

# Involvement of the mRNA binding protein CRD-BP in the regulation of metastatic melanoma cell proliferation and invasion by hypoxia

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## Summary

We have previously shown that the mRNA binding protein CRD-BP is overexpressed in human melanomas, where it promotes cell survival and resistance to chemotherapy. The present study investigates the role of hypoxia, a common characteristic of the tumor microenvironment, in the regulation of CRD-BP expression and melanoma cell responses. We found that hypoxia increases CRD-BP levels in metastatic melanoma cell lines but not in melanocytes or primary melanoma cells. Hypoxic stimulation transcriptionally regulates CRD-BP by facilitating the acetylation of histones within the CRD-BP gene and by modulating the extent of HIF1 $\alpha$  binding to the CRD-BP promoter. Hypoxia significantly enhances the proliferative and invasive potential of metastatic melanoma cells but not that of normal or primary melanoma cells. Furthermore, inhibition of CRD-BP impairs the ability of metastatic cells to proliferate and invade in response to hypoxia. These findings identify CRD-BP as a novel effector of hypoxic responses that is relevant for the selection of metastatic cells. This work also describes a previously unknown role for CRD-BP in the regulation of melanoma cell invasion and highlights the importance of the hypoxic microenvironment in determining cell fate.

**Key words:** CRD-BP, Hypoxia, Melanoma

## Introduction

Melanoma is the sixth most common cancer in the United States and the majority of disease-related deaths are attributed to the aggressive malignant stage (Jemal et al., 2011; Wilkerson, 2011). Growing evidence suggests that hypoxia is an important determinant of malignant progression and disease outcome. Indeed, several studies have demonstrated a strong association between the development of metastatic disease and the proportion of hypoxic cells in primary tumors (Hockel et al., 1996; Milosevic et al., 2004). Furthermore, intratumoral hypoxia predisposes to disease recurrence and decreases overall patient survival (Milosevic et al., 2004). Hence, identifying the specific mechanisms through which hypoxia affects melanoma cell behavior is essential to help uncover novel therapeutic targets against melanoma.

Hypoxia inducible factor 1 alpha (HIF1 $\alpha$ ) is a well characterized effector of hypoxic signals. Under low oxygen conditions, HIF1 $\alpha$  is translocated into the nucleus, where it heterodimerizes with the constitutively expressed HIF1 $\beta$  and recruits transcriptional co-activators such as CBP/p300, SCR-1 and TIF2 (Semenza, 2007). This HIF1 complex drives the transcription of numerous target genes such as drug transporters, pro-angiogenic molecules and cell motility enhancers (Semenza, 2007). HIF1 $\alpha$  is highly expressed in multiple malignancies including melanoma, breast and colorectal cancer, where it is associated with poor prognosis (Keith et al., 2012). Interestingly, the mRNA binding molecule coding region-determinant binding protein (CRD-BP) is also overexpressed in these cancers.

Furthermore, CRD-BP and HIF1 $\alpha$  similarly regulate cellular behavior through their interaction with molecules such as Gli1 and MDR-1 (Comerford et al., 2002; Sparanese and Lee, 2007; Noubissi et al., 2009; Wang et al., 2010b). However, it is not known whether HIF1 $\alpha$  and CRD-BP work in concert or independently to govern melanoma cell responses.

CRD-BP is increased during development, where it promotes embryonic growth, but its levels are low or undetectable after birth (Hansen et al., 2004; Tessier et al., 2004). Reactivation of CRD-BP in adult tissue promotes tumor formation and is associated with poor prognosis, indicating a role for this molecule in tumorigenesis and cancer progression (Tessier et al., 2004; Dimitriadis et al., 2007). CRD-BP functions in normal and disease states by binding to target mRNAs and influencing their stability, translation and cellular localization. Nevertheless, the specific mechanisms regulating CRD-BP levels in melanoma have not been elucidated. In this report we reveal that hypoxia promotes epigenetic modifications in the CRD-BP gene, enhances the interaction of HIF1 $\alpha$  with the CRD-BP promoter and increases CRD-BP expression in metastatic melanoma cells but not in melanocytes or primary melanoma cells. These hypoxia-mediated increases in CRD-BP levels contribute to the enhancement of malignant melanoma cell proliferation and invasion. Taken together, these findings highlight a previously unknown CRD-BP signaling network and suggest that hypoxia exerts selective pressure for the more aggressive metastatic cell phenotype through the regulation of CRD-BP expression.

