The tumour suppressor C/EBP δ inhibits FBXW7 expression and promotes mammary tumour metastasis

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Inflammation and hypoxia are known to promote the metastatic progression of tumours. The CCAAT/enhancer-binding protein- δ (C/EBP δ , CEBPD) is an inflammatory response gene and candidate tumour suppressor, but its physiological role in tumorigenesis *in vivo* is unknown. Here, we demonstrate a tumour suppressor function of C/EBP δ using transgenic mice overexpressing the Neu/Her2/ERBB2 proto-oncogene in the mammary gland. Unexpectedly, this study also revealed that C/EBP δ is necessary for efficient tumour metastasis. We show that C/EBP δ is induced by hypoxia in tumours *in vivo* and in breast tumour cells *in vitro*, and that C/EBP δ -deficient cells exhibit reduced glycolytic metabolism and cell viability under hypoxia. C/EBP δ supports CXCR4 expression. On the other hand, C/EBP δ directly inhibits expression of the tumour suppressor F-box and WD repeat-domain containing 7 gene (FBXW7, FBW7, AGO, Cdc4), encoding an F-box protein that promotes degradation of the mammalian target of rapamycin (mTOR). Consequently, C/EBP δ enhances mTOR/AKT/S6K1 signalling and augments translation and activity of hypoxia-inducible factor-1 α (HIF-1 α), which is necessary for hypoxia adaptation. This work provides new insight into the mechanisms by which metastasis-promoting signals are induced specifically under hypoxia.

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Introduction

Aerobic life depends on oxygen, and suboptimal levels of O₂ trigger cellular responses to cope transiently with hypoxia. These include decrease in oxygen consumption through metabolic adaptation such as reduced mitochondrial activity and enhanced glycolysis, and increase in oxygen delivery through induced angiogenesis. Similar mechanisms are also adopted by tumour cells even under normoxia, which is one reason why hypoxia is thought to promote tumourigenesis (Weidemann and Johnson, 2008). Most of the responses to hypoxia are governed by heterodimeric transcription factors called hypoxia-inducible factors (HIFs). HIFs consist of two subunits: a constitutive β -subunit and one of three oxygen-dependently regulated α -subunits. The most studied hypoxia-inducible transcription factor is HIF-1 α . Collectively, most data point to a role of HIF-1 α in promoting tumourigenesis (Zhou *et al*, 2006; Yee Koh *et al*, 2008; Semenza, 2010). Under normoxia, the HIF-1 α protein is hydroxylated by ubiquitous iron- and O₂-dependent prolyl hydroxylases (PHDs) that target HIF-1 α for proteasomal degradation. Hence, reduced levels of oxygen, as found in solid tumours, stabilize the HIF-1 α subunit because of inactivation of PHDs (Wei and Yu, 2007). However, oncogenes and growth factors also contribute to upregulation of HIF-1 α expression, in part through stimulation of translation by a PI3K/AKT/mTOR-dependent pathway (Semenza, 2003; Yee Koh *et al*, 2008). In addition to supporting hypoxia adaptation, HIF-1 α contributes to cell migration and metastasis. Thus, hypoxia in tumours promotes selection of tumour cells with aggressive phenotype (Zhou *et al*, 2006). Mice deficient in mammary epithelial expression of HIF-1 α exhibit reduced tumour growth and significantly fewer lung metastases (Liao *et al*, 2007).

HIF-1 α expression and activity have also been documented in the immune system and injured tissue in which it participates in inflammatory responses (Frede *et al*, 2007; Zinkernagel *et al*, 2007). The transcription factor CCAAT/enhancer-binding protein- δ (C/EBP δ) is another inflammatory response gene that is induced by bacterial lipopolysaccharide in most cell types and tissues, and amplifies inflammatory signalling pathways (Ramji and Foka, 2002; Litvak *et al*, 2009). In this regard, C/EBP δ expression and function parallels that of HIF-1 α . However, in contrast to HIF-1 α , C/EBP δ has tumour suppressor-like activities. Loss of C/EBP δ causes genomic instability in mouse embryo fibroblasts (MEFs) *in vitro* (Huang *et al*, 2004). C/EBP δ promotes apoptosis during involution of the mouse mammary gland (Thangaraju *et al*, 2005) and inhibits the growth of human cell lines from several tumour types including breast *in vitro* (Gery *et al*, 2005; Agrawal *et al*, 2007; Pawar *et al*, 2010). In support of a tumour suppressor function *in vivo*, C/EBP δ protein expression correlates with low-grade histology and disease-free survival of meningioma patients (Barresi *et al*,

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2009), and C/EBP δ was among a 70-gene signature predicting better survival of breast cancer patients (Naderi *et al*, 2007). Furthermore, the C/EBP δ gene was found to be methylated in a significant number of acute myelomonocytic leukaemia, cervical and hepatocellular carcinomas and a subset of breast tumours (Gery *et al*, 2005; Tang *et al*, 2005; Agrawal *et al*, 2007; Ko *et al*, 2008), and its expression is downregulated with breast tumour progression (Porter *et al*, 2001). Although C/EBP δ contains a classical transcription activation domain, it can act both as an activator and an inhibitor of gene promoters (Lacorte *et al*, 1997; Okazaki *et al*, 2002; Ramji and Foka, 2002; Wang *et al*, 2006; Lai *et al*, 2008; Turgeon *et al*, 2008).

In this study, we have used transgenic technology to assess the role of C/EBP δ in mammary tumourigenesis *in vivo*. This approach confirmed a tumour suppressor function of C/EBP δ while also uncovering an unexpected pro-metastatic activity. We found that C/EBP δ supports HIF-1 α translation through inhibition of F-box and WD repeat-domain containing 7 gene (FBXW7) expression and, as a result, stabilization of the mammalian target of rapamycin (mTOR) protein. These data reveal a novel role for C/EBP δ in integration of hypoxia and inflammatory responses to promote tumour metastasis.

Results

Loss of C/EBP δ increases mammary tumour multiplicity and decreases lung metastasis

To assess the potential tumour suppressor function of C/EBP δ *in vivo*, we used MMTV-c-Neu transgenic mice. These mice express rat tyrosine kinase receptor Neu (ERBB2/HER2) specifically in mammary epithelial cells to mimic the over-expression of ERBB2 seen in over 30% of human breast cancers (Guy *et al*, 1992). This mouse model generates stochastic, slow-onset mammary tumours in female mice before the age of 1 year (Guy *et al*, 1992). In mice with a germ-line deletion of the C/EBP δ gene (Sterneck *et al*, 1998), tumour multiplicity was significantly increased (Figure 1A). C/EBP δ -deficient (knock out, KO) mice bore 50% more tumours than wild-type (WT) or heterozygous controls at the study end point, which was defined by the size of the primary tumour. Survival based on study end point was similar between genotypes (Supplementary Figure S1), and the tumours of different groups of mice were histologically and morphologically comparable and typical of Neu morphology (Guy *et al*, 1992). Given that the study end point was defined by the size of the primary tumour, this implies that loss of C/EBP δ does not affect the growth rate of the tumours. Rather, more tumours are initiated in the mutant mice with or during the time of primary tumour growth. Thus, we infer that C/EBP δ may inhibit the probability of tumour initiation and/or the number of target cells. Regardless of the mechanism, these data represent the first animal model to show that C/EBP δ acts as a tumour suppressor.

Mammary tumours of this transgenic model metastasize to the lung (Guy *et al*, 1992). For metastasis-bearing mice, the average metastatic burden was 0.4% of the total lung area and not affected by genotype ($P=0.1455$, by two-sample *t*-test; $n=8$ KO, 16 WT mice). Surprisingly, C/EBP δ KO animals were less likely to develop lung metastases, with only 40% of the C/EBP δ -mutant mice harbouring metastases, compared with 80% of the WT group (Figure 1B). As, in a

clinical setting, metastases are the primary cause of death from breast cancer, a 50% reduction or delay in the incidence of metastasis is an important outcome. Collectively, these results demonstrate that C/EBP δ has dual activity in mammary tumourigenesis: on the one hand, C/EBP δ inhibits primary tumour development and on the other hand, it augments metastasis formation.

