OPEN

Citation: Oncogenesis (2014) **3**, e108; doi:10.1038/oncsis.2014.21

www.nature.com/oncsis

ORIGINAL ARTICLE

Selective CREB-dependent cyclin expression mediated by the PI3K and MAPK pathways supports glioma cell proliferation

P Daniel[1], G Filiz[1], DV Brown[1], F Hollande[1], M Gonzales[1,2], G D'Abaco[3], N Papalexis[4], WA Phillips[5,6], J Malaterre[6,7], RG Ramsay[6,7] and T Mantamadiotis[1,4]

The cyclic-AMP response element binding (CREB) protein has been shown to have a pivotal role in cell survival and cell proliferation. Transgenic rodent models have revealed a role for CREB in higher-order brain functions, such as memory and drug addiction behaviors. CREB overexpression in transgenic animals imparts oncogenic properties on cells in various tissues, and aberrant CREB expression is associated with tumours. It is the central position of CREB, downstream from key developmental and growth signalling pathways, which gives CREB this ability to influence a spectrum of cellular activities, such as cell survival, growth and differentiation, in both normal and cancer cells. We show that CREB is highly expressed and constitutively activated in patient glioma tissue and that this activation closely correlates with tumour grade. The mechanism by which CREB regulates glioblastoma (GBM) tumour cell proliferation involves activities downstream from both the mitogen-activated protein kinase and phosphoinositide 3-kinase (PI3K) pathways that then modulate the expression of three key cell cycle factors, cyclin B, D and proliferating cell nuclear antigen (PCNA). Cyclin D1 is highly CREB-dependent, whereas cyclin B1 and PCNA are co-regulated by both CREB-dependent and -independent mechanisms. The precise regulatory network involved appears to differ depending on the tumour-suppressor phosphatase and tensin homolog status of the GBM cells, which in turn allows CREB to regulate the activity of the PI3K itself. Given that CREB sits at the hub of key cancer cell signalling pathways, understanding the role of glioma-specific CREB function may lead to improved novel combinatorial anti-tumour therapies, which can complement existing PI3K-specific drugs undergoing early phase clinical trials.

Oncogenesis (2014) **3,** e108; doi:10.1038/oncsis.2014.21; published online 30 June 2014

INTRODUCTION

Patients diagnosed with malignant glioblastoma (GBM) show a median survival of 14 months; a statistic largely unchanged over the past decades. For gliomas, the only drug used as part of the standard therapy is the DNA alkylating/methylating agent temozolomide, which has led to an improvement in median overall survival, ranging between 0 and 7 months, depending on the methylation status of the patient's DNA repair gene, MGMT.[1] Attempts at targeting specific factors in GBM have so far been unsuccessful, with such attempts exemplified by clinical trials conducted recently: the use of the promising angiogenesis inhibitor bevacizumab (Avastin)[2] or a combination of bevacizumab and a phosphoinositide 3-kinase (PI3K) pathway inhibitor provided no benefit to patients.[3] Therefore there is a need to develop better approaches for treating gliomas to improve patient survival. To progress the discovery and testing of novel drugs and combinations of drugs, the understanding of the molecular genetic mechanisms and factors driving GBM development, growth and drug resistance must be clarified. Among the factors and pathways implicated in glioma development and growth, the kinases PI3K and mitogen-activated protein kinase (MAPK) are among the most studied. Highlighting the critical role of these kinases in cancer, $>80\%$ of GBM patients harbour alterations such as epidermal growth factor receptor (EGFR) amplification, EGFRvIII-activating mutation and/or downstream PIK3CA-activating mutations or phosphatase and tensin homolog (PTEN) deletions,[4] contributing to the hyperactivation of the downstream effectors such as extracellular signal–regulated kinase and AKT, key drivers of pathogenesis in GBM.[5] Although aspects of the immediate upstream and downstream components of these pathways are relatively well understood, the feedback loops and nuclear target networks controlling these pathways in GBM biology are not as well defined.

As many cancer signalling pathways converge on nuclear transcription factors, which then orchestrate the expression of a tumour-promoting transcriptome, targeting these transcription factors in combination with upstream-activating factors may be an attractive approach. Indeed, this has come to the fore in terms of emerging anti-tumour strategies[6–9] In cancer cells, one of the transcription factors that sit at the hub of tumour cell signalling pathways is the cyclic-AMP response element binding (CREB) protein, a serine/threonine kinase-regulated transcription factor in which phosphorylation of CREB in the N-terminal kinase-inducible domain recruits transcriptional co-activators such as CREB-binding protein and transducers of regulated CREB activity to activate CREB target gene transcription.[10,11] CREB has been implicated in the growth and progression of multiple cancers, including

[1]Department of Pathology, The University of Melbourne, Parkville, Victoria, Australia; [2]Department of Anatomical Pathology, The Royal Melbourne Hospital, Parkville, Victoria, Australia; [3]NICTA Victorian Research Laboratories, Centre for Neural Engineering, The University of Melbourne, Carlton, Australia; [4]Laboratory of Physiology, Faculty of Medicine, University of Patras, Patras, Greece; [5]Surgical Oncology Research Laboratory, Peter MacCullum Cancer Centre, Melbourne, Victoria, Australia; [6]Sir Peter MacCallum Department of Oncology, The University of Melbourne, Parkville, Victoria, Australia and [7]Differentiation and Transcription Laboratory, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia. Correspondence: Dr T Mantamadiotis, Department of Pathology, Faculty of Medicine, Dentistry and Health Sciences, The University of Melbourne, Parkville, Victoria 3010, Australia.
E-mail: theom@unimelb.edu.au
Received 3 February 2014; revised 29 April 2014; accepted 15 May 2014

leukemia,[12,13] colorectal cancer[14] and breast cancer.[15] CREB lies at the hub of multiple oncogenic signalling pathways initiated by ligand-growth factor receptor interactions such as the platelet-derived growth factor receptor and EGFR receptor tyrosine kinases and is activated by numerous downstream kinase pathways, including PI3K and MAPK, and deactivated by tumour suppressors, including PTEN. In melanoma cells harbouring BRAF(V600E) mutations, CREB is a key factor involved in MAPK pathway-mediated drug resistance[16] In GBM, CREB function appears to regulate growth of tumour cells via transcriptional control of miRNA-23a[17] and Nf-1.[18] Previous work has shown that loss of CREB function during brain development leads to neuronal death[19,20] and that CREB is required for efficient neuronal stem and progenitor cell proliferation[21,22] Based on these findings, we hypothesized that CREB activation is a critical step in gliomagenesis and that CREB function sits downstream of key cancer signalling pathways.

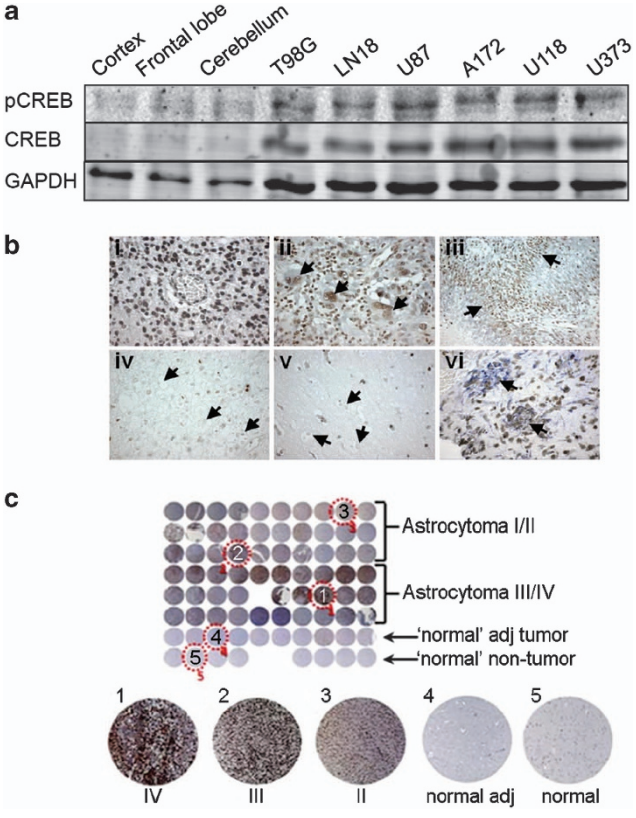

Figure 1. CREB is hyperactivated in glioma cell lines and tissue. (**a**) Cells from a range of human glioma cell lines were lysed and analysed from CREB expression and activation. All GBM cell lines tested showed an abundance of CREB protein, which was phosphorylated/activated. Non-tumour mouse brain tissue shows expression of CREB but no detectable pCREB. (**b**) Immunohisto-chemical analysis of human GBM tissue specimens show differential pCREB expression. (i) pCREB-positive tumour cells surround a tumour blood vessel; (ii) Giant cells in GBM show moderate-to-strong pCREB labelling; (iii) pseudopalidaing areas (arrows) in GBM show dense pCREB-positive tumour cells; (iv) and (v) two different patient tumours featuring oligodendroglial features show weak pCREB expression (arrows indicate oligodendroglial tumour cells); and (vi) double-labelling immunohistochemistry showed dense pCREB-positive cells (brown) surrounding foci with strong nestin expression (blue). (**c**) Brain tumour tissue microarray (US Biomax) with pCREB immunostaining. Top image shows the complete array with tumour grade layout indicated and cores magnified in the lower panel.

