# C/EBPα expression is associated with homeostasis of the gastric epithelium and with gastric carcinogenesis

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Transcription factors from the CCAAT/enhancer-binding protein (C/EBP) family are fundamental for the control of differentiation and proliferation of many adult tissues. C/EBPα has a crucial role in inducing terminal differentiation and is an established tumor suppressor gene in several cancer models. The objective of this study was to analyze the putative role of C/EBPα in gastric carcinoma (GC). We analyzed the expression of C/EBPα in normal and neoplastic gastric tissues, and assessed the role of C/EBPα on proliferation and differentiation of GC cells. In normal gastric mucosa, C/EBPα is expressed in the foveolar epithelium and co-localizes with the gastric differentiation marker trefoil factor 1 (TFF1). The expression of C/EBPα was found to be lost in 30% of GC cases. To evaluate the role of C/EBPα in cell proliferation and differentiation, we transfected GC cells with a full-length C/EBPα protein. We observed a significant decrease in proliferation in C/EBPα-transfected cells. This was accompanied by a decrease in Cyclin D1, an increase in P27 expression, and an increased expression of TFF1. Finally, we showed that inhibition of the Ras/MAPK pathway leads to increased C/EBPα and TFF1 expression, and decreased cell proliferation and cyclin D1 expression in GC cells. Our results suggest that C/EBPα (together with other members of the C/EBP family) has an active role in the control of differentiation and proliferation in normal gastric mucosa. In GC, loss of C/EBPα may be associated with the switch from a cellular differentiation to a cellular proliferation program, presumably as a consequence of Ras/MAPK pathway activation.

Gastric carcinoma (GC) is still one of the most common cancers worldwide, despite its decreasing incidence in the developed countries. The continued inflammation of the gastric epithelium by chronic *Helicobacter pylori* infection is a major contributor to carcinogenesis, most likely by promoting disruption of the balance between proliferation and differentiation in the regenerating inflamed mucosa. Although this process has been well characterized phenotipically, the main molecular players in gastric neoplastic transformation are largely unknown.[1]

Proteins of the CCAAT/enhancer-binding protein (C/EBP) family are important transcription factors that link gene expression to proliferation/differentiation control.[2] We have recently shown that C/EBPβ is over-expressed in pre-neoplastic lesions and GC.[3] Most notably, C/EBPβ over-expression is associated with loss of trefoil factor 1 (TFF1), an established differentiation marker, and a putative gastric tumor suppressor.[4]

Members of the C/EBP family are known to heterodimerize among themselves, giving rise to different functional transcriptional complexes. Moreover, they often act with a high degree of coordination.[5] This is well demonstrated in adipogenesis, where sequential expression of different C/EBP members underlies the process of differentiation from pre-adipocytes to fully mature adipocytes.[6] After the differentiation stimulus is given, C/EBPβ is expressed in immature pre-adipocytes and primes cells to differentiate by inducing C/EBPα expression.[7] Once active, C/EBPα drastically reduces cell proliferation, and promotes the expression of peroxisome proliferator-activated receptor γ.[8] In this and other models, C/EBPα is a crucial effector of lineage commitment and terminal differentiation programs.

The disruption of these programs has been shown to be oncogenic in several cellular contexts. For instance, C/EBPα is a consensual tumor suppressor in acute myeloid leukemia

where deleterious mutations have been described in a proportion of cases.[9] C/EBPα may also have a role in other cancer models[10] such as lung cancer, where it was found downregulated by methylation.[11,12] However, the expression pattern and functional relevance of C/EBPα in normal stomach and in GC has never been described.

In this study, we characterized the expression of C/EBPα in the normal gastric mucosa and in GC. Furthermore, we investigated the effects of expressing C/EBPα in GC cells, and aimed at clarifying the link between pathways of C/EBPα modulation and gastric carcinogenesis.

## MATERIALS AND METHODS
### Tissue Material
Surgical specimens from 54 GC were resected and diagnosed at Hospital S. João/Faculty of Medicine, Porto, Portugal. Tissue fragments were fixed in 10% formaldehyde and embedded in paraffin. Serial sections of 3 μm were obtained from each block and used for routine staining with hematoxylin and eosin and immunohistochemistry.

The procedures followed in this study were in accordance with the institutional ethical standards. All the samples enrolled in this study were delinked and unidentified from their donors.