## Results and Discussion

### Hypoxia differentially regulates CRD-BP in melanoma cells

Through Western blotting, we found that CRD-BP protein levels are greatly induced in metastatic melanoma cells (Skmel2), but not in melanocytes (NHEM) or primary melanoma cells (WM35), subjected to hypoxia (Fig. 1A). Similar results were observed in five other melanoma cell lines of human and mouse origin (supplementary material Fig. S1A). Thus, hypoxia differentially regulates CRD-BP in melanoma cells, depending on the cellular phenotype. Because HIF1 $\alpha$  is a major transcriptional mediator of hypoxic stimuli (Majmundar et al., 2010), HIF1 $\alpha$  protein levels were also assessed. Hypoxia increases HIF1 $\alpha$  expression in all cell lines studied (Fig. 1A; supplementary material Fig. S1A), suggesting that this transcription factor contributes to the transduction of hypoxic signals in these cells.

Real-time PCR analysis shows that hypoxic stimulation increases CRD-BP mRNA in metastatic melanoma cells but not in melanocytes or primary melanoma cells (Fig. 1B; supplementary material Fig. S1B). Thus, hypoxia-mediated changes in CRD-BP expression occur both at the mRNA and protein levels. Pretreatment of Skmel2 cells with actinomycin D, an inhibitor of transcription, significantly decreases the ability of hypoxia to induce CRD-BP mRNA expression (Fig. 1B), suggesting that hypoxia regulates CRD-BP at the transcriptional level.

### Binding of HIF1 $\alpha$ to the CRD-BP promoter is important for the transcriptional activation of CRD-BP by hypoxia

We next assessed the effect of hypoxia on CRD-BP promoter activity by transfecting cells with a CRD-BP promoter-driven luciferase construct (CRD-BP-Luc). Our results show that hypoxia significantly enhances CRD-BP-promoter driven luciferase activity in Skmel2 cells but not in NHEM or WM35 cells (Fig. 2A). These results coincide with changes observed in CRD-BP expression and suggest that hypoxia induces CRD-BP in metastatic melanoma cells through the modulation of its promoter.

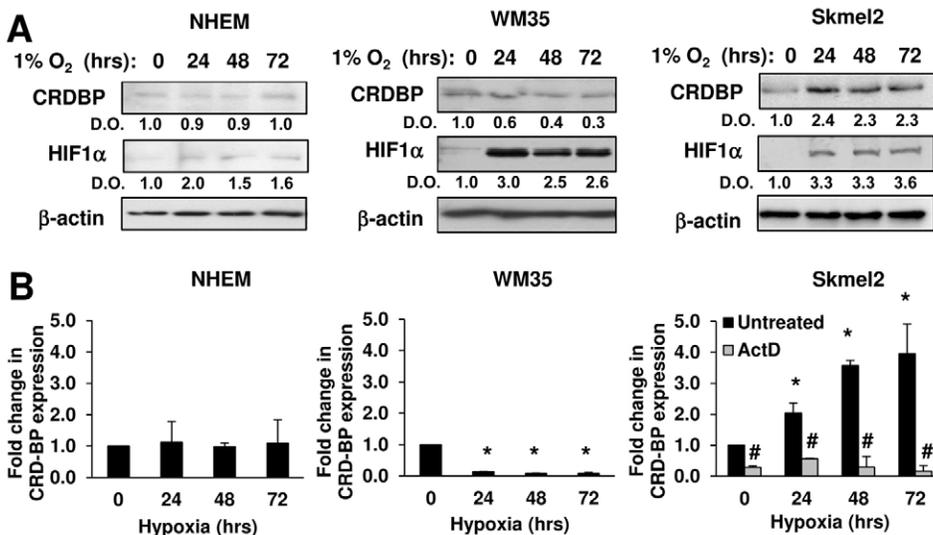
In silico analysis identified three putative HIF1 binding sites within the human CRD-BP promoter (supplementary material Fig. S2), raising the possibility of a direct regulation of CRD-BP