We show that CREB expression and activation correlates with glioma grade and that activation occurs via both the PI3K and MAPK pathways and that CREB promotes glioma cell proliferation. Furthermore, we show that the mechanism by which CREB promotes glioma cell proliferation includes the regulation of key cell cycle factors cyclin B1, D1 and proliferating cell nuclear antigen (PCNA).

RESULTS

CREB is constitutively activated in human GBM cells lines and human brain tumours exhibit grade-dependent pCREB expression

Although CREB is expressed throughout the normal brain during all stages of life, constitutively phosphorylated CREB (pCREB) is progressively restricted to neurogenic zones. It is within these zones that neural stem/progenitor cell (NSPC) populations reside and CREB gene/expression disruption studies in mice and zebrafish show that NSPC proliferation and survival depends on CREB function.[21,22] Based on data showing that CREB has oncogenic roles in the hemopoietic system[12] and other tissues[23] and as the CREB functions required by NSPCs may be important in GBM biology, we explored CREB expression, activation and function in brain tumour tissue and cells. Assessment of CREB expression and activation in human GBM tumour cell lines using CREB and pCREB antibodies revealed that GBM tumour lines showed robust CREB and pCREB expression. Non-tumour brain tissue exhibited weaker total CREB expression and undetectable levels of pCREB (Figure 1a).

Using whole GBM surgical tumour specimens prepared from formalin-fixed, paraffin-embedded tissue, we examined the morphological landscape of a further 15 patient specimens diagnosed with grade IV GBM. GBM tumour morphologies associated with pCREB expression are shown in Figures 1b-i–vi. pCREB expression was evident in most tumour cells examined with a clustering of strong pCREB expression in highly vascularized tumour regions (Figure 1b-i) and in regions with strong nestin expression (Figure 1b-vi). pCREB-positive cell density and expression was high in hypercellular pseudopalisading regions adjacent to necrotic regions, where migrating proliferating tumour cells are seen (Figure 1b-iii, arrows). Of note, tumour morphologies corresponding to GBM regions associated with less malignant characteristics exhibited weaker pCREB expression. Giant cells in GBM showed relatively weaker pCREB expression (Figure 1b-ii, arrows), and cells in tumour regions with oligodendroglial features, characterized by the so-called 'chicken wire' or 'fried egg' morphology showed the weakest pCREB expression (Figures 1b-iv and -v, arrows).

Interrogation of glioma tissue microarray specimens harbouring 40 patient glioma tissue cores (3 mm diameter) in duplicate showed that while 'normal' non-tumour and 'normal tissue adjacent to tumour' showed little-to-no CREB activation. By contrast, tumour tissue exhibited robust grade-dependent levels of pCREB expression (Figure 1c). Grade II tumours showed the lowest levels of pCREB, and WHO grade IV tissues exhibited the strongest pCREB positivity. Both pCREB intensity and the number of cells expressing pCREB correlated with tumour grade, where grade IV GBM showed the most intense and highest density of pCREB-positive cells. Using a '+' scale where '+' is just detectable staining/1% to <5% of cell/field positive and '+++++' is strong dark staining/almost 100% cells/field positive, grade II tumours showed between zero and '++'; grade III tumours showed between '+' and '+++' and grade IV/GBM showed between '+++' and '+++++'. Three oligodendroglioma specimens showed '+', '+' and '++', while all 'adjacent to tumour' and 'normal' brain tissue showed zero staining.

Using the GBM cell line T98G to examine CREB activation, we show that pCREB coincides with cells in cycle. Specifically, pCREB is present in cells undergoing S-phase of the cell cycle as shown by co-labelling with PCNA and newly divided cells labelled with the

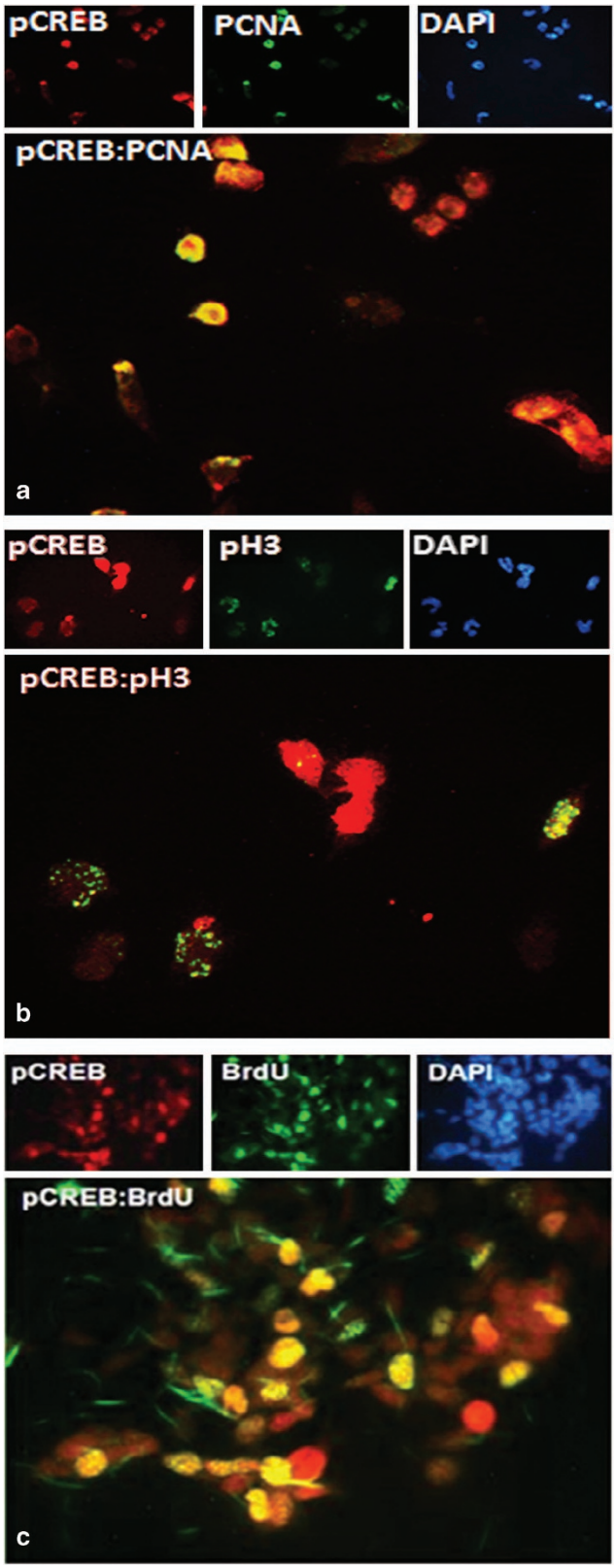

Figure 2. CREB is activated in dividing cells. T98G cells were analysed for the colocalization of pCREB(Ser 133) with markers of cell proliferation: (**a**) PCNA, (**b**) phospho-histone (pH3), nd (**c**) BrdU.

nucleotide analogue bromodeoxyuridine (BrdU; Figure 2). In contrast, cells labelled with the M-phase marker phospho-histone 3 (pH3) demonstrated poor colocalization to pCREB-positive cells.

PI3K and MAPK but not protein kinase A (PKA) signalling pathways modulate CREB activity in GBM cells

Many signalling pathways relevant to GBM converge onto CREB, suggesting that constitutive CREB activation as observed in GBM is the result of cooperation between multiple upstream factors. To determine which pathways activate CREB, we evaluated cells grown in stimulatory and non-stimulatory conditions for differences in kinase activation. Several serine/threonine kinases that are known to phosphorylate CREB at Serine133 were investigated. Analysis of cell lines T98G, LN18 and U118 demonstrated activation of CREB, AKT and MAPK upon serum stimulation. By contrast, we did not observe activation of PKA, an activator of CREB in other cell types (Figure 3a), showing that there is a selective use of upstream components regulating CREB activity.

