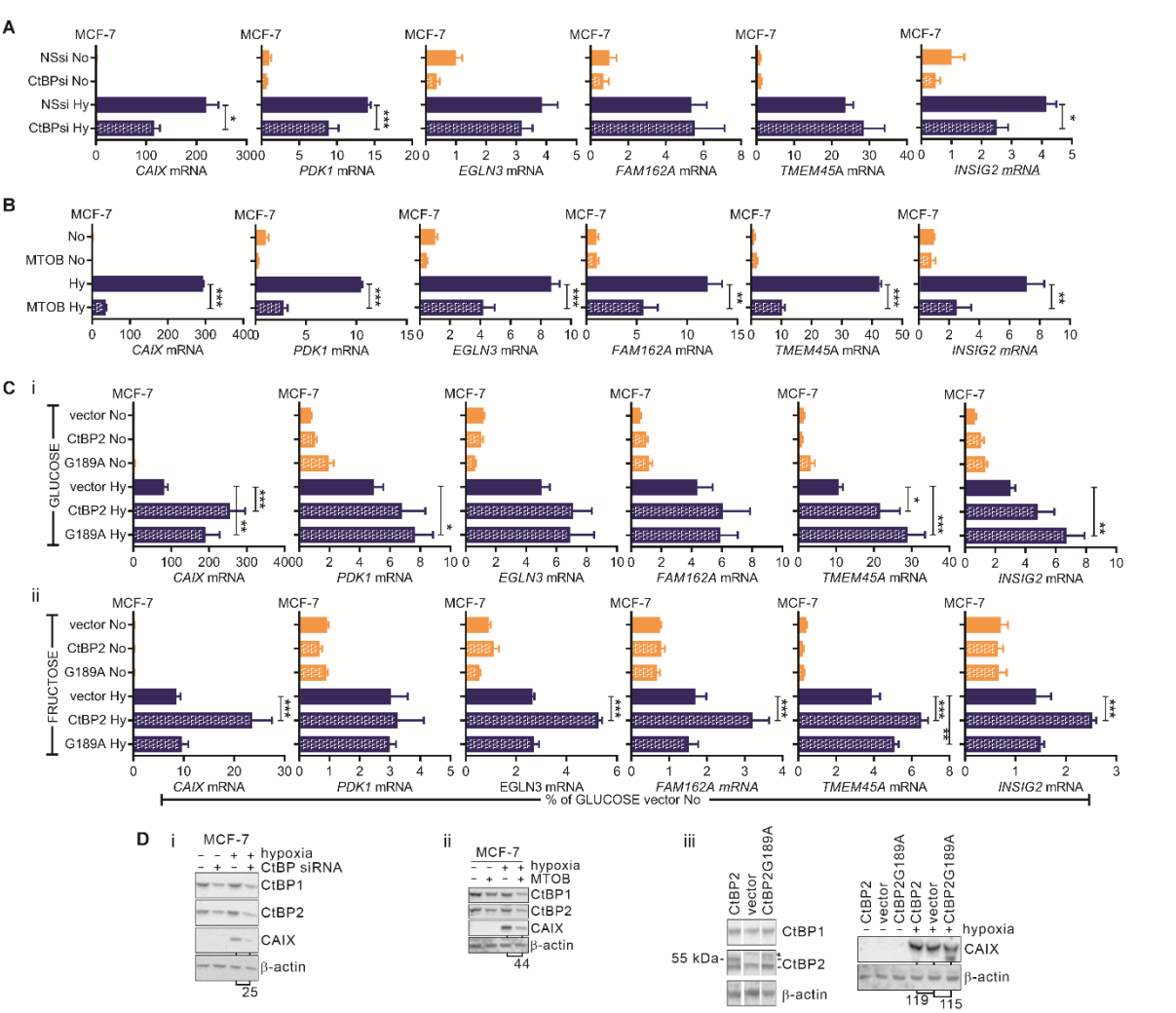

Figure 3: The influence of CtBPs on the hypoxia-induced expression of HIF-target genes. **A:** MCF-7 cells were transfected with siRNA targeting *CTBP1* and *CTBP2*, or non-silencing siRNA. 48 h later cells were cultured in normoxia (No) or 1% oxygen (hypoxia, Hy) for a further 18 h prior to the analysis of HIF target gene mRNA abundance. See (D) for demonstration of the efficacy of the siRNA. **B:** MCF-7 cells were treated with 4 mM MTOB for 48h. Cells were then cultured for a further 18 h under normoxia or hypoxia prior to the analysis of HIF target gene mRNA abundance. **C:** MCF-7 cell clones stably transfected with either empty vector, his-CtBP2 or his-CtBP2^{G189A} were adapted to grow in either (i) 25 mM glucose or (ii) 10 mM fructose. Cells were cultured in normoxia or 1% oxygen for 18 h prior to the analysis of HIF target gene mRNA abundance. **D:** Representative western blots demonstrating the effects of manipulating CtBP expression on hypoxia-induced CAIX protein expression. (i) Experimental conditions as in (A). (ii) Experimental conditions as in (B). (iii) left hand panel - expression of CtBPs in the MCF-7 clones (upper CtBP2 band is his-tagged, * = non-specific, noncontiguous bands from the same blot are shown); right hand panel experimental conditions as in (Ci) (fructose-cultured cells were not analysed due to the low CAIX protein induction shown in Figure 1Dii).