transcription by HIF1 $\alpha$ . To assess this, each HIF1 binding site within the CRD-BP-Luc construct was subjected to site-directed mutagenesis. Individual mutation of HIF binding site 1 or 3 partially decreases CRD-BP-promoter driven luciferase activity following hypoxic stimulation of Skmel2 cells (Fig. 2A). Concurrent mutation of HIF binding sites 1 and 3 diminishes luciferase activity close to basal levels (Fig. 2A), indicating that these sites contribute to the regulation of CRD-BP transcription by hypoxia. This is the first study to our knowledge demonstrating a direct relationship between the availability of HIF1 $\alpha$  binding sites and the activation of the CRD-BP gene in response to hypoxia.

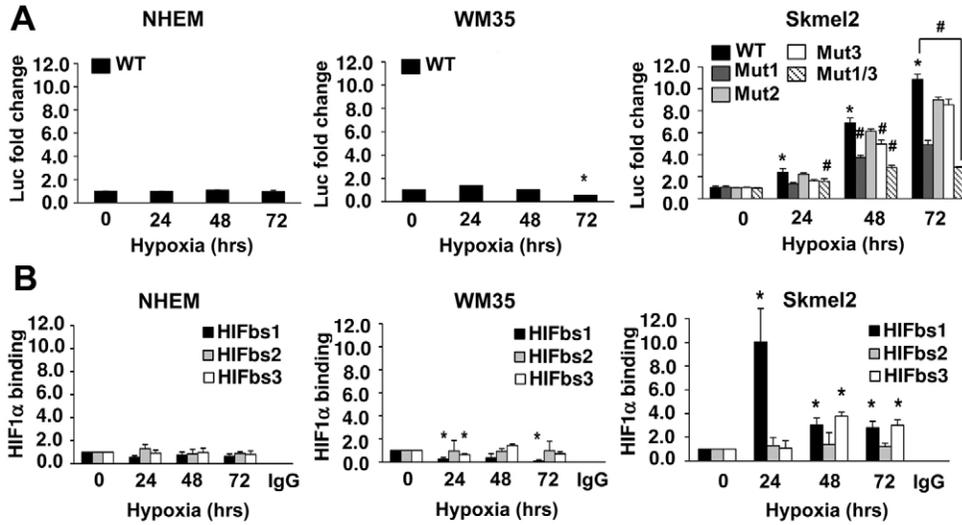
To confirm that HIF1 $\alpha$  directly interacts with the CRD-BP promoter we performed chromatin immunoprecipitation (ChIP) coupled with real-time PCR. Hypoxia increases HIF1 $\alpha$  binding to sites 1 and 3 within the CRD-BP promoter of Skmel2 cells but has no effect or even decreases HIF1 $\alpha$  binding in NHEM or WM35 cells (Fig. 2B). These data demonstrate that hypoxia differentially regulates HIF1 $\alpha$  binding to the CRD-BP promoter depending on the cell phenotype. Thus, even though HIF1 $\alpha$  is overexpressed in all cell lines studied following hypoxia, additional mechanisms may promote or prevent binding of HIF1 $\alpha$  to the CRD-BP promoter.

### Hypoxic induction of CRD-BP is dependent on the acetylation status of histones at the CRD-BP promoter

Because hypoxia has been shown to regulate gene expression by post-translationally modifying promoter-bound histones (Newton et al., 2010; Wang et al., 2010a), we next examined the role of histone acetylation in the modulation of CRD-BP transcription by hypoxia. For this, we performed ChIP analysis in combination with real-time PCR and utilized specific primers spanning the HIF1 binding sites within the CRD-BP promoter. We found that Skmel2 cells, but not NHEM or WM35 cells, exhibit enhanced histone H3 and H4 acetylation at HIF1 binding sites 1 and 3 in response to hypoxia (Fig. 3A,B). These changes are not due to alterations in histone-DNA binding patterns as total histone H3 and H4 levels bound to the CRD-BP promoter are not affected by hypoxia (supplementary material Fig. S3). Differences in histone acetylation in melanoma cells coincide with changes in HIF1 $\alpha$