To link the specificity of AKT and MAPK activation with CREB activation, the PI3K pathway inhibitor LY294002 and the MAPK pathway inhibitor U0126 were used on T98G and U118 (Figures 3b and c). These cell lines were chosen for further analysis for two reasons. First, they showed the greatest CREB-dependent change in proliferation (Figures 4b and c), and second, these cell lines differ in the tumour-suppressor PTEN status, where T98G has intact PTEN function and U118 is PTEN deficient. One of the PTEN's major functions is to act as the major phosphatase that inhibits the activation of the PI3K pathway[24] Significant variability was seen between the cell lines tested with respect to the contribution of either the PI3K or MAPK pathways on CREB activation. In U118 cells, inhibition of the PI3K pathway resulted in no change in CREB activation, while inhibition of the MAPK pathway resulted in knockdown of CREB activation to basal 'serum-deprived' levels, suggesting that MAPK signalling is the predominant CREB activation pathway in U118 cells (Figures 3c and e). By contrast, inhibition of either or both the PI3K or MAPK pathways in T98G cells did not result in significant changes in CREB activation (Figures 3b and d), highlighting the diversity and complex interdependence between signalling pathways involved in CREB activation in GBM cells.

As a further and independent measure of the contribution of the PI3K pathway to CREB activation, we used a mutant NSPC that has a constitutively activated PI3K pathway, due to the combined conditional mutation of the *Pik3ca* gene, which encodes for the major catalytic p110alpha subunit of PI3K, and has a conditional loss of tumour-suppressor PTEN. These NSPCs were derived from a conditional mutant mouse shown to develop tumours in various tissues tested.[25–27] CREB activation (pCREB) was significantly elevated along with AKT activation (pAKT), compared with parental NSPCs from the same mouse in which the PI3K pathway mutations were not activated (Figure 3f).

CREB function contributes to GBM cell proliferation

As CREB is strongly associated with proliferative zones within the normal adult brain, we examined the effect of CREB knockdown on GBM cell proliferation. We tested siRNA-mediated CREB knockdown in five human GBM cell lines (T98G, U118, U373, ANGM-CSS, U87), which represent cells with an array of GBM-associated genetic alterations. The combined use of three CREB siRNAs targeting different mRNA regions allowed robust CREB expression knockdown ($>90\%$) over at least 120 h (Figure 4a) in T98G cells. All five cell lines tested showed a reduction in cell proliferation ($n=1$, not shown). Further experiments were performed with selected cell lines as indicated. The greatest reduction in proliferation was seen in cell lines T98G and U118, with a 58% and 52% reduction compared with scrambled siRNA-transfected controls after 120 h (Figures 4b and c). As we have

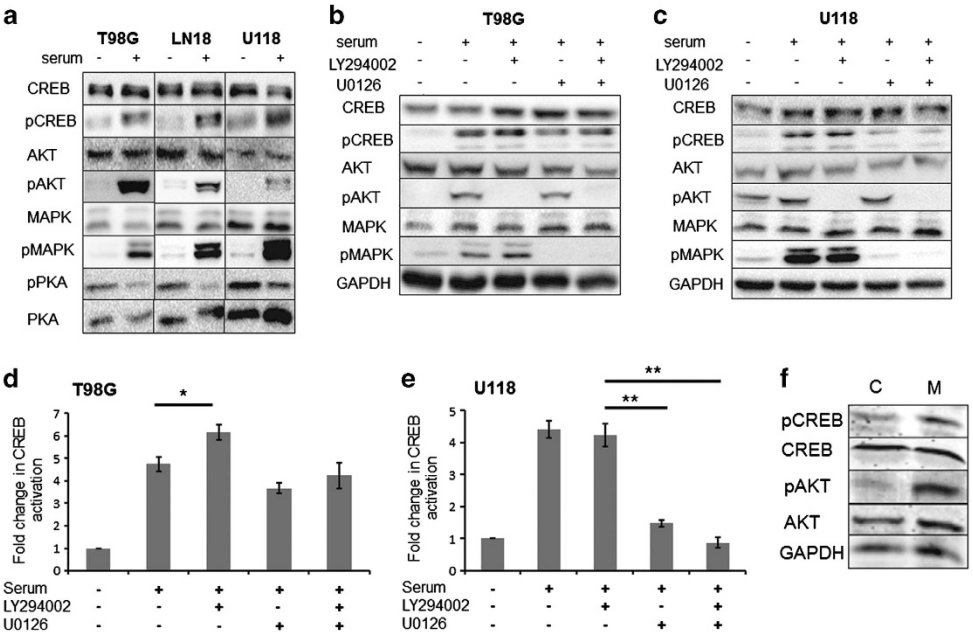

Figure 3. The PI3K and MAPK-dependent signalling pathways but not the PKA pathway activate nuclear CREB in glioma cells. (**a**) GBM cell lines T98G, LN18 and U118 were serum starved for 24 h then exposed to serum and protein lysate immunoblotted for the indicated antibodies. (**b**, **c**) GBM cell lines T98G and U118 were pretreated with a PI3K inhibitor LY294002, a MAPK inhibitor U0126 or both inhibitors, and then exposed to serum, protein lysate collected after 4 h and analysed for the presence of the indicated antibodies (**d**, **e**) Quantification of the effects of inhibitor combinations on pCREB levels in T98G and U118 GBM cells. $^{*}P<0.05$, $^{**}P<0.005$. (**f**) Western blotting showing the level of pCREB and pAKT in mouse NSPCs grown as neurospheres, with an activated Pik3ca^{H1047R} mutation and PTEN deletion (M) or parental NSPCs with 'wild-type' Pik3ca and PTEN (C). All western blottings were performed at least three times, and where applicable, total CREB, AKT and MAPK were imaged, then membranes were stripped and reprobed for pCREB, pAKT and pMAPK detection.

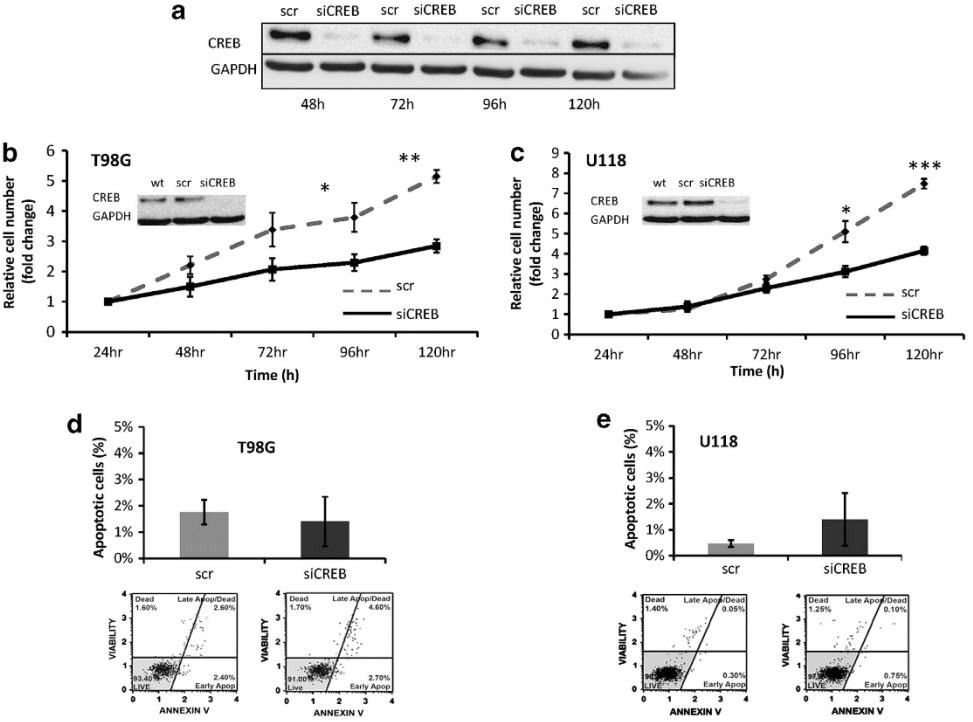

Figure 4. CREB is required for efficient human glioma cell line proliferation. (**a**) T98G cells were transfected with either CREB1-specific siRNA (siCREB) or scramble siRNA and then lysate analysed by western blotting for efficiency of CREB knockdown. $^{*}P=0.05$, $^{**}P=0.005$, $^{***}P=0.0005$. (**b**, **c**) Scrambled or siCREB was transfected into GBM cell lines T98G and U118, and proliferation was analysed every 24 h using an MTT assay. (**d**, **e**) Scrambled or siCREB was transfected into GBM cell lines T98G and U118, and apoptosis was determined by FACs determination of AnnexinV uptake.

previously shown that loss of CREB function in zebrafish and mouse brain as well as mouse neural NSPCs leads to compromized cell survival, we tested whether this was the case in GBM cells. Notably, cell survival was not affected by CREB knockdown using two different assay systems, measuring lactate dehydrogenase released from dead cells and measuring early apoptosis by measuring appearance and accumulation of Annexin V (Figures 4d and e) following CREB knockdown. Thus reduced cell numbers can be attributed to reduced proliferation.