### Immunohistochemistry
Tissue sections were first treated with 10 mmol/l citrate buffer (pH 6.0) for 40 min at 99°C. Unspecific endogenous peroxidase activity was eliminated with a Hydrogen Peroxide Block solution (Labvision, UK) for 10 min. After washing, slides were incubated with monoclonal mouse antibody anti-C/EBPα (1:300, Cell Signaling Technology, MA, USA) or C/EBPβ (1:100, Santa Cruz Biotechnology, CA, USA) 1 h at room temperature (RT). Sections were then washed and incubated with Dako Real Emvision/HRP Rabbit/Mouse solution (DAKO, Denmark) for 30 min (RT). The slides were then developed for 10 min in Dako Real diaminobenzidine (DAB) (0.05%, DAKO) and sections counterstained with hematoxylin, dehydrated, and mounted. For immunofluorescence, after the primary C/EBPα antibody incubation, sections were incubated with a biotinylated secondary antibody and signal was obtained with Alexa Fluor (Molecular probes, Invitrogen, CA, USA) incubation.

For double TFF1 and C/EBPα staining, two independent reactions were performed on the same slides. Sections were blocked for 15 min in 10% BSA with anti-mouse serum and incubated overnight in monoclonal antibody anti-C/EBPα (1:100, Cell Signaling Technology). After washing, samples were incubated with anti-rabbit secondary antibody (1:200, DAKO) for 30 min and washed again. A final 1 h incubation with avidine-biotin-peroxidase (1:100, DAKO) was performed. Slides were then developed with DAB (DAKO). After a washing step of 30 min in PBS at 60°C, slides were again incubated overnight with monoclonal antibody anti-TFF1 (1:100, Zymed, CA, USA) and developed with

alkaline phosphatase (DAKO) and Fast Red (Sigma-Aldrich, MO, USA).

Slides were reviewed by a pathologist, tumors were classified according to Laurén, and the sections were semi-quantitatively scored according to the intensity of staining when compared with the positive control: intense staining was classified as III; moderate intensity as II; and weak intensity or negativity as I. Cases were classified as 'downregulated' when >50% of the tumor cells were classified as I. All washing steps were performed in PBS buffer. Normal gastric mucosa was used as a positive control, and negative controls were performed by substitution of the primary antibody with immunoglobulins of the same class and concentration.

### Cell Culture, Transfections, and Blotting
AGS and MKN28 cells were grown in RPMI medium with 10% FBS (GIBCO, Invitrogen, CA, USA). AGS cells were grown until 60–80% confluence in six-well plates, and then transfected using 3 μg of Plenti-C/EBPα expression vector with an appropriate TFX-50 (Promega, WI, USA) concentration and volume. For western blot analysis, cells were scrapped in PBS and then lysed in RIPA buffer with protease and phosphatase inhibitors. A measure of 40 μg of total protein were loaded into acrylamide gels and separated by electrophoresis. The proteins were then transferred to Hybond membranes (Amersham Biosciences, UK). For dot blot, 20 μg of denatured protein extract were directly pipeted into Hybond membranes. After blocking, blots where incubated 1 h with primary antibodies anti-C/EBPα (1:100, Cell Signaling), anti-P27 and anti Cyclin D1 (1:100, Santa Cruz Biotechnology), anti-tubulin (1:15 000, Sigma-Aldrich), and in the case of the dot blot with anti-TFF1 (1:100, Zymed) in PBS plus 5% non-fat dried milk and 0.5% tween-20. The blots were then washed three times in the same solution and incubated 45 min with an HRP-conjugated secondary antibody (1:1000, Santa Cruz Biotechnology) in PBS 0.5% tween-20. Blots were then washed three times in PBS 0.5% tween-20 and signal was detected with chemiluminescence using ECL (Amersham Biosciences). For MAPK inhibition experiments, MKN28 cells were grown until 50–60% confluence and treated for 24 h with 10 μM SB239063 or PD98059 (Sigma-Aldrich).

### BRDU Incorporation Assay and Immunocytochemistry
AGS cells were harvested in 24-well plates with glass slides, and transfected using TFX50 (Invitrogen) with empty vector and full-length C/EBPα expression vectors in OPTIMEM medium (GIBCO). After 1 h, complete RPMI medium was added and cells were left growing for 48 h. MKN28 cells were grown in six-well plates with glass slides and treated with MAPK inhibitors as described above. After incubating 1 h in 5-bromo-2′-deoxy-uridine (BRDU), cells in the glass slides were fixed in 4% paraformaldehyde, washed with PBS two times, and quenched by incubation with 2 M HCl for 20 min. After washing, slides were incubated with anti-BRDU

antibody (1:100, DAKO) for 1 h. For simple immuno-cytochemistry, MKN28 cells treated and untreated with MAPK inhibitors were blocked in PBS with 4% BSA and incubated in C/EBPα (1:100, Cell Signaling) antibody for 1 h. In both procedures, cells were finally incubated with secondary anti-mouse FITC (1:100, DAKO)-conjugated antibody for 30 min. After washing, cells were mounted in vectashield (Vector Laboratories) with DAPI blue and scored for BRDU incorporation or C/EBPα expression on a fluorescence microscope.