C/EBP δ is required for hypoxic HIF-1 α accumulation and hypoxia adaptation

As previous reports documented reduced C/EBP δ mRNA expression with progression of breast tumourigenesis (Porter *et al*, 2001), we first asked when and where C/EBP δ was expressed. Immunohistochemistry of mature nulliparous mouse mammary glands, which—in contrast to human breast—contain mostly ductal epithelium, at different stages of the oestrous cycle revealed low levels of C/EBP δ protein in very few epithelial cells (data not shown). However, analysis of tumour tissue revealed significant, but regionally restricted, expression of C/EBP δ within the tumours. These regions often surrounded necrotic centres of the tumours typical of hypoxic regions. Indeed, analysis of parallel sections revealed that C/EBP δ expression correlated with hypoxic areas, which were visualized by immunohistochemistry of pimonidazole-derived protein modifications (Figure 1C). Because tumour hypoxia is strongly associated with increased metastasis formation (Sullivan and Graham, 2007) and the pro-metastatic activity of C/EBP δ is shared with the HIF-1 α protein (Liao *et al*, 2007), we next examined whether C/EBP δ modulated the expression of HIF-1 α . In a subset of C/EBP δ -expressing tumour areas, we could also detect expression of HIF-1 α (data not shown). For quantitative analysis of HIF-1 α expression, we exposed isolated primary mammary tumour cells to 1% oxygen or the iron chelator desferrioxamine (DFX), which inhibits PHD enzymes and thereby stabilizes HIF- α proteins (Hirsila *et al*, 2005). While HIF-1 α protein was highly induced in WT primary tumour cells under these treatments, cells lacking C/EBP δ exhibited significantly reduced HIF-1 α protein levels (Figure 1D). Expression of the HIF-1 β protein subunit is not regulated by hypoxia (Wei and Yu, 2007), and its levels were not affected in either cell type. This experiment confirmed that C/EBP δ expression was induced in WT cells by hypoxia or DFX (Figure 1D). We also examined expression of the HIF-1 target gene *CXCR4*, a chemokine receptor, which strongly promotes metastatic progression of tumours (Gelmini *et al*, 2008). Indeed, CXCR4 protein expression was induced in WT, but not in KO, primary tumour cells (Figure 1D). Reduced CXCR4 expression was consistent with reduced HIF-1 α levels in C/EBP δ -deficient cells. These data reveal a novel function for C/EBP δ in amplifying HIF-1 α expression and HIF-1 activity, which is consistent with the pro-metastatic activity of both these factors. The profound absence of CXCR4 expression in C/EBP δ -deficient cells even under normoxia led us to examine whether this gene was directly regulated by C/EBP δ . Indeed, chromatin immunoprecipitation (ChIP) and RNA analyses in MCF-7 cells support a direct role for C/EBP δ in CXCR4 expression (Supplementary Figure S2), which may contribute to its pro-metastatic function.

Because of the hypoxia-induced expression of C/EBP δ , we further focused on its role in the HIF-1 pathway.

Figure 1 Analysis of the role of C/EBP δ in mammary tumorigenesis and tumour cells. (A) C/EBP δ is a mammary tumour suppressor. Average number of tumours (mean $\pm$ s.e.m.) per mouse at the study end point, which was determined by the size of the primary tumour (+/+ WT, $n = 22$; +/- Het, $n = 15$; -/- KO, $n = 17$). * $P = 0.043$ and ** $P = 0.0065$ by Poisson regression analysis compared with tumour frequency in KO mice. (B) C/EBP δ promotes lung metastasis. Incidence of lung metastasis in the tumour-bearing mice shown in A. Depicted is the percentage of mice with metastasis regardless of the number or size of metastases per lung (see text for analysis of metastatic load). * $P = 0.028$ by odds ratio analysis and $P = 0.018$ by Fisher exact test. (C) C/EBP δ is induced by hypoxia *in vivo*. Immunohistochemistry of parallel sections of an MMTV-Neu mammary tumour stained with anti-hypoxyprobe and anti-C/EBP δ antibodies. Original magnification: $\times 400$. (D) Loss of C/EBP δ reduces HIF-1 α and CXCR4 expression. Primary MMTV-Neu tumour cells isolated from C/EBP δ WT or KO mice were cultured at 1% or at 20% O $_2$ in the presence of 200 μ M DFX for 8 h followed by western analysis of whole cell extracts with antibodies against the indicated proteins. C/EBP δ expression was analysed in nuclear extracts (NE), because it is not detectable in whole cell extracts of these cells. (E) C/EBP δ is necessary for glycolytic adaptation. Analysis of glucose and lactate levels in culture medium of primary tumour cells after 24 h at 20 or 1% O $_2$. (F) C/EBP δ promotes cell survival under hypoxia. Cell viability of primary tumour cells was analysed by MTT assay after 48 h at 1% O $_2$ relative to cells cultured at 20% O $_2$. (E, F) Results are mean $\pm$ s.e.m. of three experiments with independent preparations of primary tumour cells. * $P < 0.05$, ** $P < 0.001$; NS, not significant.

Because HIF-1 promotes a switch to glycolytic metabolism to maintain energy homeostasis and survival under hypoxia (Semenza, 2010), we assessed the role of C/EBP δ in the glycolytic response. In primary WT tumour cells *in vitro*, hypoxia stimulated the uptake of glucose and the release of lactate (Figure 1E). Loss of C/EBP δ significantly blunted both these responses (Figure 1E). Having demonstrated that C/EBP δ was required for glycolytic metabolism, we further investigated the role of C/EBP δ in cell survival. As shown in Figure 1F, C/EBP δ KO primary tumour cells were more sensitive to hypoxia than WT cells, indicating that C/EBP δ promotes survival of tumour cells under hypoxia.

To assess whether the role of C/EBP δ in hypoxia response is also relevant for human cells, we examined the effect of acute C/EBP δ depletion on HIF-1 α protein accumulation under hypoxia or in response to the chemical HIF-1 α stabilizers DFX or cobalt chloride. In the MCF-7 breast adenocarcinoma cell line, all three treatments induced not only HIF-1 α but also C/EBP δ protein expression (Figure 2A). RNAi-mediated depletion of endogenous C/EBP δ protein reduced HIF-1 α protein expression under hypoxia and HIF-1 α -stabilizing conditions. These results demonstrate that induction of the C/EBP δ protein under hypoxia also occurs in human breast tumour cells, and that it is necessary for efficient expression of the HIF-1 α protein and HIF-1 activity.

Interestingly, while C/EBP δ is downregulated in many cancers (see Introduction), analysis of publicly available expression data at <http://www.ONCOMINE.org> showed that C/EBP δ is overexpressed in glioblastoma (data not shown). Advanced glioblastoma is characterized by significant tumour hypoxia associated with highly invasive properties dependent on HIF-1 (Kaur *et al*, 2005). Indeed, we found that the human glioblastoma cell line U251 expressed readily detectable basal levels of C/EBP δ that were not further induced by DFX (Figure 2B). As seen in MCF-7 cells (Figure 2A), RNAi-mediated knockdown of C/EBP δ in U251 cells significantly reduced HIF-1 α protein levels in response to DFX (Figure 2B). The U251-HRE cell line used here contains a HIF-dependent luciferase reporter gene that is induced by hypoxia (Rapisarda *et al*, 2002). In accordance with reduced HIF-1 α protein levels, knockdown of C/EBP δ diminished HIF-dependent reporter gene activity in response to DFX (Figure 2C). Taken together, these data demonstrate that C/EBP δ is a hypoxia response gene in certain mouse and human tumour cells. Under all conditions tested, C/EBP δ was required for efficient expression of the HIF-1 α protein. Through analysis and reconstitution of MEFs derived from C/EBP δ -null mutant mice, we could demonstrate the causal relationship of C/EBP δ and HIF-1 α expression, and that the reduced HIF-1 α protein levels in C/EBP δ -null cells significantly

Figure 2 C/EBP δ is required for HIF-1 α expression in human tumour cells. **(A)** MCF-7 breast tumour cells were transfected with shRNA against C/EBP δ or GFP as negative control and, 24 h later, was exposed to 1% O₂, 100 μ M DFX or 100 μ M CoCl₂ for 16 h. Nuclear extracts were analysed by western analysis, as indicated. **(B)** U251 glioblastoma cells harbouring an HRE-luciferase reporter were transfected as in **A**. After 2 days, cells were treated with 100 μ M DFX for 16 h followed by western analysis of the indicated proteins. **(C)** U251 cells were treated as in **B** and whole cell lysates were analysed for luciferase reporter activity. Reporter activity (mean $\pm$ s.e.m. of three independent experiments) is shown relative to untreated control samples. *P < 0.05.

impaired expression of the hypoxia response genes *Vegf-c* and *Glut1*, which are critical for the establishment of pro-metastatic conditions (Supplementary Figure S3).