CREB knockdown leads to aberrant GBM cell cycle kinetics due to inhibition of cell cycle factor expression

To determine the mechanism by which CREB regulates GBM cell proliferation, cell cycle parameters were measured using DNA content by flow cytometry in T98G and U118 cells, which were the cell lines showing the greatest CREB-dependent decrease in proliferation. CREB knockdown increased the proportion of cells in G0/G1 phase in both cell lines tested (Figures 5a and b). siCREB T98G cells also showed significantly reduced proportions in cell cycle phase distribution in S- and G2/M phases compared with control cells, whereas siCREB U118 cells exhibited increased G0/G1 and fewer G2/M phase cells only. To explore the molecular basis underlying disruption of the cell cycle, we measured the expression of cell cycle/proliferation factors previously reported to be transcriptionally regulated by CREB, including cyclins B1, D1[28] and PCNA.[14,29] Protein expression of cyclins B1, D1 and PCNA was assessed in T98G and U118 cells in CREB knockdown or scrambled control siRNA-treated GBM cell lines (Figures 5c and d). Cells were serum deprived for 24 h before the addition of serum to synchronize cell cycle activation, and cyclin and PCNA protein levels were determined every 12 h, over 48 h. The dynamics of expression seen was consistent with the reported cyclin and PCNA expression profiles for mammalian cells, where PCNA and cyclin D1 are consistently expressed throughout the cell cycle while cyclin B1 expression peaks at G2/M.[30] Upon treatment with serum (triggering cell cycle entry), we observed inhibition of protein expression of cyclin B1, cyclin D1 and PCNA in both T98G and U118 cell lines as early as 12 h (not shown) with maximal inhibition reached at 24 h, compared with no-serum cells (Figures 5c and d). T98G cells showed a consistent and almost complete block in cyclin D1, cyclin B1 and PCNA expression over 48 h in CREB knockdown cells, in contrast to U118 cells, which exhibited a more modest inhibition of cyclin D1, over 48 h. In U118 cells, cylin B1 protein expression was maximally inhibited at 24 h, but little effect was seen in PCNA expression (Figure 5d). Moreover, in U118 cells cyclin B1 inhibition was not sustained, with expression approaching control levels beyond 24 h. Given that cyclin B1, D1 and PCNA harbour cAMP-resonsive elements (CREB-binding sequences) in their promoters (see Supplementary Data), we tested whether CREB exerted its influence on these target genes directly; we performed reverse transcriptase–PCR (RT–PCR) at 24 h following siCREB treatment to measure mRNA levels of cyclin B1, D1 and PCNA. CREB knockdown robustly inhibited cyclin D1 mRNA expression in both T98G and U118 cells, while cyclin B1 mRNA expression was significantly reduced in T98G cells only. Surprisingly, PCNA mRNA expression was unaffected by CREB knockdown in both cell lines (Figures 5e and f), implying that CREB exerts its affect on PCNA protein expression indirectly. The transcriptional influence CREB exerts in the expression of these genes reflects the context of the cAMP-resonsive elements in their respective promoters (see Supplementary Data), with cyclin D1 showing the best context with a full (8-base pair consensus) cAMP-resonsive element positioned closest to the transcription start site.

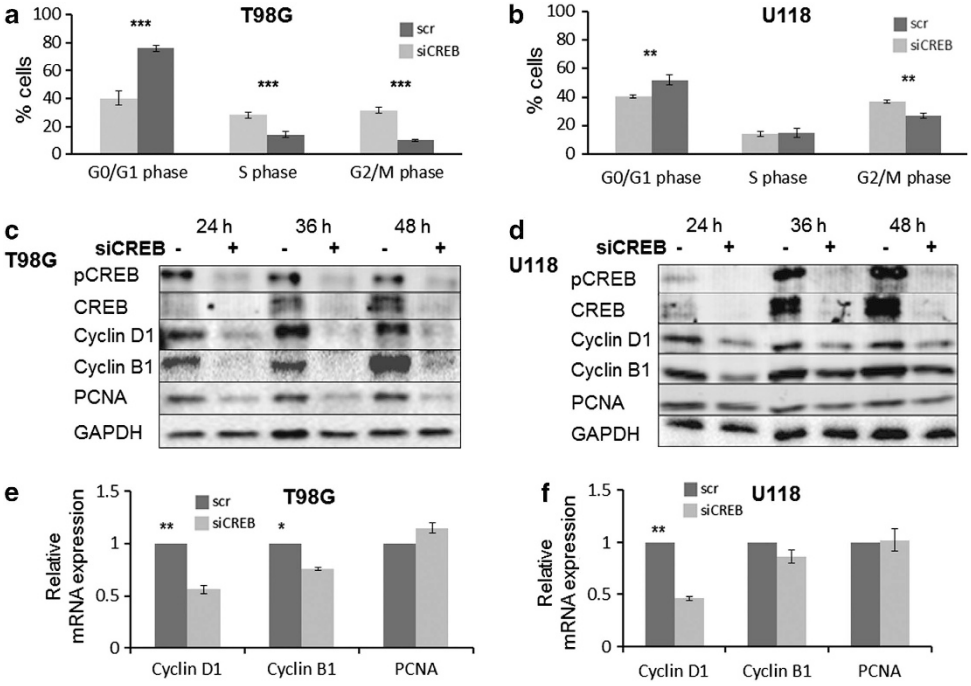

Figure 5. Knockdown of CREB alters human GBM cell cycle kinetics through regulation of cyclin B1, cyclin D1 and PCNA. (**a**, **b**) Synchronized GBM cell lines T98G and U118 were treated with either siCREB or scramble control siRNA and then stimulated with serum for 24 h before being analysed for cell cycle proportions using DNA-content analysis. (**c**, **d**) Synchronized GBM cell lines T98G and U118 were treated with either siCREB or scramble control siRNA, stimulated with serum and harvested every 12 h for analysis for cyclin B1, cyclin D1 and PCNA expression. (**e**, **f**) Synchronized GBM cell lines T98G and U118 were treated with either siCREB or scrambled control siRNA and then stimulated with serum for 24 h before harvesting and analysis using qRT–PCR for CCNB1, CCND1 and PCNA mRNA expression. ***$P<0.0005$, **$P<0.005$, *$P<0.05$. All western blottings were performed at least three times, and blottings for total CREB assay were imaged, then membranes were stripped and reprobed for pCREB detection.

Selective CREB regulation of cell cycle factors by canonical and non-canonical interations with the PI3K pathway

To investigate the mechanism of the differential regulation of cyclin D1, cyclin B1 and PCNA by CREB in T98G compared with U118 cells, we looked at whether CREB knockdown affected upstream signalling components, given that previous studies have shown a link between CREB and upstream signalling components of the PI3K and MAPK pathways, such as insulin growth factor receptor (IGFR) and insulin receptor substrate-1/2 (IRS-1/2).[31,32] Crucially, T98G and U118 differ in PTEN status (T98G PTEN$^+$ and U118 PTEN$^-$), which may account for the differential CREB-mediated regulation of cell cycle factors via interaction with the MAPK and PI3K pathways.[33,34] Treatment of T98G cells with siCREB resulted in an almost complete block of AKT activation, which was not rescued by the addition of serum (Figure 6a). In comparison, CREB knockdown in U118 cells showed reduced AKT activation only in serum-free conditions (Figure 6b). Upon the addition of serum, AKT activation in U118 cells increased to levels identical to control cells (Figure 6b).

To determine whether the cell-specific regulation of cyclin B1 and PCNA by CREB could be due to effects on AKT activation and/or PTEN status, we used the PI3K inhibitor LY294002 in conjuction with siCREB in U118 cells (Figure 6c). This experiment confirmed the dependence of cyclin D1 expression on CREB, with siCREB treatment alone showing almost complete attenuation of cyclin D1 expression. Furthermore, the expression of cyclin B1 and PCNA were found to be dependent on both CREB and PI3K signalling. Neither siCREB nor the PI3K inhibitor LY294002 alone could block cyclin B1 or PCNA expression in U118 cells.