### Inhibition of C/EBPα by siRNA

MKN28 cells were grown until 50% confluence and pre-incubated in serum-free medium. The appropriate anti-C/EBPα target sequence (100 nM) as well as scrambled control siRNA (Qiagen) were mixed with Metafectene (Biontex laboratories GmbH, Germany) in serum-free medium, incubated for 20 min and added to the cells. After overnight incubation, the medium was changed to complete RPMI and cells left to grow for 48 h, after which BRDU incorporation and protein expression analyses were performed.

### Statistical Analysis

Comparison of GC cases regarding their clinicopathological features was performed using Fisher's and $\chi^2$ test. Three independent measurements were performed for the BRDU incorporation experiments and results were compared by Student's *t*-test.

## RESULTS

### Immunohistochemical Analysis of C/EBPα Expression

In the normal mucosa of the stomach, C/EBPα staining was mostly nuclear with some residual cytoplasmic positivity and mostly localized in the mucous surface epithelium (Figure 1a). This expression pattern contrasts with that of C/EBPβ whose expression is concentrated to the neck zone
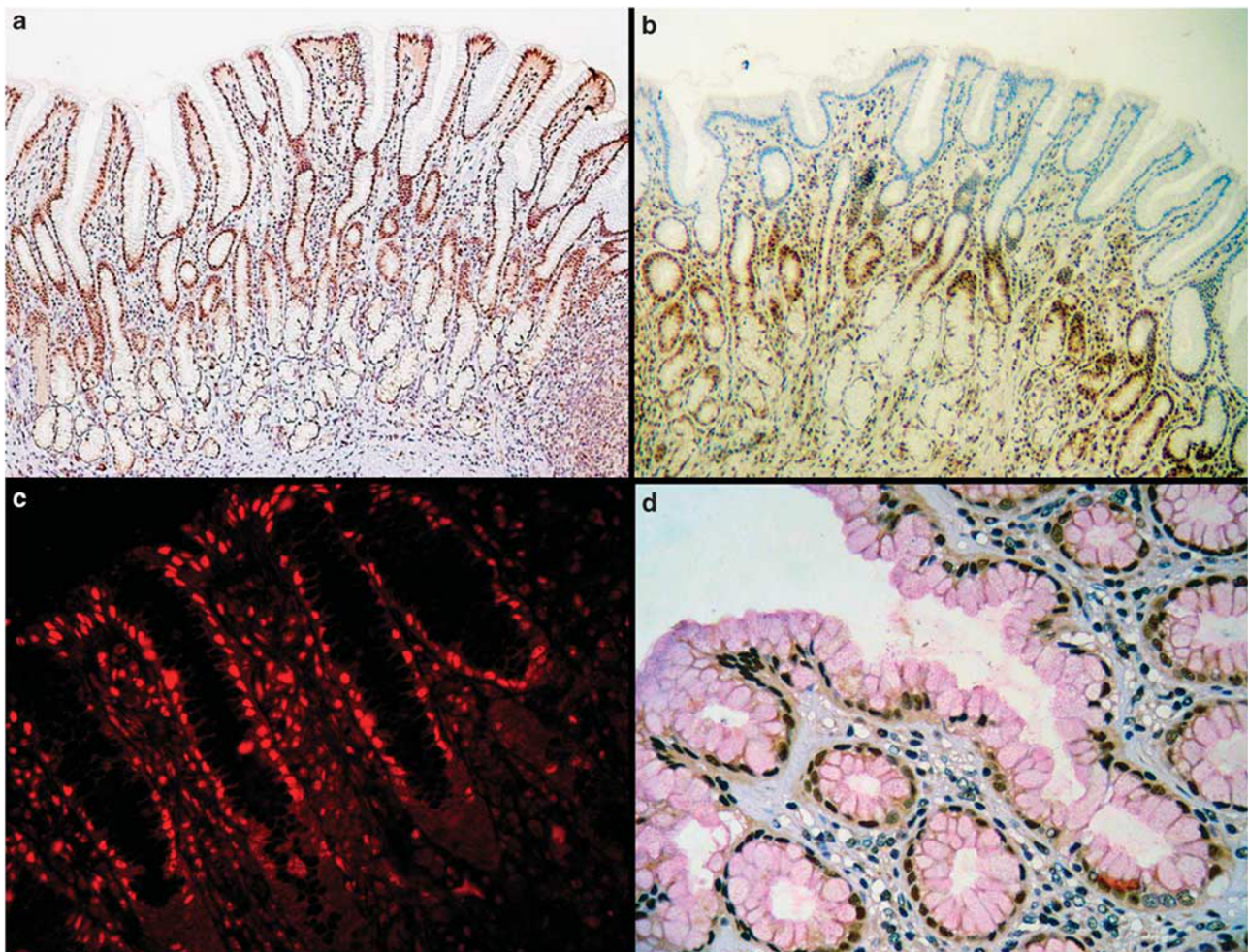