Reduced HIF-1 α expression correlates with impaired AKT signalling

Because C/EBP δ promoted HIF-1 α expression in primary tumour cells, MEFs, glioblastoma and breast tumour cells, we chose KO MEFs to study the mechanism by which C/EBP δ augments HIF-1 α expression. We found that C/EBP δ did not affect HIF-1 α mRNA expression or protein stability (Supplementary Figure S4), suggesting that C/EBP δ may promote HIF-1 α translation. Inflammatory signals, cytokines and growth factors such as insulin promote HIF-1 α translation through AKT (PKB) signalling (Semenza, 2003; Yee Koh *et al*, 2008). The serine/threonine kinase AKT is activated by phosphorylation at Ser473 by mTOR/riCTOR/LST8 of the mTORC2 complex. AKT then activates its downstream effectors including the mTORC1 complex consisting of mTOR/raptor/PRAS40, which stimulates translation through phosphorylation of the ribosomal S6 kinase 1 (S6K1) and of 4E-BP1, a repressor of the eIF4E translation initiation factor (Dann *et al*, 2007). To address the possible role of C/EBP δ in this pathway, MEFs were starved in serum-free conditions before serum was added back for acute stimulation. As shown in Figure 3A, serum stimulation led to increased phosphorylation of AKT at both Ser473 and Thr308.

Although C/EBP δ KO MEFs also exhibited serum-induced AKT phosphorylation, the absolute level of pAKT during serum-starved and stimulated conditions was significantly lower than in WT MEFs. Total AKT protein levels were comparable in WT and KO MEFs. In accordance with reduced AKT activity, serum stimulation of S6K1 phosphorylation was markedly reduced in C/EBP δ -deficient MEFs (Figure 3A). In addition, 4E-BP1 was hypophosphorylated in KO MEFs, which is indicative for hyperactivity of the repressor. These data demonstrate that C/EBP δ KO MEFs are deficient in AKT activity and, consequently, in S6K1 and 4E-BP1 activation, suggesting reduced translational activity.

Phosphorylation of GSK-3 β at Ser9 by AKT leads to inactivation of this kinase, which is an inhibitor of the translation initiation factor eIF2B and represses HIF-1 α expression (Schnitzer *et al*, 2005; Proud, 2006). Consistent with the reduced AKT activity, we found that GSK-3 β phosphorylation was decreased in the absence of C/EBP δ under serum-stimulated conditions (Figure 3A). To further characterize the role of GSK-3 β in these cells, we used LiCl, which promotes inhibitory autophosphorylation of GSK-3 β (Zhang *et al*, 2003). MEFs were treated with DFX in the presence or absence of LiCl or with NaCl as a control. As shown in Figure 3B, LiCl alone did not affect HIF-1 α protein expression, but slightly increased the response to DFX. This can be explained by the stimulation of protein translation by LiCl together with inhibition of HIF-1 α degradation by DFX. In C/EBP δ -deficient cells, LiCl substantially rescued inhibitory Ser9 phosphorylation of GSK-3 β and DFX-induced HIF-1 α protein accumulation (Figure 3B). These data confirm that, consistent with reduced AKT activity, C/EBP δ -null MEFs harbour hyperactive GSK-3 β , which contributes to the reduced HIF-1 α accumulation.

The above analyses suggest that C/EBP δ is necessary for AKT/S6K1-mediated activity. To investigate whether this pathway was indeed operating *in vivo*, we analysed phosphorylation of the ribosomal protein S6, a principal target of S6K1, in mammary tumours. Immunohistochemical analysis showed that S6 phosphorylation was reduced in hypoxic tumour regions (Figure 3C), consistent with the notion that general translational activity becomes reduced as a measure of hypoxic adaptation (Kaper *et al*, 2006). Nevertheless, it was evident that pS6 staining was less in C/EBP δ -null tissue compared with C/EBP δ WT tumours (Figure 3C). In summary, these data show that C/EBP δ promotes ribosomal S6K1 activity in tumour tissue *in vivo*.

C/EBP δ augments HIF-1 α expression through stabilization of mTOR protein

Because C/EBP δ KO MEFs exhibited reduced Ser473 phosphorylation of AKT, we hypothesized that mTORC2 function may be impaired. Indeed, we found that C/EBP δ was necessary for efficient expression of mTOR. C/EBP δ -deficient primary MEFs had reduced mTOR protein levels (Supplementary Figure S5A). Transient expression of mTOR or C/EBP δ in C/EBP δ -null MEFs reconstituted the mTOR/AKT/HIF-1 pathway (Figure 4A). Exogenous C/EBP δ augmented endogenous mTOR and HIF-1 α levels, and exogenous mTOR also recovered HIF-1 α expression in DFX-treated C/EBP δ -deficient cells (Figure 4A). The mTORC1 complex, which phosphorylates and activates S6K1, is sensitive to inhibition by rapamycin (Dann *et al*, 2007). Therefore, we used

Figure 3 C/EBP δ -deficient cells have reduced AKT and increased GSK-3 β activity. (A) C/EBP δ WT and KO MEFs were serum-starved (SS) for 16 h and then stimulated with 10% FBS (FBS) for 1 h. Whole cell extracts were prepared and immunoblotting was performed with the indicated antibodies on two membranes, as shown by the separating line. The WT and KO samples were run on the same gel, with intermediate lanes deleted. The AKT antibody used here recognizes all three isoforms of AKTs. (SE, short exposure; LE, long exposure). (B) MEFs were cultured at 20% O₂ in the presence of 100 μ M DFX and/or 25 mM LiCl or NaCl for 16 h, followed by western analysis of whole cell extracts with the indicated antibodies. (C) Representative images of immunohistochemistry on parallel sections from MMTV-Neu mammary tumours of C/EBP δ WT and KO mice stained for anti-hypoxyprobe and pS6^{S235/236}. Original magnification: $\times$ 200.

Figure 4 C/EBP δ promotes HIF-1 α protein expression through mTOR signalling. (A) Western analysis of whole cell extracts with the indicated antibodies from C/EBP δ KO MEFs 2 days after transfection with expression vectors for C/EBP δ or mTOR followed by 100 μ M DFX treatment for 16 h. The endogenous C/EBP δ protein of WT cells is not detected because exposure time was optimized for the overexpressed protein. Relative quantification of mTOR expression levels normalized to actin are shown at the bottom. (B) MEFs were pretreated with 200 nM rapamycin for 30 min followed by 100 μ M DFX for 16 h. Western analysis was performed with whole cell extracts and the indicated antibodies. (C) Western analysis of whole cell lysates from primary WT and C/EBP δ KO mammary tumour cells, or from MCF-10A cells 48 h after transfection with shRNA against C/EBP δ or GFP as control. (D) C/EBP δ WT and KO primary tumour cells or immortalized MEFs were treated with 25 μ M MG132 or DMSO control for 6 h. Whole cell extracts were analysed for expression of mTOR and β -actin. (E) C/EBP δ WT and KO MEFs were transfected with HA-ubiquitin expression vector. The next day, whole cell lysates were prepared and subjected to immunoprecipitation of mTOR followed by western analysis with antibodies for HA-tag, mTOR or FBXW7. IgG was used as negative control. (F) Western analysis of whole cell extracts from MEFs treated with 100 μ M CHX for the indicated time periods. Quantification of the mTOR/actin signal from three independent experiments is shown on the right. * P < 0.05.

rapamycin to further interrogate the role of mTOR in C/EBP δ -mediated HIF-1 α expression. Rapamycin significantly reduced HIF-1 α accumulation in response to DFX in both WT and KO MEFs (Figure 4B) and in glioblastoma cell lines under hypoxia (Supplementary Figure S5B). Similarly, the increase in DFX-induced HIF-1 α in KO MEFs after rescue with exogenous C/EBP δ was abolished by rapamycin (Figure 4B). Collectively, these data show that C/EBP δ augments HIF-1 α expression through mTORC1. Next, we confirmed that primary mammary tumour cells lacking C/EBP δ also exhibited reduced mTOR expression, which was accompanied by decreased phosphorylation of AKT, S6K1 and GSK-3 β (Figure 4C). The causal relationship of C/EBP δ and mTOR expression levels was further confirmed in the MCF-10A human breast epithelial cell line. Depletion of endogenous C/EBP δ protein by RNAi resulted in reduced mTOR protein levels (Figure 4C, bottom panel). Collectively, these findings show that reduced mTOR protein levels are the reason for an impaired AKT/S6K1 signalling pathway in C/EBP δ -null cells that culminates in reduced HIF-1 α protein expression. These data are in agreement with the lower levels of HIF-1 α protein and HIF-1 target genes in C/EBP δ -mutant tumour cells, which are impaired in their glycolytic metabolism and metastatic activity (Figure 1).