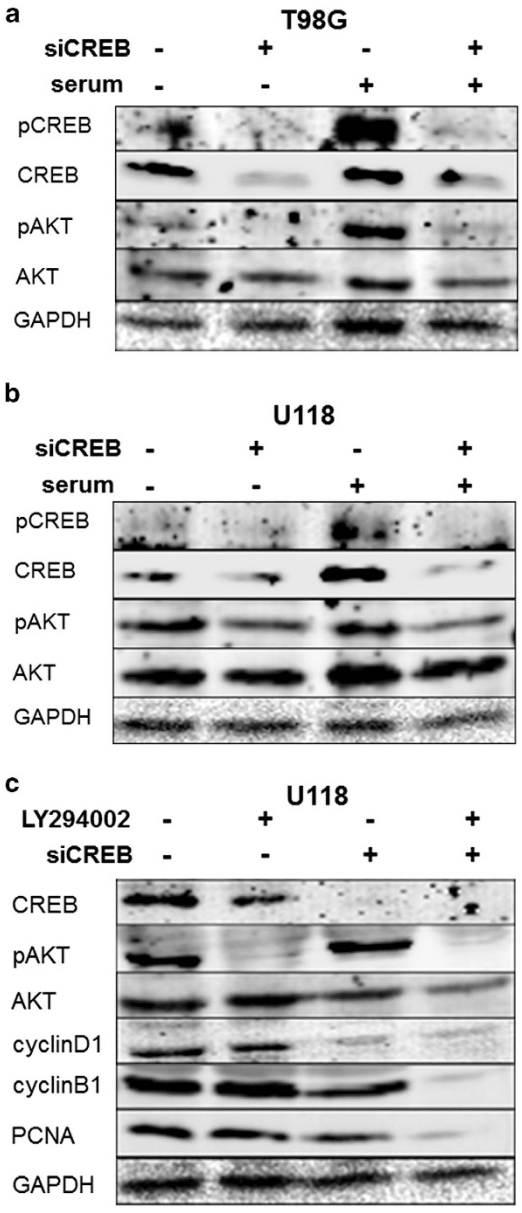

Figure 6. Selective dependence of cyclin expression on CREB is determined by the PI3K pathway and tumour-suppressor PTEN status in glioma cells. (**a**, **b**) T98G and U118 cells were treated with either scramble or siCREB for 24 h before exposure to serum-free or serum conditions for 12 h. Cell lysate was then analysed for markers of PI3K activation. (**c**) U118 cells were treated with either LY294002, siCREB or both, and cell lysate was then immunoblotted for cyclin B1, cyclin D1 and PCNA expression changes. All western blottings were performed at least three times, and where applicable, total CREB and AKT were imaged, and then membranes were stripped and reprobed for pCREB and pAKT detection.

DISCUSSION

CREB-dependent brain functions have been extensively explored with most previous studies focussed on neurological functions ranging from learning and memory,[35] opiate withdrawal[36] to feeding behavior.[37] Other work has shown that CREB is critical for neuronal survival and that CREB disruption *in vivo* leads to a neurodegenerative phenotype[20] and reduction in neural stem cell survival and proliferation[21,22] In parallel, a body of evidence has accumulated showing that CREB imparts oncogenic properties on cells outside the central nervous system.[23,38] We focussed our investigations on defining CREB-dependent mechanisms orchestrating malignant GBM cell proliferation. Analysis of a panel of human GBM cell lines shows that CREB is not only highly expressed but also constitutively hyperactivated (Figure 1a). This is in contrast to non-tumour brain tissue, which show highly regulated and transient activation, dependent on external stimuli.[11] Cells in adult mouse brain showing robust constitutive phosphorylated CREB expression are enriched in the neurogenic zones,[22,39] so the constitutive expression of pCREB in GBM tumour cells is consistent with the generally held view that tumour cells exhibit immature stem/progenitor cell-like characteristics. In GBM tissue examined, pCREB was evident in many GBM hallmark morphological features, including near tumour angiogenic regions (Figure 1b-i), peripheral to necrotic foci characterized by pseudopalisading regions (Figure 1b-iii). This is interesting as GBM regions with pseudopalisading features harbour migrating tumour cells adjacent to hypoxic necrotic regions with high levels of pro-angiogenic factors, including hypoxia-inducible factor-1, vascular endothelial growth factor and interleukin-8, which are all CREB target genes.[40–42] CREB may therefore be intimately involved in regulating the angiogenic tumour niche in GBM. In one GBM tumour examined, strong pCREB-positive cells were clustered in foci exhibiting strong nestin co-labelling (Figure 1b-vi). Nestin, an intermediate filament protein, is highly expressed in immature NSPCs and, similar to pCREB, is associated with brain tumour grade[43–45] This suggests that CREB may contribute to immature tumour cell biology by regulating CREB target genes involved in tumour cell growth and suppression of differentiation/promotion of immaturity; this is supported by studies showing that PI3K regulation of CREB is crucial for NSPC function.[46] Our data also shows that less aggressive GBM tumour subtypes, known to be associated with better survival, express lower levels of activated CREB. Giant cell GBM and oligodendroglial regions in GBM tissue show low to almost no pCREB labelling in most tumour cells (Figures 1b-iv and -v). It may be that pCREB is a biomarker associated with GBM malignancy and may also actively promote malignant properties in the more aggressive types of brain cancer.

These putative roles of CREB relate to its position in a complex signalling network where CREB sits at a convergence point of multiple oncogenic signalling pathways and is activated by multiple factors, including the PI3K and MAPK pathways which are essential contributors to the cancer phenotype. As such, CREB activation may be reflective of oncogenic pathway activation. Use of the PI3K inhibitor LY294002 and MAPK inhibitor U0126 to dissect the contribution of these core pathways established a causal relationship between CREB activation and the PI3K and MAPK pathways; however, our data also demonstrate that CREB is activated by multiple pathways in GBM and that these pathways vary between GBM cell lines (Figures 3b and c). The regulation of CREB by the PI3K and MAPK pathways appears to be complex and there is some redundancy in this regulation. Furthermore, CREB is able to regulate cell proliferation (Figure 4), an important characteristic of GBM cells to which multiple pathways are able to contribute.[47–49] Taken together, our data suggest that disruption of oncogenic functions regulated by the PI3K and MAPK pathways in GBM may be achieved by direct targeting of CREB rather than the redundant signalling pathways.

One mechanism by which CREB regulates proliferation is via modulation of the cell cycle, a previously reported role conserved across multiple cancer and non-cancer cell types.[28,50,51] Knockdown of CREB in GBM cell lines T98G and U118 resulted in attenuation of the cell cycle however the effect on cell cycle progression was much greater in T98G than U118. The sensitivity to CREB knockdown differed in the two cells tested, with CREB loss having a greater effect on cyclin expression in T98G cells compared with U118 (Figures 5a and b). Cyclin D1 exhibited robust CREB dependence in both cell lines, whereas CREB knockdown reduced expression of cyclin B1 and PCNA in T98G but not in U118 cells (Figures 5c and d). Cyclin D1, cyclin B1 and PCNA are known markers of poor survival in a multitude of cancers, such as hepatocellular carcinoma,[52] anorectal malignant melanoma[53] epithelial ovarian carcinoma[54] and breast cancer.[55]

Differences observed in the regulation of cell cycle genes by CREB are likely due to differences in the genetic background of GBM where PTEN status was most notable in the GBM cell lines tested ($T98G^{PTEN+}$, $U118^{PTEN-}$). The presence or absence of PTEN can influence many cellular responses, including proliferation, with PTEN not only acting as a negative regulator of the canonical PI3K pathway but influencing many other PI3K-independent effects, including roles in cell cycle and DNA integrity.[56] Mechanistically, a difference in PTEN status likely explains the ability for CREB to differentially regulate AKT activity in T98G and U118 (Figures 6a and b). A feedback loop involving CREB and AKT may occur through direct CREB-dependent regulation of insulin receptor substrate 2 (IRS1/2) expression, adaptor proteins downstream of IGFR/IR which directly associates with the p85 subunit of PI3K to activate the PI3K/AKT pathway.[57] In the presence of PTEN, the activation of the PI3K pathway is antagonized, resulting in the upregulation of IR/IGFR/IRS1/IRS[58] in an attempt to maintain AKT activation. Importantly, CREB has been shown to regulate the expression of IRS components,[59] suggesting a mechanism by which CREB is able to regulate the activity of AKT. In T98G cells, the presence of PTEN would inhibit the phosphorylation of IRS1, while the knockdown of CREB would cause loss of IRS2, leading to the sustained downregulation of PI3K pathway (Figure 6a). By contrast, U118 cells that are PTEN null would have no PTEN-dependent inhibitory influence on IRS1, thus allowing a serum-dependent increase in PI3K activity (Figure 6b). In line with this, the consequence of CREB loss on cell cycle progression and regulation of cell cycle genes cyclin B1 and PCNA was dependent on the regulation of PI3K by CREB in a PTEN-dependent manner. Importantly, co-inhibition of CREB and PI3K activity was necessary for the downregulation of cyclin B1 and PCNA (Figure 6c), highlighting the key role for CREB in promoting cell proliferation, even when the PI3K pathway is inhibited. This has considerable therapeutic significance, as redundancy in oncogenic signalling networks is a major mechanism by which the clinical impact of targeted therapies is negated. Indeed, recent reports have demonstrated that CREB has a major role mediating resistance to targeted therapies in melanoma,[16] which when considered with the data presented here, highlights the need for further investigation of CREB and PI3K pathway target genes in tumour cells.