**Figure 1** C/EBP expression in normal gastric mucosa. (**a**) C/EBPα immunostaining in non-neoplastic mucosa, showing strong expression in the superficial epithelium. (**b**) C/EBPβ expression in normal gastric mucosa of the antrum, showing strong localization in the neck zone. (**c**) C/EBPα immunofluorescence, showing expression in differentiated gastric foveolae, and few positive cells toward the neck zone. (**d**) C/EBPα (brown) and TFF1 (red) double staining, showing co-expression of the two proteins in gastric foveolae.
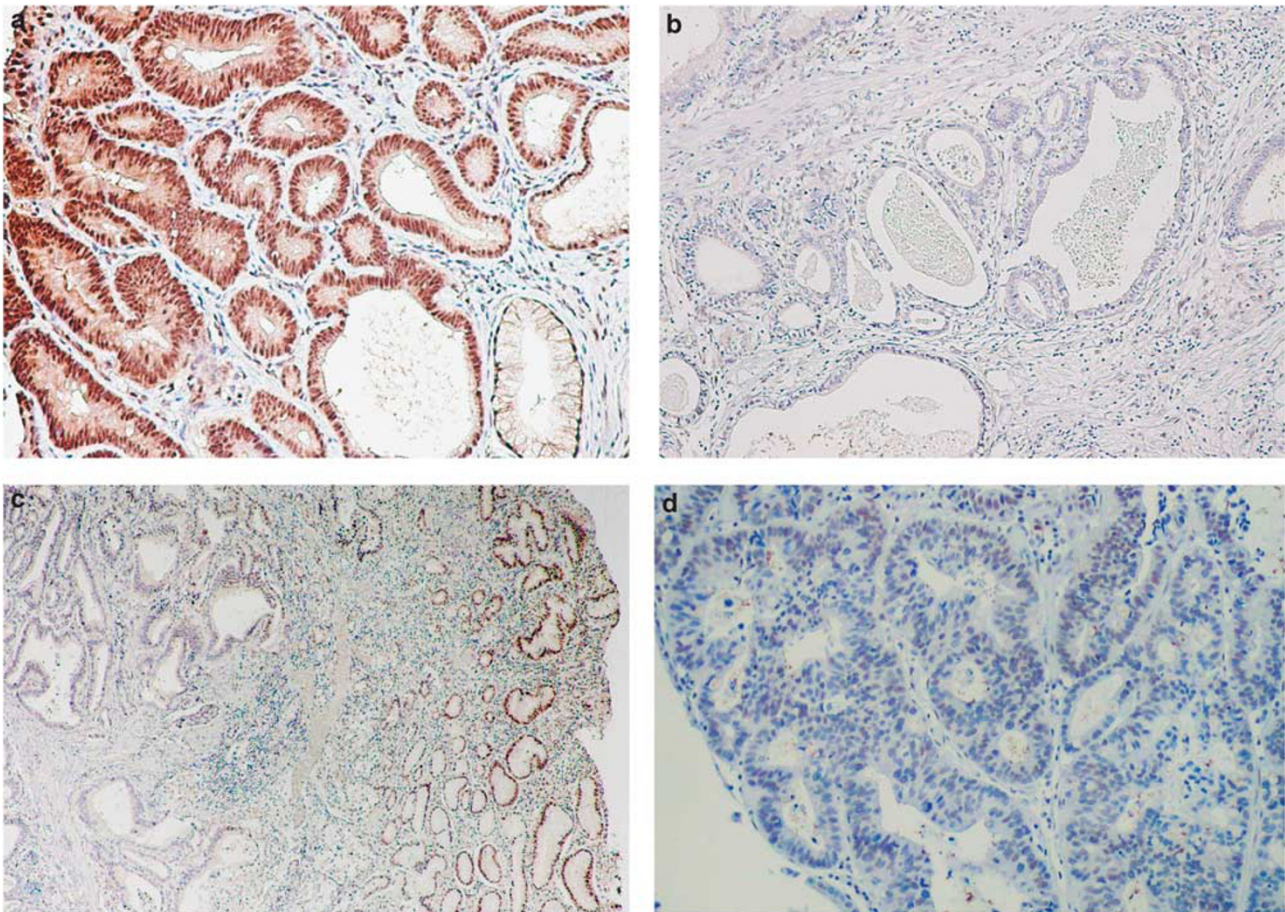
**Figure 2** C/EBPα staining in intestinal-type GC. (**a**) C/EBPα positive tumor. (**b**) Tumor showing complete loss of C/EBPα expression. (**c**) GC displaying downregulation of C/EBPa expression (positive cells to the right are located in non-neoplastic gastric epithelium). (**d**) GC negative for C/EBPα expression.