Analysis of mTOR mRNA revealed comparable levels between C/EBP δ WT and KO primary MEFs and tumour cells (data not shown), suggesting that C/EBP δ regulates mTOR expression post-transcriptionally. The stability of the mTOR protein can be modulated by mechanisms that include ubiquitin-mediated proteasomal degradation (Mao *et al*, 2008). Therefore, we assessed the effect of proteasome

inhibition by MG132 in primary tumour cells and MEFs, and found that the mTOR protein was stabilized specifically in cells lacking C/EBP δ (Figure 4D). Next, we examined the ubiquitination status of mTOR after transfection of HA-tagged ubiquitin into WT and KO MEFs. In agreement with reduced mTOR levels, we detected increased mTOR polyubiquitination in C/EBP δ -null MEFs compared with WT MEFs (Figure 4E). Because mTOR protein stability can be regulated by the tumour suppressor FBXW7 (Mao *et al*, 2008), we also assessed expression of this protein. The FBXW7 gene gives rise to three protein isoforms, with the longest α -isoform being predominantly expressed (Welcker and Clurman, 2008). Consistent with the greater levels of polyubiquitinated mTOR, C/EBP δ -null cells contained more FBXW7 protein than WT cells (Figure 4E). Moreover, inhibition of protein synthesis confirmed that mTOR protein was degraded more quickly in C/EBP δ -deficient MEFs ($t_{1/2}$, ~12 h) compared with control cells ($t_{1/2}$, ~20 h) (Figure 4F). Collectively, these data show that C/EBP δ promotes stability of the mTOR protein.

C/EBP δ enhances mTOR protein stability through inhibition of FBXW7 expression

As seen in MEFs (Figure 4E–F), an inverse correlation of C/EBP δ and FBXW7 protein expression was also observed in human MCF-10A, MCF-7 and U251 glioblastoma cell lines. MCF-7 cells expressed more FBXW7 but less C/EBP δ (and mTOR), compared with MCF-10A or U251 cells (Figure 5A). Overexpression of C/EBP δ in MCF-7 cells resulted in down-regulation of FBXW7, and mTOR expression was induced as predicted (Figure 5B). Moreover, expression of another FBXW7 target, the oncogenic Aurora A kinase (Fujii *et al*,

Figure 5 C/EBP δ augments mTOR and HIF-1 α expression by direct inhibition of FBXW7 expression. (A) Inverse correlation of C/EBP δ and FBXW7. Western analysis of whole cell extracts from MCF-7, MCF-10A and U251 cells with the indicated antibodies. (B) Ectopic C/EBP δ downregulates FBXW7 protein levels. Western analysis of whole cell extracts from MCF-7 cells 48 h after transfection with vector or C/EBP δ expression construct. (C) FBXW7 mRNA and protein analysis in C/EBP δ WT and KO MEFs and in primary tumour cells. *Left*: qRT-PCR analysis of FBXW7 mRNA levels (mean $\pm$ s.e.m., normalized to actin) of three independent experiments. WT levels were set at 1. **** P < 0.001. *Right*: Western analysis of whole cell lysates. (D) C/EBP δ depletion increases FBXW7 expression. MCF-10A cells were nucleofected with shRNA constructs against GFP or C/EBP δ , harvested 2 days later and analysed as in C. **** P < 0.001. (E) C/EBP δ targets the FBXW7 α promoter. Schematic of the FBXW7 α promoter with the sequence of the C/EBP-binding site and the position of the primers for ChIP analysis. MCF-7 cells were treated with 100 μ M DFX for 2 h. The chromatin was immunoprecipitated with two different C/EBP δ antibodies (M17, Icon) or IgG as control, and amplified with primers for the FBXW7 α or ZBRK1 promoter (Lin *et al*, 2010) as negative control. (F) FBXW7 depletion increases HIF-1 α expression. C/EBP δ KO MEFs were nucleofected with siRNA against FBXW7 or GFP as control. After 24 h 100 μ M DFX was added for 16 h. Western analysis was carried out with whole cell extracts as indicated.

2006), was also induced by C/EBP δ (Figure 5B). This effect was dependent on an intact DNA-binding domain of C/EBP δ (Supplementary Figure S6A). Furthermore, the related proteins C/EBP α and C/EBP β had no effect on FBXW7 protein levels (Supplementary Figure S6A). Collectively, these data show that C/EBP δ downregulates the tumour suppressor FBXW7 and induces expression of its oncogenic targets mTOR and Aurora A.

Because C/EBP δ is a transcription factor, we assessed the effect of C/EBP δ on *FBXW7* mRNA expression. As shown in Figure 5C, C/EBP δ KO primary tumour cells contained 3.5-fold increased *FBXW7* mRNA levels compared with WT cells. C/EBP δ KO MEFs exhibited a more modest but statistically significant two-fold increase. Consistent with these and previous results, FBXW7 protein levels were elevated in C/EBP δ -deficient cells and associated with reduced mTOR protein expression (Figure 5C). The causal relationship of C/EBP δ expression and FBXW7 downregulation was further confirmed by RNAi depletion of endogenous C/EBP δ protein in MCF-10A and U251 cells, which resulted in increased *FBXW7* mRNA and protein levels and concomitantly reduced mTOR protein levels (Figure 5D and Supplementary Figure S6B). Analysis of the *FBXW7* α promoter revealed a potential C/EBP-binding site in the proximal promoter (Figure 5E), which was confirmed by electrophoretic mobility shift assay (Supplementary Figure S6C). ChIP assays with MCF-7 cells demonstrated that endogenous C/EBP δ binds to the *FBXW7* α promoter (Figure 5E). Consistent with DFX-induced C/EBP δ protein expression in these cells (Figure 2A), specific binding to the *FBXW7* α promoter was significantly enhanced after DFX exposure (Figure 5E). These results showed that C/EBP δ directly binds to the *FBXW7* α promoter. We then cloned ~1.3 kb of the human *FBXW7* α promoter into a luciferase reporter construct and mutated the C/EBP-binding site. Reporter activity from this construct was inhibited by C/EBP δ but not when the C/EBP site was mutated (Supplementary Figure S6D). Taken together, these results show that C/EBP δ directly inhibits *FBXW7* gene expression by functioning as a transcriptional repressor.

Recently, it was reported that the viral E1A oncogene inhibits FBXW7 activity (Isobe *et al*, 2009). Interestingly, C/EBP δ KO MEFs that harbour E1A exhibited normal mTOR levels and DFX-induced HIF-1 α expression compared with WT controls, despite elevated FBXW7 expression (Supplementary Figure S7). To further confirm the causal relationship between FBXW7 inhibition and HIF-1 α expression, we silenced FBXW7 expression in C/EBP δ -null MEFs. Indeed, this was sufficient to rescue mTOR protein levels and DFX-induced HIF-1 α expression in the C/EBP δ -deficient cells (Figure 5F). Collectively, these data demonstrate that C/EBP δ controls mTOR and HIF-1 α protein levels through inhibition of FBXW7 expression.

Discussion

In this study, we have found that C/EBP δ protects mammary glands from Neu/ERBB2-initiated tumour formation, while on the other hand augmenting metastatic tumour progression. A role of C/EBP δ in activation of the *CXCR4* gene, which is a known pro-metastatic agent (Gelmini *et al*, 2008), may in part contribute to its metastasis-promoting activity. Furthermore, we have shown that C/EBP δ is a

hypoxia response gene augmenting HIF-1 activity through mTOR stabilization. This is achieved by inhibiting the expression of FBXW7, a tumour suppressor that belongs to the F-box protein family and targets substrates for ubiquitin-dependent proteasomal degradation. In addition, C/EBP δ is an inflammatory response gene activated by STAT3 (Thangaraju *et al*, 2005) and NF- κ B (Litvak *et al*, 2009). The latter also induces HIF-1 α at the level of gene transcription (Rius *et al*, 2008). Therefore, this study suggests that C/EBP δ serves to integrate and amplify hypoxia and inflammatory signalling by promoting the mTOR/AKT/HIF-1 pathway through inhibition of FBXW7 (Figure 6). This function within the solid tumour facilitates tumour cell survival and is a plausible explanation for the unexpected pro-metastatic activity of C/EBP δ , despite being a tumour suppressor at earlier stages of tumour development. Our data also suggest that clinical application of mTOR inhibitors (Strimpakos *et al*, 2009) may have particular value in the prevention of metastatic disease and in combination with anti-angiogenic agents.