Overall, this study shows that pCREB is a marker of glioma grade and that CREB activation in GBM cells appears to modulate GBM tumour cell proliferation through the integration of signals from PI3K and MAPK pathways (Figure 7). We also show that CREB acts as a master transcriptional regulator of cyclin D1 in GBM. This observation is consistent with the spectrum of tumour cell types where CREB has been shown to regulate cyclin D1 expression, including malignant T-cells[60] gastric cancer cells,[61] breast cancer cells,[62] embryonic carcinoma cells[63] and prostate cancer cells. Furthermore, we establish that CREB may be part of a complex signalling feedback loop modulating the expression of cell cycle factors. Given the roles CREB has in regulating GBM cell proliferation and the convergence of multiple redundant cancer signalling pathways upon CREB, the use of anti-tumour therapeutic approaches incorporating CREB inhibitors would be expected to provide more effective anti-tumour responses and

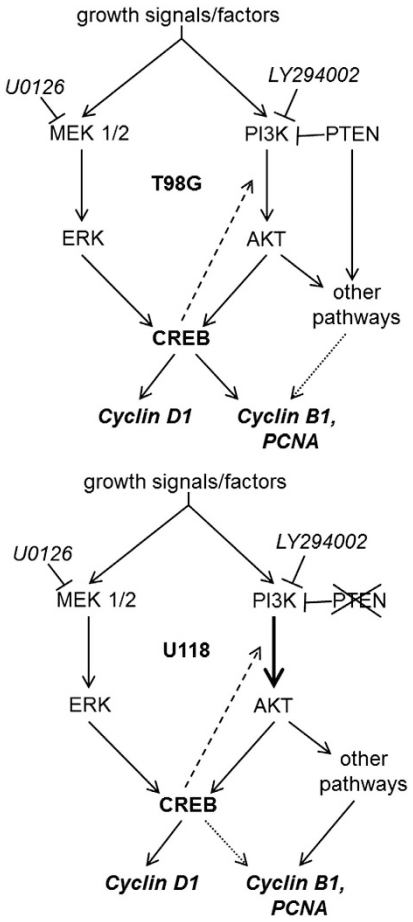

Figure 7. CREB-dependent cell cycle factor expression signalling network in GBM tumour cells. CREB lies downstream of both the PI3K and MAPK pathways and modulates cell cycle factor expression. Cyclin D1 is completely dependent on CREB activity while cyclin B1 and PCNA appear to require activation of both CREB and non-CREB mechanisms. The width of the arrow indicates the relative strength of the signal. The broken arrows pointing from CREB to AKT indicates the putative CREB-dependent regulation of the PI3K pathway.

may bypass the inherent redundancy and associated drug resistance that promote tumour cell proliferation.

MATERIALS AND METHODS

Cell culture and treatments

Human GBM cell lines (T98G, LN18 and U118) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained at 37 °C and 5% CO_2 in complete media (Dulbecco's Modified Eagle Medium (DMEM) F12 + GlutaMAX (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Fisher Biotech, Wembley, WA, Australia) and penicillin/streptomycin (Invitrogen) diluted to 50 units/ml of penicillin and 50 μg/ml of streptomycin. Cells were synchronized before treatment with serum and/or inhibitors by serum starvation for 24 h. Inhibitor experiments were performed on serum-starved cells using 25 μM of LY294002 (Genesearch) and/or U0126 (Genesearch, Danvers, MA, USA) or the equivalent concentration of dimethyl sulphoxide. Cells were exposed to treatments for 30 min before the addition of serum and then harvested as necessary.

siRNA transfection

Twenty picomolar CREB1-specific siRNA or scramble siRNA (Santa Cruz, Dallas, TX, USA) was incubated with 2 μl/ml Lipofectamine RNAiMax (Invitrogen) in unsupplemented DMEM F12 for 20 min at 37 °C to allow complex formation. Cells were incubated with siRNAs overnight at 37 °C before washing in phosphate-buffered saline (PBS) and culturing in complete media (as above).

MTT and FACS analysis

Cell lines were transfected with CREB siRNA or scramble siRNA and then plated onto 96-well dishes in complete media the next day. Cell growth was determined every 24 h by incubation with MTT reagent (Invitrogen) at 1.2 mM final concentration, for 4 h before lysis in MTT lysis buffer (isopropanol, 1% Triton-X, 1 drop glacial HCl/50 ml). Absorbance at 570 nm was then measured using a Wallac 1420 VICTOR2 plate reader (GMI, Ramsey, MN, USA).

Cell cycle and AnnexinV analysis was performed on siRNA-transfected cells using the MUSE cell analyser (Millipore, Billerica, MA, USA). For AnnexinV experiments, cells were harvested 48 h after transfection, resuspended in 100 μl complete media and 100 μl AnnexinV buffer solution (Millipore), incubated for 15 min at room temperature and then analysed on the MUSE. For cell cycle analysis, siRNA-transfected cells were serum starved for 24 h to synchronize cells before the addition of complete media. Twenty-four hours after the addition of complete media, cells were harvested and then fixed in ice-cold 80% ethanol overnight. Cells were then washed and incubated with cell cycle assay buffer (Millipore) for 30 min before being analysed on a MUSE cell analyser (Millipore).

Protein extraction and western blotting

Total cellular protein was extracted using RIPA buffer (150 mM sodium chloride, 1% Triton-X, 0.5% sodium deoxycholate, 50 mM Tris, pH 8.0) supplemented with Pierce complete protease and phosphatase inhibitor tablet (Pierce, Rockford, IL, USA) and then protein quantified using a BCA protein assay kit (Pierce). In all, 15–20 μg of protein was run on a 10% polyacrylamide gel by electrophoresis and then transferred onto a polyvinylidene difluoride membrane (Millipore) before being blocked in 1% Tween-20 Tris-buffered saline pH 6.8 (TBS-T) supplemented with 2% skim milk. Membranes were probed for specific proteins using antibodies as listed below.

Immunohistochemistry

Fixed (4% buffered paraformaldehyde) paraffin-embedded tissue was used to section at 7 μm and then dewaxed with ethanol and histolene. Immunohistochemical labelling was performed using antibodies as listed below, with overnight incubation of primary antibodies at 4 °C, followed by 1 h incubation of secondary antibodies and solutions for colour development provided in a VectoLabs Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) used according to the manufacturer's instructions. Double labelling immunohistochemistry was performed sequentially, each primary antibody being individually incubated and developed.

Quantitative RT–PCR (qRT–PCR)

Synchronized cells were treated with either scrambled (control) or CREB-specific siRNA and then treated with serum for 24 h. Cellular RNA was harvested by the addition of 300 μl of Trizol (Ambion, Foster City, CA, USA) directly to the plate before addition of 100 μl of chloroform (Sigma, St Louis, MO, USA) and subsequent centrifugation for 15 min at 12 000 r.p.m. for phase separation. The upper RNA phase was collected before being passed through a NucleoSpin RNA Clean-up XS column (Macherey-Nagel, D-52313, Düren, Germany). Eight hundred nanograms of RNA was reverse transcribed using a Superscript III first-strand reverse transfection kit (Invitrogen) and then amplified with primers specific to CCND1 (Forward-5′-GATCAAGTGTGACCCGGACTG-3′; Reverse-5′-CCTTGGGGTCCATGTTCT GC-3′), CCNB1 (Forward-5′-TGGTGAATGGACACCAACTC-3′; Reverse-5′-TTC TTAGCCAGGTGCTGCAT-3′) or PCNA (Forward-5′-GCCCTGGTTCTGGAGGT AAC-3′; Reverse-5′-TAGCTGGTTTCGGCTTCAGG-3′). Amplification was performed on a LightCycler480 (Roche, Basel, Switzerland) using LightCycler 480 SYBR Green I Mastermix (Roche) under the following conditions: preincubation 50 °C for 2 min, 95 °C for 2 min, amplification at 95 °C for 15 s and then at 55–65 °C for 30 s.

Immunofluorescence

Cells were cultured on poly-d-lysine and fibronectin-coated glass coverslips and then incubated with or without BrdU (3 μg/ml) for 4 h before being fixed in 4% paraformaldehyde (Sigma) for 15 min on ice. Cells were then washed in PBS, and BrdU slides were incubated in HCl (1 N) for 10 min. Cells were permeabilized and blocked in staining buffer 1 (DPBS supplemented with 0.1% triton-X, 10% fetal calf serum) for 1 h before incubation with primary antibodies overnight at 4 °C. Slides were then washed 3 × in DPBS and then co-labelled with Alexa-Fluor secondary antibodies (Invitrogen) for 1 h. Slides were washed in PBS again before a 10-min incubation with 100 ng/ml DAPI solution (Sigma). Slides were then washed a final time in distilled water and then mounted with Fluorogel aqueous anti-fade media (ProSciTech, Townsville City, QLD, Australia).