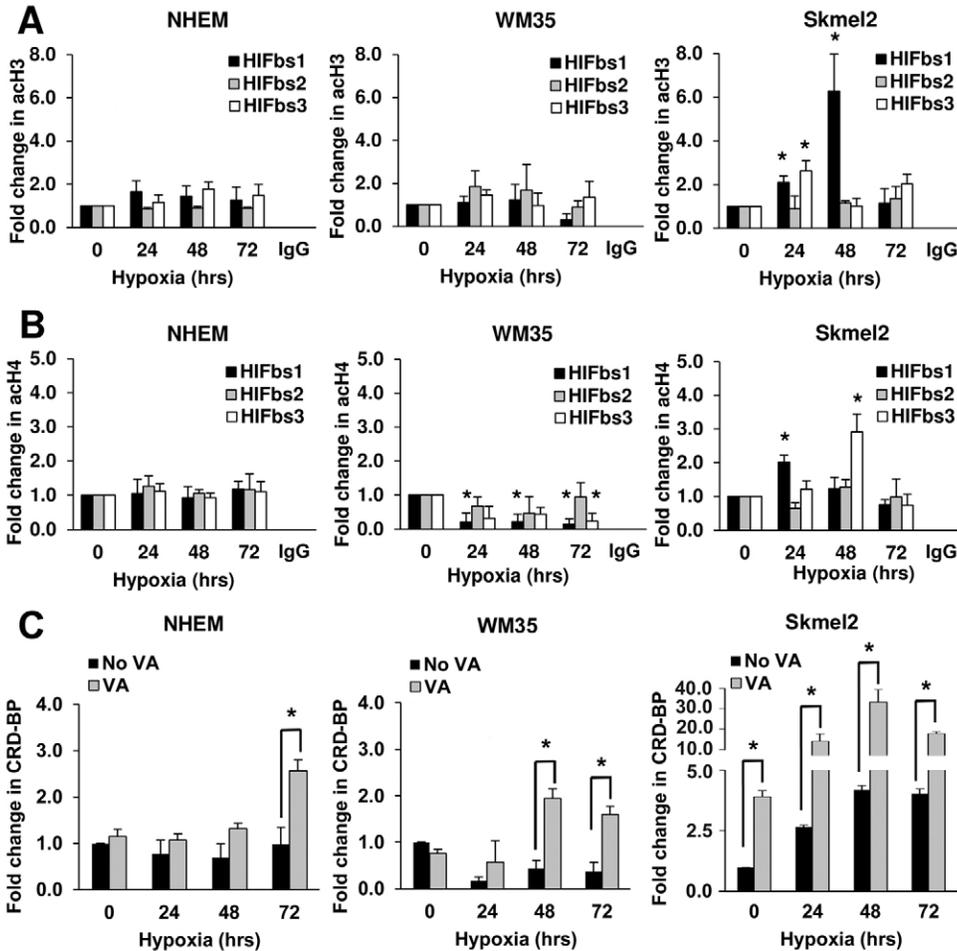
**Fig. 1. Hypoxia regulates CRD-BP expression in melanoma cells but not in melanocytes.** (A) Lysates from hypoxia-treated cells were probed by western blotting with the antibodies shown on the left. D.O. denotes relative densitometry values. (B) Cells pretreated with or without actinomycin D were subjected to hypoxia followed by real-time PCR analysis for CRD-BP. The data are displayed as mean fold-change values  $\pm$  s.d. \* $P$ <0.05, compared with 0 hours of hypoxia. # $P$ <0.05, compared with untreated cells (no actinomycin D).