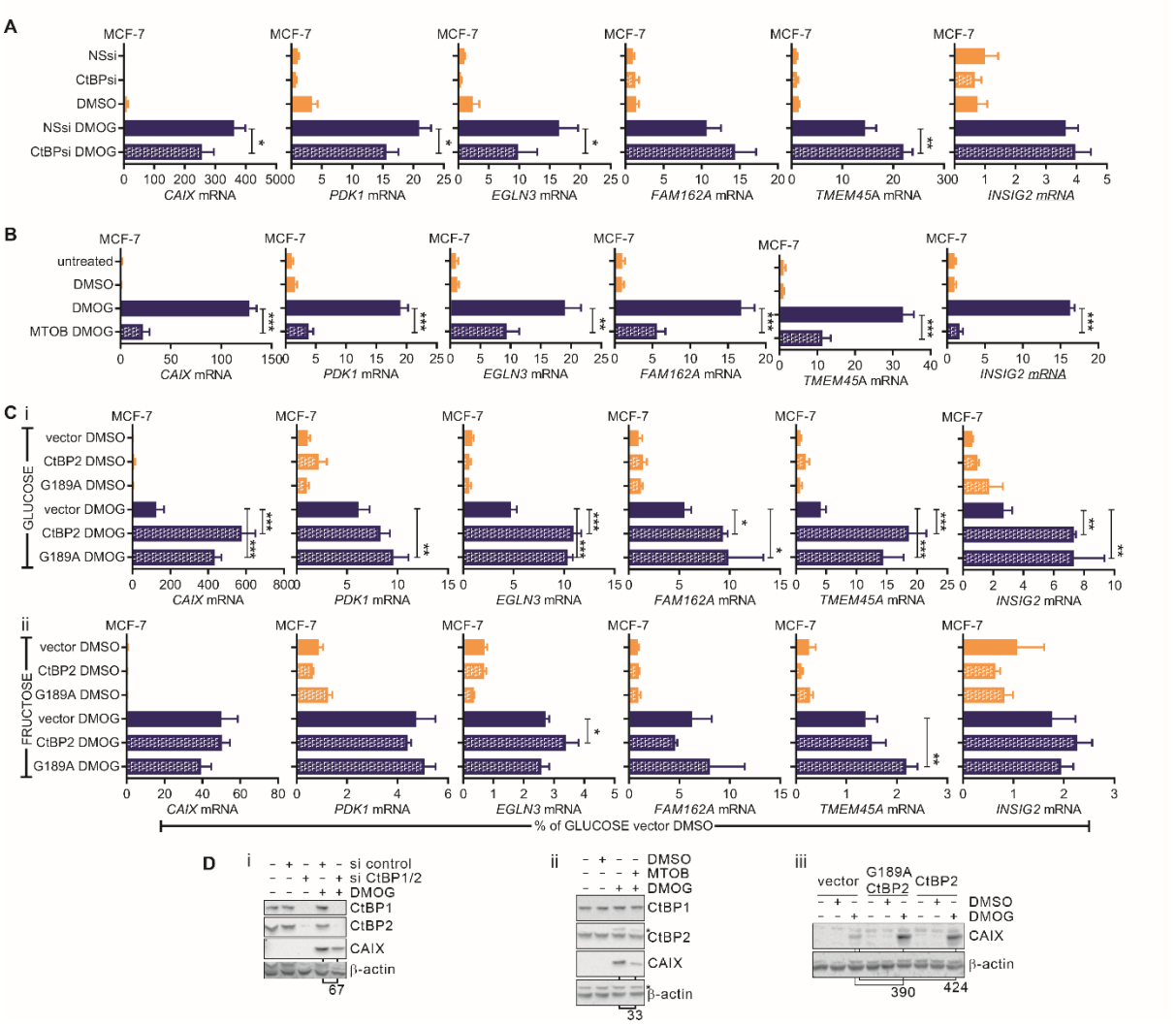

Figure 4: The impact of CtBPs on the regulation of HIF-target genes by the HIF-activating agent DMOG. A: MCF-7 cells were transfected with siRNA targeting CtBP1 and CtBP2, or non-targeting siRNA. 48 h later cells were cultured in DMSO carrier or 1 mM DMOG for a further 18 h prior to the analysis of HIF target gene mRNA abundance. See (D) for demonstration of the efficacy of the siRNA. **B:** MCF-7 cells were treated with 4 mM MTOB for 48h. DMSO control or 1 mM DMOG was then added, and cells were cultured for a further 18 h prior to the analysis of HIF target gene mRNA abundance. **C:** MCF-7 cells clones stably transfected with either empty vector, his-CtBP2 or his-CtBP2^{G189A} were adapted to grow in either (i) 25 mM glucose or (ii) 10 mM fructose. Cells were cultured in DMSO carrier or 1 mM DMOG for 18 h prior to the analysis of HIF target gene mRNA abundance. **D:** Representative western blots demonstrating the effects of manipulating CtBP expression on DMOG-induced CAIX protein expression. (i) Experimental conditions as in (A). (ii) Experimental conditions as in (B). (iii) Experimental conditions as in (Ci).

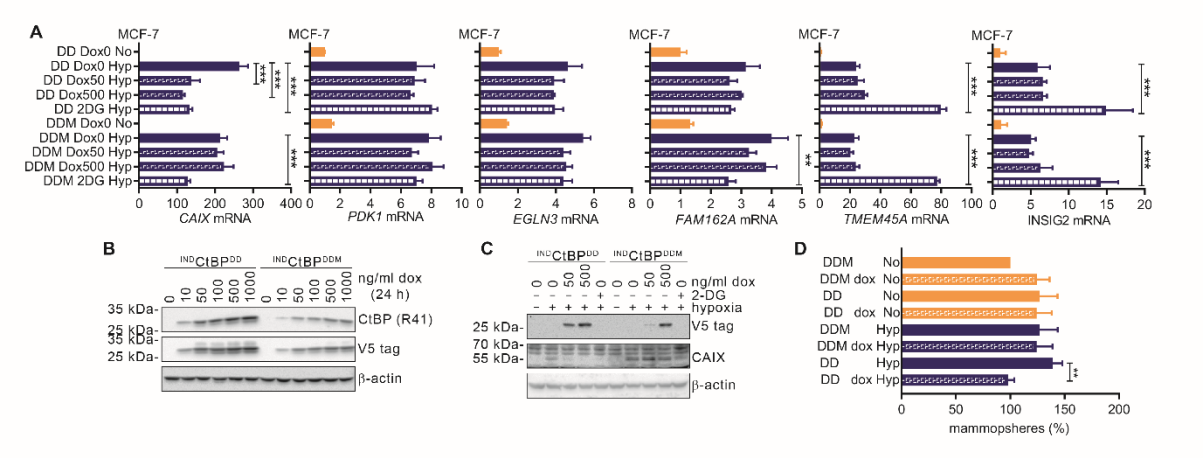