Antibodies used

The antibodies used were CREB (Genesearch no. 9197L), pCREB Serine 133 (Genesearch no. 9197), BrdU (Genesearch no. 5292), pH3 (Genesearch no. 9701S), AKT (Genesearch no. 9272), pAKT Threonine 308 (Genesearch no. 9275S), MAPK (Genesearch no. 9102), pMAPK Threonine 202/Tyrosine 204 (Genesearch no. 9101), PKA-C (Genesearch no. 4782), pPKA-C Threonine197 (Genesearch no. 4781), Cyclin D1 (Abcam no. ab95281), Cyclin B1 (Abcam no. ab2949), PCNA (Genesearch no. 2586), and GAPDH (Genesearch no. 2118S). All primary antibodies were used at 1:2000 dilutions for western blottings and 1:100 for immunofluorescence or immunohistochemistry. Blots were then developed with enhanced chemiluminescence reagent (Bio-Rad, Hercules, CA, USA) and imaging performed on a Microchemi (DNR BioImaging Systems, South San Francisco, CA, USA).

Statistical analysis

Data are presented as mean ± s.e.m. of at least three independent experiments. *P*-values were determined using a pairwise two-tailed Student's *t*-test, with $P < 0.05$ considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank the members of the Department of Pathology, TheUniversityofMelbourne, Parkville, Victoria, Australia for the use of equipment and reagents, especially Dr Genevieve Evin, Dr Vicki Lawson, Laura Ellett and Janine James. We also thank Professor Paul Waring for financial support for PD, GF and DVB and the Cell Signaling Laboratory. Part of this work was also supported by an EU-FP7-PEOPLE Marie Curie IRG (Neurogencreb 231032) grant to TM. WAP, RGR and JM were supported, in part, by project grants from the National Health and Medical Research Council of Australia.

REFERENCES

1 Hegi ME, Diserens A-C, Gorlia T, Hamou MF, de Tribolet N, Weller M *et al.* MGMT gene silencing and benefit from temozolomide in glioblastoma. *New Engl J Med* 2005; **352**: 997–1003.
2 Gilbert MR, Dignam J, Won M, Bluementhal DT, Vogelbaum MA, Aldape KD *et al.* RTOG 0825: Phase III double-blind placebo-controlled trial evaluating bevacizumab (Bev) in patients (Pts) with newly diagnosed glioblastoma (GBM). *J Clin Oncol* 2013; **31**: Suppl(abstr 1).
3 Lassen U, Sorensen M, Gaziel TB, Hasselbalch B, Poulsen HS. Phase II study of bevacizumab and temsirolimus combination therapy for recurrent glioblastoma multiforme. *Anticancer Res* 2013; **33**: 1657–1660.
4 Gao BA, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO *et al.* Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal* 2013; **6**: pl1.
5 Vitucci M, Karpinich NO, Bash RE, Werneke AM, Schmid RS, White KK *et al.* Cooperativity between MAPK and PI3K signaling activation is required for glioblastoma pathogenesis. *Neuro Oncol* 2013; **15**: 1317–1329.
6 Grossmann TN, Yeh JT, Bowman BR, Chu Q, Moellering RE, Verdine GL. Inhibition of oncogenic Wnt signaling through direct targeting of beta-catenin. *Proc Natl Acad Sci USA* 2012; **109**: 17942–17947.
7 Mertz JA, Conery AR, Bryant BM, Sandy P, Balasubramanian S, Mele DA *et al.* Targeting MYC dependence in cancer by inhibiting BET bromodomains. *Proc Natl Acad Sci USA* 2011; **108**: 16669–16674.
8 Soucek L, Whitfield J, Martins CP, Finch AJ, Murphy DJ, Sodir NM *et al.* Modelling Myc inhibition as a cancer therapy. *Nature* 2008; **455**: 679–683.
9 Sukhdeo K, Mani M, Zhang Y, Dutta J, Yasui H, Rooney MD *et al.* Targeting the beta-catenin/TCF transcriptional complex in the treatment of multiple myeloma. *Proc Natl Acad Sci USA* 2007; **104**: 7516–7521.
10 Mayr B, Montminy M. Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat Rev Mol Cell Biol* 2001; **2**: 599–609.
11 Shaywitz AJ, Greenberg ME. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annu Rev Biochem* 1999; **68**: 821–861.
12 Cheng JC, Kinjo K, Judelson DR, Chang J, Wu WS, Schmid I *et al.* CREB is a critical regulator of normal hematopoiesis and leukemogenesis. *Blood* 2008; **111**: 1182–1192.
13 Shankar DB, Sakamoto KM. The role of cyclic-AMP binding protein (CREB) in leukemia cell proliferation and acute leukemias. *Leuk Lymphoma* 2004; **45**: 265–270.
14 Sampurno S, Bijenhof A, Cheasley D, Xu H, Robine S, Hilton D *et al.* The Myb-p300-CREB axis modulates intestine homeostasis, radiosensitivity and tumorigenesis. *Cell Death Dis* 2013; **4**: e605.
15 Fan CF, Mao XY, Wang EH. Elevated p-CREB-2 (ser 245) expression is potentially associated with carcinogenesis and development of breast carcinoma. *Mol Med Rep* 2012; **5**: 357–362.
16 Johannessen CM, Johnson LA, Piccioni F, Townes A, Frederick DT, Donahue MK *et al.* A melanocyte lineage program confers resistance to MAP kinase pathway inhibition. *Nature* 2013; **504**: 138–142.
17 Tan X, Wang S, Zhu L, Wu C, Yin B, Zhao J *et al.* cAMP response element-binding protein promotes gliomagenesis by modulating the expression of oncogenic microRNA-23a. *Proc Natl Acad Sci USA* 2012; **109**: 15805–15810.
18 Tan X, Wang S, Yang B, Yin B, Chao T, Zhao J *et al.* The CREB-miR-9 negative feedback minicircuitry coordinates the migration and proliferation of glioma cells. *PLoS ONE* 2012; **7**: e49570.
19 Lonze BE, Riccio A, Cohen S, Ginty DD. Apoptosis, axonal growth defects, and degeneration of peripheral neurons in mice lacking CREB. *Neuron* 2002; **34**: 371–385.
20 Mantamadiotis T, Lemberger T, Bleckmann SC, Kern H, Kretz O, Martin Villalba A *et al.* Disruption of CREB function in brain leads to neurodegeneration. *Nat Genet* 2002; **31**: 47–54.
21 Dworkin S, Heath JK, deJong-Curtain TA, Hogan BM, Lieschke GJ, Malaterre J *et al.* CREB activity modulates neural cell proliferation, midbrain-hindbrain organization and patterning in zebrafish. *Dev Biol* 2007; **307**: 127–141.
22 Dworkin S, Malaterre J, Hollande F, Darcy PK, Ramsay RG, Mantamadiotis T. cAMP response element binding protein is required for mouse neural progenitor cell survival and expansion. *Stem Cells* 2009; **27**: 1347–1357.
23 Siu YT, Jin DY. CREB-a real culprit in oncogenesis. *FEBS J* 2007; **274**: 3224–3232.
24 Gary DS, Mattson MP. PTEN regulates Akt kinase activity in hippocampal neurons and increases their sensitivity to glutamate and apoptosis. *Neuromolecular Med* 2002; **2**: 261–269.
25 Kinross KM, Montgomery KG, Kleinschmidt M, Waring P, Ivetac I, Tikoo A *et al.* An activating Pik3ca mutation coupled with Pten loss is sufficient to initiate ovarian tumorigenesis in mice. *J Clin Invest* 2012; **122**: 553–557.
26 Tikoo A, Roh V, Montgomery KG, Ivetac I, Waring P, Pelzer R *et al.* Physiological levels of Pik3ca(H1047R) mutation in the mouse mammary gland results in ductal hyperplasia and formation of ERalpha-positive tumors. *PLoS ONE* 2012; **7**: e36924.
27 Trejo CL, Green S, Marsh V, Collisson EA, Iezza G, Phillips WA *et al.* Mutationally activated PIK3CA(H1047R) cooperates with BRAF(V600E) to promote lung cancer progression. *Cancer Res* 2013; **73**: 6448–6461.
28 Wang P, Huang S, Wang F, Ren Y, Hehir M, Wang X *et al.* Cyclic AMP-response element regulated cell cycle arrests in cancer cells. *PLoS ONE* 2013; **8**: e65661.
29 Catalano S, Giordano C, Rizza P, Gu G, Barone I, Bonofiglio D *et al.* Evidence that leptin through STAT and CREB signaling enhances cyclin D1 expression and promotes human endometrial cancer proliferation. *J Cell Physiol* 2009; **218**: 490–500.
30 Weinberg RA. *The Biology of Cancer*. Garland Science: New York, NY, USA, 2007.
31 Zuloaga R, Fuentes EN, Molina A, Valdes JA. The cAMP response element binding protein (CREB) is activated by insulin-like growth factor-1 (IGF-1) and regulates myostatin gene expression in skeletal myoblast. *Biochem Biophys Res Commun* 2013; **440**: 258–264.
32 Liu B, Barbosa-Sampaio H, Jones PM, Persaud SJ, Muller DS. The CaMK4/CREB/IRS-2 cascade stimulates proliferation and inhibits apoptosis of beta-cells. *PLoS ONE* 2012; **7**: e45711.
33 Fatrai S, Elghazi L, Balcazar N, Cras-Méneur C, Krits I, Kiyokawa H *et al.* Akt induces beta-cell proliferation by regulating cyclin D1, cyclin D2, and p21 levels and cyclin-dependent kinase-4 activity. *Diabetes* 2006; **55**: 318–325.
34 Roberts EC, Shapiro PS, Nahreini TS, Pages G, Pouyssegur J, Ahn NG. Distinct cell cycle timing requirements for extracellular signal-regulated kinase and phosphoinositide 3-kinase signaling pathways in somatic cell mitosis. *Mol Cell Biol* 2002; **22**: 7226–7241.
35 Kandel ER. The molecular biology of memory: cAMP, PKA, CRE, CREB-1, CREB-2, and CPEB. *Mol Brain* 2012; **5**: 14.
36 Carlezon Jr WA, Duman RS, Nestler EJ. The many faces of CREB. *Trends Neurosci* 2005; **28**: 436–445.
37 Fusco S, Ripoli C, Podda MV, Ranieri SC, Leone L, Toietta G *et al.* A role for neuronal cAMP responsive-element binding (CREB)-1 in brain responses to calorie restriction. *Proc Natl Acad Sci USA* 2012; **109**: 621–626.
38 Conkright MD, Montminy M. CREB: the unindicted cancer co-conspirator. *Trends Cell Biol* 2005; **15**: 457–459.
39 Nakagawa S, Kim JE, Lee R, Malberg JE, Chen J, Steffen C *et al.* Regulation of neurogenesis in adult mouse hippocampus by cAMP and the cAMP response element-binding protein. *J Neurosci* 2002; **22**: 3673–3682.
40 Iourgenko V, Zhang W, Mickanin C, Daly I, Jiang C, Hexham JM *et al.* Identification of a family of cAMP response element-binding protein coactivators by genome-scale functional analysis in mammalian cells. *Proc Natl Acad Sci USA* 2003; **100**: 12147–12152.
41 Kvietikova I, Wenger RH, Marti HH, Gassmann M. The transcription factors ATF-1 and CREB-1 bind constitutively to the hypoxia-inducible factor-1 (HIF-1) DNA recognition site. *Nucleic Acids Res* 1995; **23**: 4542–4550.
42 Wu D, Zhau HE, Huang WC, Iqbal S, Habib FK, Sartor O *et al.* cAMP-responsive element-binding protein regulates vascular endothelial growth factor expression: implication in human prostate cancer bone metastasis. *Oncogene* 2007; **26**: 5070–5077.
43 Hlobilkova A, Ehrmann J, Knizetova P, Krejci V, Kalita O, Kolar Z. Analysis of VEGF, Flt-1, Flk-1, nestin and MMP-9 in relation to astrocytoma pathogenesis and progression. *Neoplasma* 2009; **56**: 284–290.
44 Ishiwata T, Teduka K, Yamamoto T, Kawahara K, Matsuda Y, Naito Z. Neuroepithelial stem cell marker nestin regulates the migration, invasion and growth of human gliomas. *Oncol Rep* 2011; **26**: 91–99.
45 Kitai R, Horita R, Sato K, Yoshida K, Arishima H, Higashino Y *et al.* Nestin expression in astrocytic tumors delineates tumor infiltration. *Brain Tumor Pathol* 2010; **27**: 17–21.
46 Peltier J, O'Neill A, Schaffer DV. PI3K/Akt and CREB regulate adult neural hippocampal progenitor proliferation and differentiation. *Dev Neurobiol* 2007; **67**: 1348–1361.
47 Molina JR, Hayashi Y, Stephens C, Georgescu MM. Invasive glioblastoma cells acquire stemness and increased Akt activation. *Neoplasia* 2010; **12**: 453–463.
48 Zohrabian VM, Forzani B, Chau Z, Murali R, Jhanwar-Uniyal M. Rho/ROCK and MAPK signaling pathways are involved in glioblastoma cell migration and proliferation. *Anticancer Res* 2009; **29**: 119–123.
49 Uht RM, Amos S, Martin PM, Riggan AE, Hussaini IM. The protein kinase C-eta isoform induces proliferation in glioblastoma cell lines through an ERK/Elk-1 pathway. *Oncogene* 2007; **26**: 2885–2893.
50 Yang C, Li X, Wang Y, Zhao L, Chen W. Long non-coding RNA UCA1 regulated cell cycle distribution via CREB through PI3-K dependent pathway in bladder carcinoma cells. *Gene* 2012; **496**: 8–16.
51 Stewart R, Flechner L, Montminy M, Berdeaux R. CREB is activated by muscle injury and promotes muscle regeneration. *PLoS ONE* 2011; **6**: e24714.