As we employed mice with a germ-line deletion of C/EBP δ , we do not rule out the potential contribution of stromal and systemic factors to tumour initiation or metastasis. However, the C/EBP δ gene is activated by the STAT3 transcription factor, and loss of STAT3 expression, specifically in Neu/ERBB2 mouse mammary tumours, reduced C/EBP δ expression in the epithelial cells and significantly impaired tumour metastasis (Ranger *et al*, 2009). Deletion of HIF-1 α from mouse mammary epithelial cells resulted in reduced lung tumour metastases, demonstrating the role of tumour cell intrinsic HIF-1 activity in metastatic progression (Liao *et al*, 2007). Furthermore, isolated C/EBP δ -deficient tumour cells exhibited reduced expression of not only HIF-1 α but also the highly pro-metastatic gene *CXCR4*. Taken together, these data support a mammary epithelial cell intrinsic role of C/EBP δ in metastasis through its role in promoting HIF-1 α expression and hypoxic adaptation within the solid tumour.

C/EBP δ has been considered a tumour suppressor protein based on (a) growth inhibitory activity *in vitro*, (b) pro-

Figure 6 Model showing how C/EBP δ may integrate and amplify hypoxia and inflammatory signals to promote HIF-1 α protein expression, hypoxic adaptation and tumour metastasis. Colours indicate effects mediated by regulation of mRNA expression (blue), protein translation (green), protein stability (red) or protein activity (black). The mTOR protein is part of both TORC1 and TORC2 complexes. The dashed line indicates that the mechanism by which hypoxia induces C/EBP δ expression remains to be determined.

apoptotic activity *in vivo*, (c) a role in promoting genomic stability, (d) correlation of its expression in tumours with improved patient survival and (e) downregulation of its expression in tumours (see Introduction). This study confirms for the first time the tumour suppressor activity of C/EBP δ in an animal model of mammary tumourigenesis. A specific increase in tumour multiplicity of C/EBP δ -null mice suggests a protective function early in tumour development. This function may be due to its pro-apoptotic activity in the mammary gland, its role in genome stability and/or its general growth inhibitory properties. Two recent reports from our group provide further mechanisms by which C/EBP δ may act as a tumour suppressor. We showed that C/EBP δ promotes APC/C-mediated degradation of cyclin D1 by indirectly upregulating Cdc27 expression (Pawar *et al*, 2010). Cyclin D1 ablation was shown to protect mice from Neu-mediated mammary tumourigenesis (Yu *et al*, 2001). Yet, C/EBP δ -null mammary glands or tumours did not exhibit elevated cyclin D1 expression or reduced expression of Cdc27 (data not shown). However, Cdc27 protein was already barely detectable in C/EBP δ WT tissue, perhaps because C/EBP δ may not promote Cdc27 expression in these cells. Recently, it was shown that cyclin D1 is required specifically in the mammary stem cells to promote tumourigenesis (Jeselsohn *et al*, 2010). Therefore, we can still speculate that an effect of C/EBP δ deficiency on cyclin D1 expression in stem cells, which evaded detection by our analyses, may contribute to its role as a tumour suppressor. In addition, we reported that C/EBP δ promotes DNA repair by the Fanconi anaemia (FA) pathway by facilitating nuclear translocation of the FANCD2 protein (Wang *et al*, 2010). Mutations in the FA pathway are related to breast cancer risk (Levy-Lahad, 2010). Furthermore, nuclear localization of FANCD2 is typical of benign breast tumours, and loss of nuclear localization predicts poor outcome for patients (Rudland *et al*, 2010). Thus, C/EBP δ may act as a tumour suppressor through its role in the FA DNA repair pathway.

The tumour suppressor function of C/EBP δ is counter-intuitive given its role in mTOR and HIF-1 α expression. However, one should bear in mind that C/EBP δ is not normally expressed at high levels. Its specific function in HIF-1 α expression may be most relevant under acute and particular circumstances such as hypoxic and proinflammatory conditions. On the other hand, although HIF-1 α is a well-established tumour promoter (Melillo, 2007; Semenza 2010), some studies also support a tumour suppressor function of HIF-1. In certain cancers, HIF-1 activity can be associated with better patient survival (Semenza, 2003). In breast cancer patients, diffuse, as opposed to perinecrotic, HIF-1 α expression was associated with better survival (Vleugel *et al*, 2005). Recent findings suggest that HIF-1 restricts the initial phases of tumour development, as its target *mir-210* represses establishment of tumour xenografts (Huang *et al*, 2009). These findings corroborate previous studies, showing that loss of HIF-1 α accelerated growth of embryonic stem cell-derived tumours (Carmeliet *et al*, 1998) or of astrocytoma when grown in blood-vessel-rich brain areas (Blouw *et al*, 2003). Furthermore, HIF-1 α reduced tumour growth in a renal cell carcinoma xenograft model (Raval *et al*, 2005). Several studies highlighted the pivotal function of HIF-1 α in cell cycle arrest or apoptosis under hypoxic conditions (Goda *et al*, 2003; Greijer and van der Wall, 2004; Koshiji *et al*,

2004). In this aspect, the function of HIF-1 is reminiscent of that of C/EBP δ (Johnson, 2005). In summary, HIF-1 has differential roles in tumour progression. Under select circumstance, HIF-1 could also function as a tumour suppressor, and it is plausible that C/EBP δ may act as a tumour suppressor in part through its role in HIF-1 α expression.

Interestingly, our data also revealed a Yin-Yang relationship of C/EBP α and C/EBP δ with respect to HIF-1 α regulation. C/EBP α inhibits HIF-1 function by competing with HIF-1 β for direct binding to HIF-1 α (Yang *et al*, 2008). In contrast, C/EBP δ enhances HIF-1 α activity through mTOR-mediated translational regulation. Thus, the two C/EBP family members affect HIF-1 function not only in opposite direction but also by different mechanisms.

Although hypoxia leads to energy deprivation, and energy-consuming processes such as translation are reduced, translation of specific genes such as HIF-1 α is induced (Yee Koh *et al*, 2008). HIF-1 α protein synthesis requires the PI3K/AKT/mTOR pathway, and defects in this pathway lead to profound attenuation of HIF-1 α protein in the majority of cancer cells studied (Abraham, 2004). Indeed, hypoxia activates the PI3K/AKT/mTOR pathway and promotes HIF-1-dependent survival of various cell types (Yee Koh *et al*, 2008; Semenza 2010). In agreement with this, our findings support a significant role of translational activity on HIF-1 α accumulation under hypoxic conditions. While oncogenic signals and hypoxia promote HIF-1 function, in part, through stimulating translation, tumour suppressors such as p53, phosphatase and tensin homologue, serine/threonine protein kinase 11 (STK11, LKB1, PJS) and von Hippel-Lindau (pVHL) negatively regulate HIF-1 activity (Semenza, 2003; Yee Koh *et al*, 2008; Shackelford *et al*, 2009). To our knowledge, C/EBP δ is the first tumour suppressor found to promote mTOR and HIF-1 activity.

C/EBP δ augments mTOR protein stability by directly inhibiting expression of FBXW7, a subunit of the SKP1/CUL1/F-box ubiquitin ligase complex that targets many positive regulators of cell cycle for degradation, and, therefore, acts as a tumour suppressor (Welcker and Clurman, 2008). Inhibition of FBXW7 gene expression by C/EBP δ is consistent with its ability to associate with transcriptional co-repressors such as HDAC1 and mSin3A, and with previous reports of target gene repression by C/EBP δ (Lacorte *et al*, 1997; Okazaki *et al*, 2002; Wang *et al*, 2006; Lai *et al*, 2008; Turgeon *et al*, 2008). Interestingly, FBXW7 itself is a tumour suppressor (Welcker and Clurman, 2008), which is consistent with its role in downregulating the mTOR/AKT/HIF-1 pathway. Most FBXW7 targets require 'priming' through phosphorylation by the GSK-3 β kinase (Crusio *et al*, 2010). Given that loss of C/EBP δ leads to hyperactive GSK-3 β , (this study and Pawar *et al*, 2010), most likely through induction of FBXW7, the pathway also triggers the priming of its substrates. The induction of C/EBP δ expression in hypoxic tumour tissue eliminates the FBXW7 break on the mTOR/AKT/HIF-1, which can explain the pro-metastatic function of C/EBP δ .