(Figure 1b). This expression pattern was confirmed using immunofluorescence, where C/EBPα staining was again found to be stronger in the foveolar and surface epithelium, with fewer positive cells observed in the neck zone (Figure 1c). As described earlier, infiltrating inflammatory cells were also found to express C/EBPα. To confirm that C/EBPα expression does correlate with the differentiation status of the gastric epithelium, we performed double staining with TFF1, a well-established gastric differentiation marker. A clear overlap was observed between TFF1 and C/EBPα in the surface epithelium (Figure 1d).

Similarly to what was observed in the normal gastric mucosa, in GC C/EBPα staining was mostly nuclear with some residual cytoplasmic positivity (Figure 2a). In GC, C/EBPα was considered downregulated in 30% of the tumors (Figures 2b–d). No statistical significant relationships were found between C/EBPα expression and any clinicopathological features of the cases (Table 1).

**Effect of C/EBPα Expression on Cell Proliferation and Differentiation**

To assess the effect of C/EBPα on the proliferation status of GC cells, we transfected the C/EBPα-negative GC cell line

AGS with an expression vector for the full-length C/EBPα gene and measured the incorporation of BRDU after 48 h. We observed that re-expression of C/EBPα on AGS cells led to a 15% reduction ($P = 0.001$) in cell proliferation in comparison with the control (Figure 3a). Conversely, inhibition of C/EBPα by siRNA in the MKN28 cell line led to an increase ($P < 0.001$) in cell proliferation in comparison with the control (Figure 3b).

To confirm this inhibitory effect of C/EBPα on proliferation, we analyzed by western blotting the expression of two cell-cycle proteins typically associated with the control of gastric epithelial cell division. We observed decreased expression of Cyclin D1, a cell-cycle inductor, and increased expression of P27, a cyclin-dependent kinase inhibitor (Figures 4a and c). Both these changes are consistent with an inhibitory effect on proliferation.

The results on the effect of C/EBPα on proliferation, together with its expression pattern in the normal gastric mucosa, suggested C/EBPα to have a role both on proliferation arrest and on the differentiation of gastric epithelial cells. That being the case, increased expression of TFF1 would be expected in the presence of higher levels of C/EBPα. In accordance with this hypothesis, after transfection of AGS

**Table 1 Relationship between the clinicopathological features of GC and C/EBPα expression scoring**

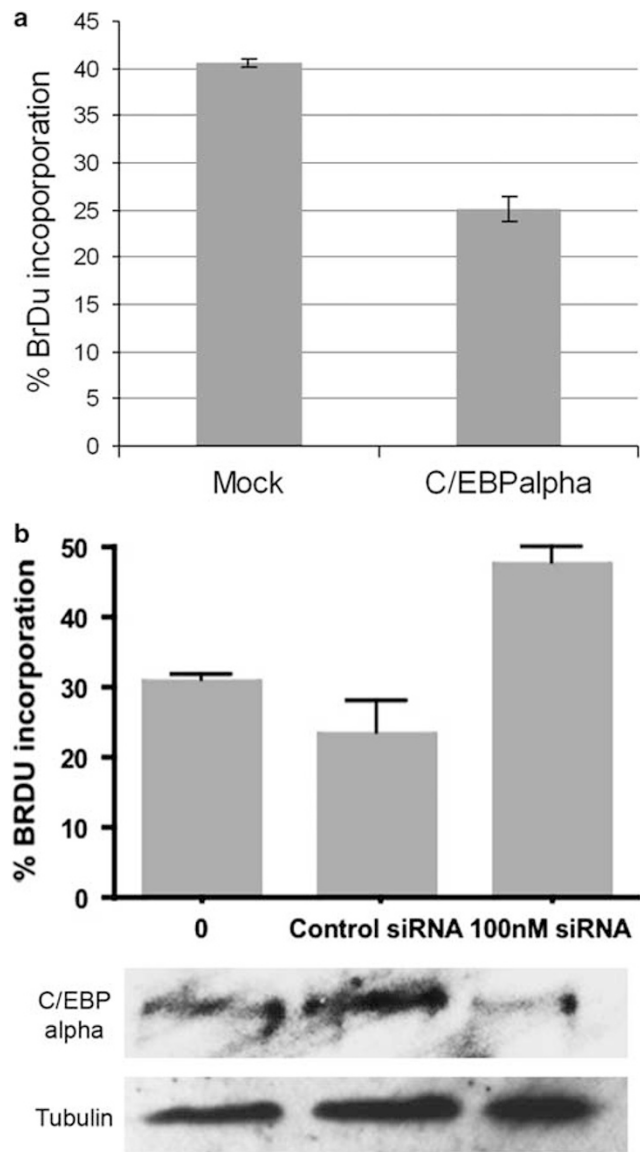| | No. of cases (%) | C/EBPα downregulation | | P-value |
| | | Yes | No | |
|---|---|---|---|---|
| *Age (years)* | | | | |
| ≤40 | 2 (4) | 1 (50) | 1 (50) | NS |
| 40–65 | 18 (36) | 2 (11.1) | 16 (88.9) | NS |
| ≥65 | 30 (60) | 12 (40) | 18 (60) | NS |
| | | | | |
| *Gender* | | | | |
| Male | 30 (60) | 11 (36.7) | 19 (63.3) | NS |
| Female | 20 (40) | 4 (20) | 16 (80) | NS |
| | | | | |
| *Histological type* | | | | |
| Intestinal | 27 (54) | 11 (40.7) | 16 (59.3) | NS |
| Diffuse | 16 (32) | 1 (6.3) | 15 (93.7) | NS |
| Atypical | 7 (14) | 3 (42.9) | 4 (57.1) | NS |
| | | | | |
| *Depth of invasion* | | | | |
| T1 | 2 (4) | 0 (0) | 2 (100) | NS |
| T2 | 26 (52) | 10 (38.5) | 16 (61.5) | NS |
| ≥T3 | 22 (44) | 5 (22.7) | 17 (77.3) | NS |
| | | | | |
| *Vascular invasion* | | | | |
| Absent | 15 (30) | 5 (33.3) | 10 (66.7) | NS |
| Present | 35 (70) | 10 (28.6) | 25 (71.4) | NS |
| | | | | |
| *Metastasis* | | | | |
| Absent | 12 (24) | 6 (50) | 6 (50) | NS |
| Present | 38 (76) | 9 (23.7) | 29 (76.3) | NS |
| | | | | |
| Total | 50 (100) | 15 (30) | 35 (70) | |