52 Stroescu C, Dragnea A, Ivanov B, Pechianu C, Herlea V, Sgarbura O *et al.* Expression of p53, Bcl-2, VEGF, Ki67 and PCNA and prognostic significance in hepatocellular carcinoma. *J Gastrointestin Liver Dis* 2008; **17**: 411–417.
53 Ben-Izhak O, Bar-Chana M, Sussman L, Dobiner V, Sandbank J, Cagnano M *et al.* Ki67 antigen and PCNA proliferation markers predict survival in anorectal malignant melanoma. *Histopathology* 2002; **41**: 519–525.
54 Barbieri F, Lorenzi P, Ragni N, Schettini G, Bruzzo C, Pedullà F *et al.* Overexpression of cyclin D1 is associated with poor survival in epithelial ovarian cancer. *Oncology* 2004; **66**: 310–315.
55 Aaltonen K, Amini RM, Heikkila P, Aittomäki K, Tamminen A, Nevanlinna H *et al.* High cyclin B1 expression is associated with poor survival in breast cancer. *Br J Cancer* 2009; **100**: 1055–1060.
56 Lobo GP, Waite KA, Planchon SM, Romigh T, Houghton JA, Eng C. ATP modulates PTEN subcellular localization in multiple cancer cell lines. *Hum Mol Genet* 2008; **17**: 2877–2885.
57 Metz HE, Houghton AM. Insulin receptor substrate regulation of phosphoinositide 3-kinase. *Clin Cancer Res* 2011; **17**: 206–211.
58 Hirashima Y, Tsuruzoe K, Kodama S, Igata M, Toyonaga T, Ueki K *et al.* Insulin down-regulates insulin receptor substrate-2 expression through the phosphatidylinositol 3-kinase/Akt pathway. *J Endocrinol* 2003; **179**: 253–266.
59 Weng LP, Smith WM, Brown JL, Eng C. PTEN inhibits insulin-stimulated MEK/MAPK activation and cell growth by blocking IRS-1 phosphorylation and IRS-1/Grb-2/Sos complex formation in a breast cancer model. *Hum Mol Genet* 2001; **10**: 605–616.
60 Kim YM, Geiger TR, Egan DI, Sharma N, Nyborg JK. The HTLV-1 tax protein cooperates with phosphorylated CREB, TORC2 and p300 to activate CRE-dependent cyclin D1 transcription. *Oncogene* 2010; **29**: 2142–2152.
61 Pradeep A, Sharma C, Sathyanarayana P, Albanese C, Fleming JV, Wang TC *et al.* Gastrin-mediated activation of cyclin D1 transcription involves beta-catenin and CREB pathways in gastric cancer cells. *Oncogene* 2004; **23**: 3689–3699.
62 Boulon S, Dantonel JC, Binet V, Vié A, Blanchard JM, Hipskind RA *et al.* Oct-1 potentiates CREB-driven cyclin D1 promoter activation via a phospho-CREB- and CREB binding protein-independent mechanism. *Mol Cell Biol* 2002; **22**: 7769–7779.
63 Kim MJ, Sun Y, Yang H, Kim NH, Jeon SH, Huh SO. Involvement of the cAMP response element binding protein, CREB, and cyclin D1 in LPA-induced proliferation of P19 embryonic carcinoma cells. *Mol Cells* 2012; **34**: 323–328.

Supplementary Information accompanies this paper on the Oncogenesis website (http://www.nature.com/oncsis)