While this study primarily employed mammary and breast tumour cells, our findings may also be relevant for brain tumours. Loss of FBXW7 expression is an important step in glioma progression (Hagedorn *et al*, 2007). In fact, malignant glioblastoma express higher levels of C/EBP δ than benign lesions (see <http://www.ONCOMINE.org>), and our study

shows that the U251 glioblastoma cell line depends on C/EBP δ for maximal hypoxia-induced HIF-1 activity.

In summary, while we demonstrated the role of C/EBP δ as a tumour suppressor in an animal model, our findings also revealed a novel role of C/EBP δ in metastasis promotion. Hence, C/EBP δ represents a new example of the 'TGF- β paradox' in cancer progression (Tian and Schiemann, 2009). Our results provide further insight into the mechanisms by which inflammatory and hypoxic signals cooperate to drive tumour progression. The discovery that a tumour suppressor can stimulate the mTOR/AKT/HIF-1 pathway by suppressing another tumour suppressor, FBXW7, reveals novel complexity of this important signalling cascade in cancer.

Materials and methods

Analysis of MMTV-Neu transgenic mice

The MMTV-c-Neu mice (Guy *et al*, 1992) were crossed with C/EBP δ -null mutant mice (Sterneck *et al*, 1998) on the FVB/NCr genetic background. Female mice were homozygous for MMTV-Neu and derived from intercrosses of C/EBP δ heterozygous mice. For the tumour study, mice were monitored twice a week for tumour development and killed with CO₂ when the primary tumour reached ~1.5 cm in diameter. At necropsy, gross lesions from all mammary glands and one inguinal mammary gland were fixed in 10% neutral-buffered formalin (NBF). Whole mounts of all mammary glands were fixed in Carnoy's solution and stained with carmine alum. Masses that were at least 4 mm in one dimension and could be confirmed by histological analysis were included in the data. For analysis of metastases, lungs were inflated with 10% NBF, separated lung lobes were paraffin embedded and three 5- μ m sections were collected 100 μ m apart and stained with hematoxylin and eosin (H and E). NCI-Frederick is accredited by AAALC International and follows the Public Health Service Policy for the care and use of laboratory animals. Animal care was provided in accordance with the procedures outlined in the 'Guide for Care and Use of Laboratory Animals' (National Research Council; 1996; National Academy Press; Washington, DC).

Statistical analysis

In many analyses, there was a concern that measurements made on clusters of mice with the same parents may be correlated with one another. In the time until tumour analysis, a random effect for these clusters was incorporated into a frailty model survival analysis that was used to compare the tumour hazard rates across the three mouse cohorts: heterozygous, knockout and WT. In the tumour multiplicity analysis, mean tumour counts were compared in a Poisson regression model that included a clusters random effect, and which also included the age of the mouse as an offset. Parameters were estimated using the generalized estimating equations method. For the analysis of the metastasis probabilities, exact conditional inference was used to compare the odds metastasis across the mouse cohorts. Pairwise comparisons of means of continuous measurements were performed using Student's *t*-tests or Wilcoxon rank-sum tests and a *P*-value of 0.05 was used to assess statistical significance.

Analysis of metastatic burden

Mice of this model developed few and predominantly intravascular metastases. To assess metastatic burden, H and E-stained lung sections were scanned digitally with the Aperio ScanScope. All metastatic lesions were annotated by the study pathologist. Image analysis was performed using the Aperio Positive Pixel Count macro that was customized to count only the tissue area. The metastatic burden was expressed as the ratio of total area of metastases over total lung area for three sections per lung. An arcsin variance-stabilizing transformation was applied to the metastasis areas, and a two-sample *t*-test was used to test for homogeneity of metastasis area.

Cell culture and treatments

Primary mammary tumour cells were isolated essentially as described (Varticovski *et al*, 2007). Briefly, the tumour tissue was minced by passage through a 40 μ m mesh and 18-gauge needle. The cells were collected by centrifugation at 400 r.p.m. for 3 min and cultured in MCF-10A cell medium. Primary MEFs and spontaneously immortalized 3T3 MEFs derived from either WT (C/ebp δ ^{+/+}) or mutant (C/ebp δ ^{-/-}) mice were obtained as described (Huang *et al*, 2004). MEFs and U251/HRE cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO₂. MCF-7 cells were grown with additional 5 mM sodium pyruvate. MCF-10A cells were cultured in DMEM:F-12 HAM medium, 10% FBS, 10 μ g/ml Insulin, 100 ng/ml cholera toxin, 0.5 μ g/ml hydrocortisone, 20 ng/ml recombinant epidermal growth factor and 1 mM calcium chloride. Primary MEFs were maintained at 5% O₂ and used at passages 1–4. A 1% O₂ incubator was used as indicated. Cell-culture-grade dimethyl sulfoxide was used as solvent control for MG132 and rapamycin.

Metabolic measurements and cell viability assay

Commercial kits were used to assay extracellular lactate and glucose, as well as cell viability (MTT). See Supplementary Information for details.

Reagents and antibodies

See Supplementary Information for details.

Transient transfections

Cells were seeded 24 h before transfection and co-transfected with C/EBP δ or mTOR expression constructs along with a GFP expression construct using Mirus TransIT-LT1 transfection reagent according to manufacturer's instructions. The total amount of DNA in each transfection was kept constant by complementation with vector control DNA. After 24 h, cells were switched to hypoxic treatment as indicated. To silence endogenous C/EBP δ , two shRNA expression constructs were used at 1:1 ratio: a pSilencer construct (Wang *et al*, 2006) and one targeted to the 5' region of C/EBP δ mRNA with the following sequence 5'-GATCCGCAGCAATCACAA GGCGGGCTTCAAGAGAGCCCGCCTTGTGATTGCTGTTTTTGCTAG CG-3' cloned into HindIII/BglII sites in the pSuper vector. FBXW7-specific siRNA (sense: 5'-GCACAGAAUUGAUACAACCTT-3', antisense: 5'-GUUAGUAUCAUUCUGUGCTG-3') was purchased from Ambion (Silencer® Pre-designed) and nucleofected at a concentration of 100 nM siRNA into 2 $\times$ 10⁶ cells per 100 μ l MEF Nucleofector solution (Amaxa Biosystems/Lonza) according to manufacturer's instructions.

Luciferase reporter assay

U251-HRE glioblastoma cells with an integrated luciferase-based HIF reporter (Rapisarda *et al*, 2002) were transfected and treated as described above. Luciferase activity was assessed using a luciferase assay kit according to manufacturer's instructions (Promega). See Supplementary Information for details on FBXW7 promoter reporter assay.

ChIP assay

The ChIP assay was performed essentially as described (Lin *et al*, 2010). See Supplementary Information for details.

Quantitative real-time PCR

Total RNA was isolated using TRIZOL (Invitrogen, CA), treated with DNase I and purified by RNeasy (Qiagen), and cDNA was synthesized using superscript reverse transcriptase III (RT) according to manufacturer's instructions (Invitrogen). PCR was conducted with Taqman gene expression primer/probe sets using the 7500 Fast Real-Time PCR instrument (Applied Biosystems). Analysis was performed using the MxPro Software (Stratagene). All probes were tested in no-RT controls and all reactions were performed in duplicates. The relative expression levels were measured using the relative quantitation $\Delta\Delta$ Ct method and normalized to β -actin.

Western analysis

Nuclear extracts were prepared according to methods described previously (Martin *et al*, 2005). For whole cell extracts, cells were harvested and washed once with cold PBS and resuspended in 50–100 μ l lysis buffer containing 50 mM Tris (pH 8.0), 0.1% NP-40,

400 mM NaCl, 10 μ g/ml phosphatase inhibitor I, 10 μ g/ml phosphatase inhibitor II and 10 μ g/ml protease inhibitor mixture. A measure of 20–50 μ g of whole cell extract or 10–20 μ g nuclear extract was loaded onto 6 or 10% SDS–polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membrane and blocked with 5% non-fat dry milk in PBS. Immunoblotting was performed using indicated primary antibodies for overnight incubation at 4°C. Following washing and incubation with the appropriate HRP-conjugated secondary antibodies, signals were visualized by enhanced chemiluminescence kit (Pierce, Rockford, IL). Quantification was done by Quantity One software (Bio-Rad). All western analysis data are representative of at least three independent biological replicates.

In vivo ubiquitination and immunoprecipitation studies

HA–ubiquitin expression constructs were transfected into MEFs using Mirus transfection reagent. After 24 h, whole cell extracts were prepared and immunoprecipitation was carried out with anti-mTOR antibodies or IgG as negative control, as described earlier (Pawar *et al*, 2010).