**Fig. 2. Hypoxia regulates HIF1 $\alpha$  binding and activation of the CRD-BP promoter.** (A) Cells were transfected with either wild-type (WT) or mutant (Mut) Luc-CRD-BP and pSV40  $\beta$ -gal. After hypoxia treatment, luciferase levels were measured by luminometry and normalized to  $\beta$ -gal. The data are expressed as mean fold-change values  $\pm$  s.d. (B) Hypoxia-treated cells were subjected to ChIP with HIF1 $\alpha$  antibody or mouse IgG as a negative control. ChIP samples were analyzed by real-time PCR using primers spanning the HIF1 binding sites within the CRD-BP promoter. Results are shown as mean fold-changes in HIF1 $\alpha$  binding  $\pm$  s.d. \* $P$ <0.05, compared with 0 hours of hypoxia. # $P$ <0.05, compared with wild-type cells.

binding to CRD-BP and alterations in CRD-BP levels (Figs 1, 2, 3). This coincides with previous studies showing that hypoxia can promote histone acetylation to induce gene activation (Wang et al., 2010a) but it can also decrease histone acetylation to repress gene expression (Newton et al., 2010).

To further assess the importance of histone acetylation in the regulation of CRD-BP by hypoxia we employed valproic acid (VA), a drug known to increase the acetylation of histone H3 and H4 in melanoma cells (Facchetti et al., 2004). Real-time PCR data show that pretreatment with VA significantly increases



**Fig. 3. Acetylation of CRD-BP-associated histones is important for hypoxia to induce CRD-BP expression.** (A,B) Cells cultured under hypoxia were subjected to ChIP analysis with an antibody directed to (A) acetylated histone H3 (ach3) or (B) acetylated histone H4 (ach4). The precipitated chromatin was quantified by real-time PCR using primers spanning the putative HIF1 binding sites. Mouse IgG was used as a negative control. The data are presented as mean fold-change values  $\pm$  s.d. \* $P$ <0.05, compared to 0 hours of hypoxia. (C) Cells treated with or without valproic acid (VA) were subjected to hypoxia and analyzed by real-time PCR for CRD-BP. Results are shown as mean fold-changes in CRD-BP mRNA levels  $\pm$  s.d., \* $P$ <0.05, compared with no valproic acid.

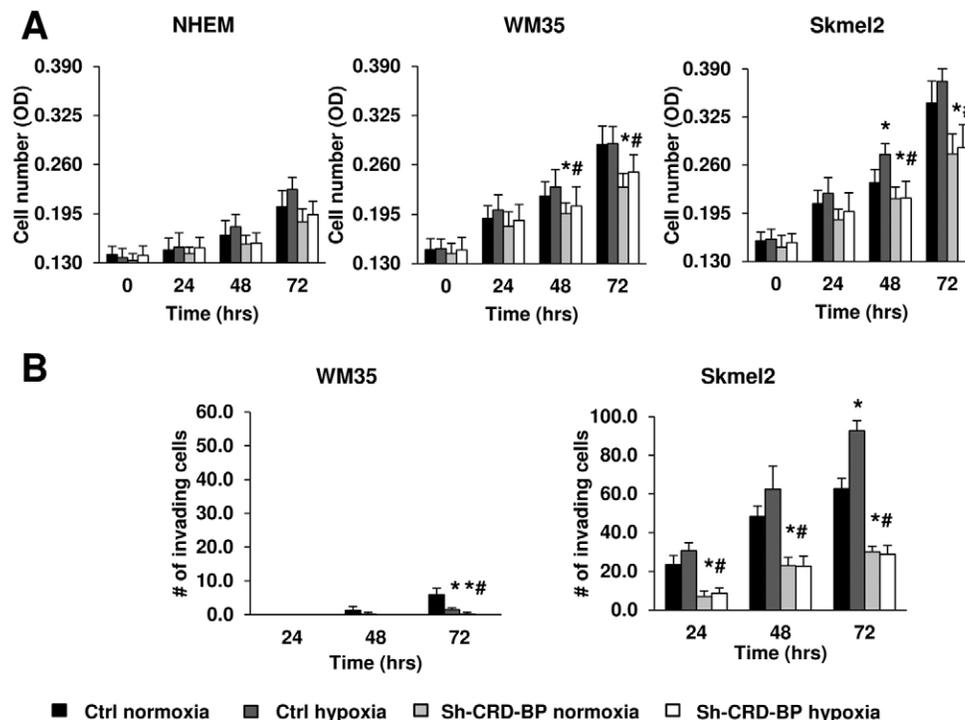
CRD-BP expression in response to hypoxia in NHEM and WM35 cells, even though hypoxia alone either has no effect or even decreases CRD-BP levels (Fig. 3C). The VA-mediated induction of CRD-BP levels in non-metastatic cells coincides with increased HIF1 binding to the CRD-BP promoter, indicating that increased histone acetylation facilitates HIF1 binding (supplementary material Fig. S4). In the case of Skmel2 cells, the presence of VA in combination with hypoxic stimulation induces CRD-BP expression 5 to 7 fold more than what is observed with hypoxia alone (Fig. 3C). Hence, the acetylation status of histones surrounding the CRD-BP promoter is an important determinant of the extent of CRD-BP expression in response to hypoxia.