NS, non-significant.

Cases are classified according to the intensity and percentage of positive cells. Cases classified as 'downregulated' present > 50% of tumor cells classified as I.



**Figure 3** BRDU incorporation assay in GC cells. (**a**) Decreased proliferation rates of C/EBPα-transfected AGS cells in comparison with the control ($P = 0.001$). (**b**) C/EBPα inhibition by siRNA leads to increased BRDU incorporation in MKN28 cells. In all, 1000 cells were counted and BRDU incorporation expressed as the rate between DAPI and BRDU positive cells. The y axis represents the % of BRDU positive cells. Error bars represent s.d. Tubulin was used as protein-loading control.

cells with the C/EBPα expression vector, we observed an increase in the expression of TFF1 (Figures 4b and c).

**Effect of MAPK Inhibitors on the Expression of C/EBPα and Cell Proliferation**

The Ras/MAPK signaling pathway is one of the most consistently altered in human cancers. In GC, the Ras/MAPK pathway is constitutively activated through mutation of several of its receptors and signal-transducing members.[13] To explore the possibility of C/EBPα regulation by the Ras/MAPK pathway in GC, we treated MKN28 cells, which express C/EBPα, with

specific p38 (SB239063) and ERK1/2 (PD98059) inhibitors. Treatment with both inhibitors led to a marked increase in C/EBPα expression and nuclear localization as detected by immunocytochemistry (Figure 5). This increase in C/EBPα expression was further confirmed by western blotting, and shown to be accompanied by an increase in TFF1 expression (Figures 6a and c). Concomitantly, we observed a decrease in cell proliferation by BRDU incorporation (Figure 6b) both in cells treated with p38 inhibitor ($P = 0.009$) and in cells treated with ERK1/2 inhibitor ($P = 0.003$). This decrease in proliferation was accompanied by a decrease in Cyclin D1 expression (Figure 6c).
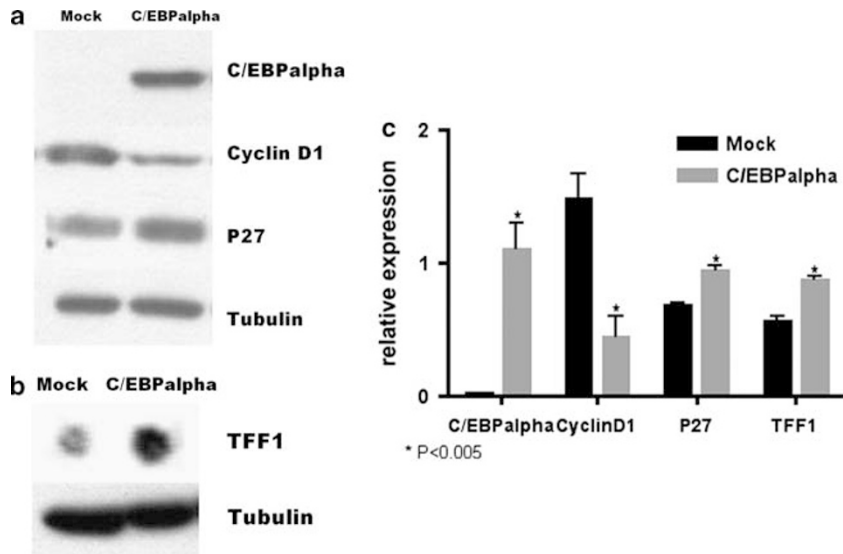
**Figure 4** Effect of C/EBPα expression on AGS cells. (**a**) Western blot for cell-cycle proteins, showing increased P27 and decreased cyclin D1 expression after transfection with C/EBPα. (**b**) Dot blot showing increased TFF1 expression in C/EBPα-transfected cells. (**c**) Expression of C/EBPα, cyclin D1, p27, and TFF1 shown as ratios to loading controls. Error bars represent s.d. *represents statistically significant differences between mock- and C/EBPα-transfected cells.
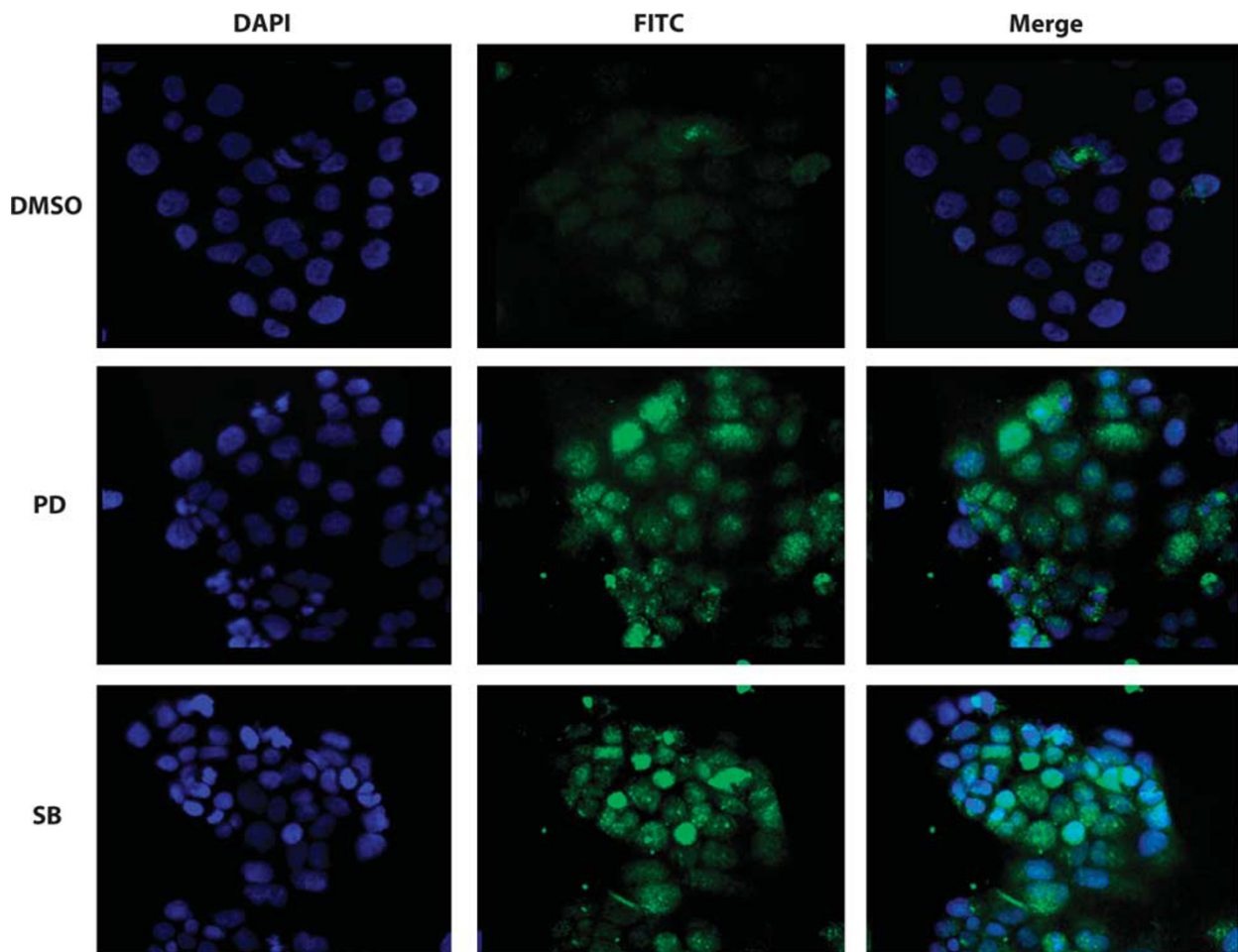