Immunohistochemistry

For immunohistochemistry of hypoxia, mice were injected with 1.5 mg pimonidazole 90 min before euthanasia. Tissue was fixed in 10% NBF for <24 h. Primary antibodies were used in the ratio 1:2000 for C/EBP δ (Rockland Immunologicals Inc, cat. no. 600-401-A61) after 10 min HIER EDTA antigen retrieval, and in the ratios 1:100 for hypoxyprobe-1 (Natural Pharmacia, HP1-1000) or 1:150 for phospho-S6 (Cell Signaling, cat. no. 2211) after 20 min HIER citrate antigen retrieval.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

References

- Abraham RT (2004) mTOR as a positive regulator of tumor cell responses to hypoxia. *Curr Top Microbiol Immunol* **279**: 299–319
- Agrawal S, Hofmann WK, Tidow N, Ehrlich M, van den Boom D, Koschmieder S, Berdel WE, Serve H, Muller-Tidow C (2007) The C/EBP δ tumor suppressor is silenced by hypermethylation in acute myeloid leukemia. *Blood* **109**: 3895–3905
- Barresi V, Vitarelli E, Cerasoli S, Barresi G (2009) The cell growth inhibitory transcription factor C/EBP δ is expressed in human meningiomas in association with low histological grade and proliferation index. *J Neurooncol* **97**: 233–240
- Blouw B, Song H, Tihan T, Bosze J, Ferrara N, Gerber HP, Johnson RS, Bergers G (2003) The hypoxic response of tumors is dependent on their microenvironment. *Cancer Cell* **4**: 133–146
- Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D, Keshert E (1998) Role of HIF-1 α in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* **394**: 485–490
- Crusio KM, King B, Reavie LB, Aifantis I (2010) The ubiquitous nature of cancer: the role of the SCF(Fbw7) complex in development and transformation. *Oncogene* **29**: 4865–4873
- Dann SG, Selvaraj A, Thomas G (2007) mTOR complex1-S6K1 signaling: at the crossroads of obesity, diabetes and cancer. *Trends Mol Med* **13**: 252–259
- Frede S, Berchner-Pfannschmidt U, Fandrey J (2007) Regulation of hypoxia-inducible factors during inflammation. *Methods Enzymol* **435**: 405–419
- Fujii Y, Yada M, Nishiyama M, Kamura T, Takahashi H, Tsunematsu R, Susaki E, Nakagawa T, Matsumoto A, Nakayama KI (2006) Fbxw7 contributes to tumor suppression by targeting multiple proteins for ubiquitin-dependent degradation. *Cancer Sci* **97**: 729–736
- Gelmini S, Mangoni M, Serio M, Romagnani P, Lazzeri E (2008) The critical role of SDF-1/CXCR4 axis in cancer and cancer stem cells metastasis. *J Endocrinol Invest* **31**: 809–819

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Conflict of interest

The authors declare that they have no conflict of interest.

- Gery S, Tanosaki S, Hofmann WK, Koppel A, Koeffler HP (2005) C/EBP δ expression in a BCR-ABL-positive cell line induces growth arrest and myeloid differentiation. *Oncogene* **24**: 1589–1597
- Goda N, Ryan HE, Khadivi B, McNulty W, Rickert RC, Johnson RS (2003) Hypoxia-inducible factor 1 α is essential for cell cycle arrest during hypoxia. *Mol Cell Biol* **23**: 359–369
- Greijer AE, van der Wall E (2004) The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis. *J Clin Pathol* **57**: 1009–1014
- Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ (1992) Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc Natl Acad Sci USA* **89**: 10578–10582
- Hagedorn M, Delugin M, Ahrades I, Allain N, Belaud-Rotureau MA, Turmo M, Prigent C, Loiseau H, Bikfalvi A, Javerzat S (2007) FBXW7/hCDC4 controls glioma cell proliferation *in vitro* and is a prognostic marker for survival in glioblastoma patients. *Cell Div* **2**: 9
- Hirsila M, Koivunen P, Xu L, Seeley T, Kivirikko KI, Myllyharju J (2005) Effect of desferrioxamine and metals on the hydroxylases in the oxygen sensing pathway. *FASEB J* **19**: 1308–1310
- Huang AM, Montagna C, Sharan S, Yajun N, Ried T, Sterneck E (2004) Loss of CCAAT/enhancer binding protein δ promotes chromosomal instability. *Oncogene* **23**: 1549–1557
- Huang X, Ding L, Bennewith KL, Tong RT, Welford SM, Ang KK, Story M, Le QT, Giaccia AJ (2009) Hypoxia-inducible mir-210 regulates normoxic gene expression involved in tumor initiation. *Mol Cell* **35**: 856–867
- Isobe T, Hattori T, Kitagawa K, Uchida C, Kotake Y, Kosugi I, Oda T, Kitagawa M (2009) Adenovirus E1A inhibits SCF(Fbw7) ubiquitin ligase. *J Biol Chem* **284**: 27766–27779
- Jeselsohn R, Brown NE, Arendt L, Klebba I, Hu MG, Kuperwasser C, Hinds PW (2010) Cyclin D1 kinase activity is required for the self-renewal of mammary stem and progenitor cells that are targets of MMTV-ErbB2 tumorigenesis. *Cancer Cell* **17**: 65–76