### Regulation of CRD-BP by hypoxia enhances malignant melanoma cell proliferation and invasion while it diminishes the invasive response of primary melanoma cells

Because hypoxia has been shown to play a role in cancer cell proliferation and motility (Schmaltz et al., 1998; Li and Lu, 2010), we assessed whether CRD-BP contributes to hypoxia-mediated changes in these responses. Cells were transfected with scrambled sh-RNA (control) or a specific sh-RNA against CRD-BP (sh-CRD-BP) in the presence or absence of hypoxia. Proliferation was assessed with the MTS assay. We found that inhibition of CRD-BP diminishes proliferation in melanoma cells and that hypoxia induces a transient increase in metastatic cell proliferation that is abrogated in the presence of sh-CRD-BP (Fig. 4A; supplementary material Fig. S5A). Therefore, CRD-BP is important for melanoma cells to proliferate and it also contributes to the enhancement of metastatic melanoma cell growth by hypoxia.

The role of hypoxia and CRD-BP in regulating melanoma cell motility was examined using matrigel invasion assays. In WM35 cells, hypoxia alone decreases invasion and inhibition of CRD-BP in the presence of hypoxia further diminishes this response (Fig. 4B; supplementary material Fig. S6). On the other hand, WM35 cells overexpressing CRD-BP and subjected to hypoxia invade at similar rates as normoxic control cells (supplementary material Fig. S5B). In metastatic cells, hypoxia significantly increases cell invasion in controls after 72 hours. In the presence of sh-CRD-BP, metastatic melanoma cells not only invade less compared to controls but are also unresponsive to hypoxia (Fig. 4B; supplementary material Fig. S5C and Fig. S6). Our sh-CRD-BP construct is specific against human CRD-BP as expression of an RNAi-resistant form of CRD-BP rescues the invasive phenotype (supplementary material Fig. S5D). These findings indicate that hypoxia regulates CRD-BP to enhance metastatic melanoma cell invasion while inhibiting that of primary melanoma cells. Although CRD-BP has been shown to participate in the regulation of cancer cell migration (Stöhr et al., 2012), this is the first study demonstrating that CRD-BP is important for the invasion of melanoma cells through an extracellular matrix, suggesting that CRD-BP overexpression may contribute to the spread of cancer cells.

In summary, we report that CRD-BP is a novel hypoxia-responsive gene and identify a previously unknown interaction between CRD-BP and HIF1 $\alpha$  that is dependent on the acetylation status of the CRD-BP promoter. This work also describes a relevant role for CRD-BP in relaying hypoxic signals. Furthermore, hypoxia-mediated induction of CRD-BP acts as a mechanism to selectively enhance cell proliferation and invasion in metastatic melanoma cells but not in melanocytes or primary melanoma cells. Therefore, targeted disruption of CRD-BP may



**Fig. 4. CRD-BP is important for the regulation of proliferation and invasion by hypoxia.** (A) Cells were transfected with scrambled sh-RNA (Ctrl) or sh-RNA against CRD-BP (Sh-CRD-BP) and cultured under normoxia or hypoxia for 0–72 hours. Cell proliferation was assessed by the MTS assay. The data are shown as mean absorbance values  $\pm$  s.d. (B) Cells were transfected as for A and placed into tri-dimensional invasion chambers and subjected to normoxia or hypoxia for 24–72 hours. Invading cells were stained and visualized by microscopy. Results are shown as the mean number of invading cells  $\pm$  s.d. \* $P$ <0.05, compared with control normoxia; # $P$ <0.05, compared with control hypoxia.

represent a novel strategy for preventing the selection and spread of malignant cells in the permissive skin microenvironment.