Figure 5: Inhibition of CtBP dimerisation supresses CAIX expression and stem cell like cancer cell survival under hypoxic conditions. A: MCF-7 cells stably expressing the inducible CtBP dimerisation inhibitor, $^{IND}CtBP^{DD}$, or its inactive control $^{IND}CtBP^{DDM}$, were cultured in the absence/presence of the inducing agent (0.05 to 0.5 µg/mL doxycycline) for 24 h, and then cultured under normoxia or hypoxia (3% oxygen) without/with 10 mM 2-DG for 18 h prior to the analysis of HIF target gene mRNA abundance. **B**: (i) Western blot demonstrating induction of $^{IND}CtBP^{DD}$ and $^{IND}CtBP^{DDM}$ in response to increasing concentrations of doxycycline. **C:** Western blot demonstrating the effects of $^{IND}CtBP^{DD}$ and $^{IND}CtBP^{DDM}$ on the induction of CAIX protein by hypoxia. Experimental conditions as in (A). **D:** MCF-7 cells stably expressing $^{IND}CtBP^{DD}$, or its inactive control $^{IND}CtBP^{DDM}$, were cultured in the absence/presence of the inducing agent (1 µg/mL doxycycline) for 24 h, and then a further 18h in the same media under normoxia or hypoxia (3% oxygen) prior to assaying for mammosphere-forming ability. Paired t-test.