- Johnson PF (2005) Molecular stop signs: regulation of cell-cycle arrest by C/EBP transcription factors. *J Cell Sci* **118**: 2545–2555
- Kaper F, Dornhoefer N, Giaccia AJ (2006) Mutations in the PI3K/PTEN/TSC2 pathway contribute to mammalian target of rapamycin activity and increased translation under hypoxic conditions. *Cancer Res* **66**: 1561–1569
- Kaur B, Khwaja FW, Severson EA, Matheny SL, Brat DJ, Van Meir EG (2005) Hypoxia and the hypoxia-inducible-factor pathway in glioma growth and angiogenesis. *Neuro Oncol* **7**: 134–153
- Ko CY, Hsu HC, Shen MR, Chang WC, Wang JM (2008) Epigenetic Silencing of CCAAT/enhancer-binding protein {delta} activity by YY1/polycomb group/DNA methyltransferase complex. *J Biol Chem* **283**: 30919–30932
- Koshiji M, Kageyama Y, Pete EA, Horikawa I, Barrett JC, Huang LE (2004) HIF-1alpha induces cell cycle arrest by functionally counteracting Myc. *EMBO J* **23**: 1949–1956
- Lacorte JM, Beigneux A, Parant M, Chambaz J (1997) Repression of apoC-III gene expression by TNFalpha involves C/EBPdelta/NF-IL6beta via an IL-1 independent pathway. *FEBS Lett* **415**: 217–220
- Lai PH, Wang WL, Ko CY, Lee YC, Yang WM, Shen TW, Chang WC, Wang JM (2008) HDAC1/HDAC3 modulates PPARG2 transcription through the sumoylated CEBPD in hepatic lipogenesis. *Biochim Biophys Acta* **1783**: 1803–1814
- Levy-Lahad E (2010) Fanconi anemia and breast cancer susceptibility meet again. *Nat Genet* **42**: 368–369
- Liao D, Corle C, Seagroves TN, Johnson RS (2007) Hypoxia-inducible factor-1alpha is a key regulator of metastasis in a transgenic model of cancer initiation and progression. *Cancer Res* **67**: 563–572
- Lin LF, Chuang CH, Li CF, Liao CC, Cheng CP, Cheng TL, Shen MR, Tseng JT, Chang WC, Lee WH, Wang JM (2010) ZBRK1 acts as a metastatic suppressor by directly regulating MMP9 in cervical cancer. *Cancer Res* **70**: 192–201
- Litvak V, Ramsey SA, Rust AG, Zak DE, Kennedy KA, Lampano AE, Nykter M, Shmulevich I, Aderem A (2009) Function of C/EBPdelta in a regulatory circuit that discriminates between transient and persistent TLR4-induced signals. *Nat Immunol* **10**: 437–443
- Mao JH, Kim IJ, Wu D, Climent J, Kang HC, DelRosario R, Balmain A (2008) FBXW7 targets mTOR for degradation and cooperates with PTEN in tumor suppression. *Science* **321**: 1499–1502
- Martin F, Linden T, Katschinski DM, Oehme F, Flamme I, Mukhopadhyay CK, Eckhardt K, Troger J, Barth S, Camenisch G, Wenger RH (2005) Copper-dependent activation of hypoxia-inducible factor (HIF)-1: implications for ceruloplasmin regulation. *Blood* **105**: 4613–4619
- Melillo G (2007) Targeting hypoxia cell signaling for cancer therapy. *Cancer Metastasis Rev* **26**: 341–352
- Naderi A, Teschendorff AE, Barbosa-Morais NL, Pinder SE, Green AR, Powe DG, Robertson JF, Aparicio S, Ellis IO, Brenton JD, Caldas C (2007) A gene-expression signature to predict survival in breast cancer across independent data sets. *Oncogene* **26**: 1507–1516
- Okazaki K, Li J, Yu H, Fukui N, Sandell LJ (2002) CCAAT/enhancer-binding proteins beta and delta mediate the repression of gene transcription of cartilage-derived retinoic acid-sensitive protein induced by interleukin-1 beta. *J Biol Chem* **277**: 31526–31533
- Pawar SA, Roy Sarkar T, Balamurugan K, Sharan S, Wang J, Zhang Y, Dowdy SF, Huang AM, Sterneck E (2010) C/EBP{delta} targets cyclin D1 for proteasome-mediated degradation via induction of CDC27/APC3 expression. *Proc Natl Acad Sci USA* **107**: 9210–9215
- Porter DA, Krop IE, Nasser S, Sgroi D, Kaelin CM, Marks JR, Riggins G, Polyak K (2001) A sage (serial analysis of gene expression) view of breast tumor progression. *Cancer Res* **61**: 5697–5702
- Proud CG (2006) Regulation of protein synthesis by insulin. *Biochem Soc Trans* **34**: 213–216
- Ramji DP, Foka P (2002) CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochem J* **365**: 561–575
- Ranger JJ, Levy DE, Shahalizadeh S, Hallett M, Muller WJ (2009) Identification of a Stat3-dependent transcription regulatory network involved in metastatic progression. *Cancer Res* **69**: 6823–6830
- Rapisarda A, Uranchimeg B, Scudiero DA, Selby M, Sausville EA, Shoemaker RH, Melillo G (2002) Identification of small molecule inhibitors of hypoxia-inducible factor 1 transcriptional activation pathway. *Cancer Res* **62**: 4316–4324
- Raval RR, Lau KW, Tran MG, Sowter HM, Mandriota SJ, Li JL, Pugh CW, Maxwell PH, Harris AL, Ratcliffe PJ (2005) Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. *Mol Cell Biol* **25**: 5675–5686
- Rius J, Guma M, Schachtrup C, Akassoglou K, Zinkernagel AS, Nizet V, Johnson RS, Haddad GG, Karin M (2008) NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha. *Nature* **453**: 807–811
- Rudland P, Platt-Higgins A, Davies L, Rudland S, Wilson J, Aladwani A, Winstanley J, Barraclough D, Barraclough R, West C, Jones NJ (2010) Significance of the fanconi anemia FANCD2 protein in sporadic and metastatic human breast cancer. *Am J Pathol* **176**: 2935–2947
- Schnitzler SE, Schmid T, Zhou J, Eisenbrand G, Brune B (2005) Inhibition of GSK3beta by indirubins restores HIF-1alpha accumulation under prolonged periods of hypoxia/anoxia. *FEBS Lett* **579**: 529–533
- Semenza GL (2003) Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* **3**: 721–732
- Semenza GL (2010) HIF-1: upstream and downstream of cancer metabolism. *Curr Opin Genet Dev* **20**: 51–56
- Shackelford DB, Vasquez DS, Corbett J, Wu S, Leblanc M, Wu CL, Vera DR, Shaw RJ (2009) mTOR and HIF-1alpha-mediated tumor metabolism in an LKB1 mouse model of Peutz-Jeghers syndrome. *Proc Natl Acad Sci USA* **106**: 11137–11142
- Sterneck E, Paylor R, Jackson-Lewis V, Libbey M, Przedborski S, Tessarollo L, Crawley JN, Johnson PF (1998) Selectively enhanced contextual fear conditioning in mice lacking the transcriptional regulator CCAAT/enhancer binding protein delta. *Proc Natl Acad Sci USA* **95**: 10908–10913
- Strimpakos AS, Karapanagiotou EM, Saif MW, Syrigos KN (2009) The role of mTOR in the management of solid tumors: an overview. *Cancer Treat Rev* **35**: 148–159
- Sullivan R, Graham CH (2007) Hypoxia-driven selection of the metastatic phenotype. *Cancer Metastasis Rev* **26**: 319–331
- Tang B, Sivko GS, DeWille J (2005) Promoter methylation reduces C/EBP delta gene expression in the SUM-52PE human breast cancer cell line and in primary breast tumors. *Breast Cancer Res Treat* **95**: 161–170
- Thangaraju M, Rudelius M, Bieri B, Raffeld M, Sharan S, Hennighausen L, Huang AM, Sterneck E (2005) C/EBP delta is a critical regulator of pro-apoptotic gene expression during mammary gland involution. *Development* **132**: 4675–4685
- Tian M, Schiemann WP (2009) The TGF-beta paradox in human cancer: an update. *Future Oncol* **5**: 259–271
- Turgeon N, Valiquette C, Blais M, Routhier S, Seidman EG, Asselin C (2008) Regulation of C/EBPdelta-dependent transactivation by histone deacetylases in intestinal epithelial cells. *J Cell Biochem* **103**: 1573–1583
- Varticovski L, Hollingshead MG, Robles AI, Wu X, Cherry J, Munroe DJ, Lukes L, Anver MR, Carter JP, Borgel SD, Stotler H, Bonomi CA, Nunez NP, Hursting SD, Qiao W, Deng CX, Green JE, Hunter KW, Merlino G, Steeg PS *et al* (2007) Accelerated preclinical testing using transplanted tumors from genetically engineered mouse breast cancer models. *Clin Cancer Res* **13**: 2168–2177
- Vleugel MM, Greijer AE, Shvarts A, van der Groep P, van Berkel M, Aarbodend Y, van Tinteren H, Harris AL, van Diest PJ, van der Wall E (2005) Differential prognostic impact of hypoxia induced and diffuse HIF-1alpha expression in invasive breast cancer. *J Clin Pathol* **58**: 172–177
- Wang J, Roy Sarkar T, Zhou M, Sharan S, Ritt DA, Veenstra TD, Morrison DK, Huang AM, Sterneck E (2010) CCAAT/enhancer binding protein delta (C/EBP{delta}), CEBPD-mediated nuclear import of FANCD2 by IPO4 augment. *Proc Natl Acad Sci USA* **107**: 16131–16136
- Wang JM, Ko CY, Chen LC, Wang WL, Chang WC (2006) Functional role of NF-IL6beta and its sumoylation and acetylation modifications in promoter activation of cyclooxygenase 2 gene. *Nucleic Acids Res* **34**: 217–231
- Wei W, Yu XD (2007) Hypoxia-inducible factors: crosstalk between their protein stability and protein degradation. *Cancer Lett* **257**: 145–156
- Weidemann A, Johnson RS (2008) Biology of HIF-1alpha. *Cell Death Differ* **15**: 621–627
- Welcker M, Clurman BE (2008) FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation. *Nat Rev Cancer* **8**: 83–93

- Yang L, Jiang Y, Wu SF, Zhou MY, Wu YL, Chen GQ (2008) CCAAT/enhancer-binding protein alpha antagonizes transcriptional activity of hypoxia-inducible factor 1 alpha with direct protein-protein interaction. *Carcinogenesis* **29**: 291–298
- Yee Koh M, Spivak-Kroizman TR, Powis G (2008) HIF-1 regulation: not so easy come, easy go. *Trends Biochem Sci* **33**: 526–534
- Yu Q, Geng Y, Sicinski P (2001) Specific protection against breast cancers by cyclin D1 ablation. *Nature* **411**: 1017–1021
- Zhang F, Phiel CJ, Spece L, Gurvich N, Klein PS (2003) Inhibitory phosphorylation of glycogen synthase kinase-3 (GSK-3) in response to lithium. Evidence for autoregulation of GSK-3. *J Biol Chem* **278**: 33067–33077
- Zhou J, Schmid T, Schnitzer S, Brune B (2006) Tumor hypoxia and cancer progression. *Cancer Lett* **237**: 10–21
- Zinkernagel AS, Johnson RS, Nizet V (2007) Hypoxia inducible factor (HIF) function in innate immunity and infection. *J Mol Med* **85**: 1339–1346