## Materials and Methods

### Cell culture, expression vectors and reagents

Media were prepared and cells were transfected as previously described (Craig and Spiegelman, 2012). Cultures were maintained at 37°C with 95% ambient air and 5% CO<sub>2</sub>. For hypoxic conditions, cells were placed in an air tight chamber and gassed with 1% O<sub>2</sub>, 5% CO<sub>2</sub> and 94% N<sub>2</sub>. The pSV40 β-gal construct was obtained from Promega (Madison, WI) while the sh-CRD-BP, pcDNA-CRD-BP and CRD-BP-Luc plasmids were previously characterized (Noubissi et al., 2006; Noubissi et al., 2010). Mutant CRD-BP-Luc constructs were made by amino acid substitutions of putative HIF1α binding sites within the CRD-BP promoter. Primers employed are described in supplementary material Table S1. Actinomycin D and valproic acid were obtained from Sigma-Aldrich (St. Louis, MO).

### Western blot

Total cellular lysates were prepared using radio immunoprecipitation (RIPA) buffer. Proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane. After blocking with 3% milk, blots were probed with primary antibodies against CRD-BP, HIF1α, or β-actin (Santa Cruz Biotechnologies, Santa Cruz, CA), washed, and incubated with HRP-conjugated secondary antibodies for 1 hour. Detection was performed using ECL substrate (Perkin Elmer, Waltham, MA).

### Real-time PCR

Total RNA was prepared, cDNA was synthesized and real-time PCR was performed as previously described (Craig and Spiegelman, 2012). Primer sets are listed in supplementary material Table S2.

### Luciferase reporter assay

Cells transfected with pSV40 β-gal and CRD-BP-Luc were subjected to hypoxia and lysed using the reporter lysis buffer (Promega). Luciferase activity and β-galactosidase expression were assessed as described previously (Craig and Spiegelman, 2012). All luciferase values were normalized to β-galactosidase.

### Chromatin immunoprecipitation assay

ChIP was performed using the EZ ChIP Chromatin Immunoprecipitation Kit (Millipore, Billerica, MA) according to the manufacturer's recommendations. Briefly, treated or untreated cells were cross-linked, sonicated, and precleared with protein G agarose. 10 μl of each sample were collected as input and the remaining fractions were immunoprecipitated using antibodies against HIF1α, acetylated histone H3, acetylated histone H4, histone H3 or histone H4 (Santa Cruz Biotechnologies). Protein/DNA complexes were then eluted, reverse cross-linked and purified. The recovered DNA was subjected to real-time PCR using SYBR Green reagents (Applied Biosystems) and primers for the three putative HIF1α binding sites (supplementary material Table S3). Immunoprecipitated samples were normalized to inputs.

### MTS assay

Cells transfected with control sh-RNA or sh-CRD-BP were seeded onto 96-well plates and cultured under normoxia or hypoxia. Cell proliferation was assessed by adding 20 μl of CellTiter 96 Aqueous One solution (Promega) to cells in 100 μl of culture medium and incubating for 2 hours at 37°C. Absorbance values were recorded at 492 nm using a 96-well plate reader.

### Invasion assay

Cells transfected as indicated were seeded onto matrigel-coated 24-well inserts containing an 8 μm pore membrane (BD Biosciences, Bedford, MA). Media containing 10% FBS was placed in the bottom chamber as a chemoattractant. After attachment, cells were subjected to normoxia or hypoxia, fixed with 10% formalin and stained with 0.5% crystal violet. The amount of cells that invaded through the matrigel layer and porous membrane was assessed by light microscopy.

### Statistical analysis

Data are representative of at least 3 independent experiments and were analyzed using the 2-sample Student's *t*-test. The level of significance was established at *P* < 0.05.

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<http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.115204/-DC1>

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