**Figure 5** Treatment of MKN28 cells with p38 (SB) and ERK1/2 (PD) inhibitors leads to an increase in C/EBPα expression with nuclear localization. C/EBPα is stained green with FITC and nuclei are stained blue with DAPI for contrast.
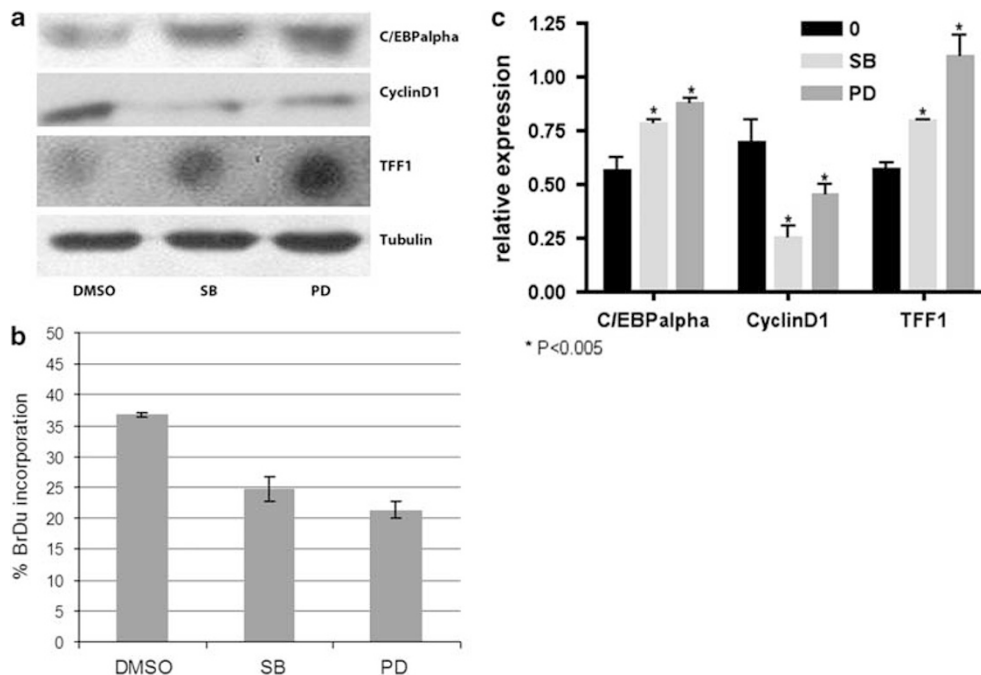
**Figure 6** Effects of the treatment of MKN28 cells with a p38 (SB) and an ERK1/2 (PD) inhibitor in cellular proliferation and differentiation. (**a**) Western blot showing that treatment of MKN28 cells with SB and PD leads to an increase in C/EBPα and TFF1 expression and to a decrease in Cyclin D1 levels. (**b**) Decrease of cell proliferation by BRDU incorporation assay of MKN28 cells treated with SB ($P = 0.009$) and PD ($P = 0.003$) inhibitors. (**c**) Expression of C/EBPα, cyclin D1, and TFF1 shown as ratios to loading controls. Error bars represent s.d. *represents statistically significant differences between treated and non-treated cells.

## DISCUSSION

We have shown that C/EBPα is expressed in the differentiated epithelial compartment of the superficial gastric mucosa. This expression pattern mirrors that described for C/EBPβ, which is expressed in the proliferative neck zone of the normal gastric mucosa. We have previously argued that C/EBPβ may have a role in maintaining a balance between proliferation and differentiation in the normal gastric mucosa.[3] In the proposed model, C/EBPβ would have a pro-proliferative activity in gastric epithelial stem-like cells. The presence of C/EBPα in differentiated cells, together with its ability to reduce cell proliferation and to upregulate the gastric differentiation marker TFF1, suggest that C/EBPβ and C/EBPα may have complementary roles in maintaining a balance between proliferation and differentiation in the normal gastric mucosa. By analogy to the model of adipogenesis,[6] one feels tempted to speculate that C/EBPβ is expressed in gastric epithelial stem-like cells and may prime gastric epithelial cells to differentiate by inducing C/EBPα expression. Once active, C/EBPα would reduce cell proliferation, and promote the expression of gastric differentiation markers such as TFF1.

C/EBPα was first described as a tumor suppressor gene in acute myeloid leukemias. In normal hematopoiesis, C/EBPα has a key role in defining cell lineages through interaction with other transcription factors. C/EBPα disruption by mutation leaves bone marrow cells in an undifferentiated, hyperproliferative state being this event causal for a large percentage of leukemias.[14] Downregulation of C/EBPα was additionally found in several epithelial tumor types, namely lung, breast, and skin carcinomas.[11,12,15,16] In all these examples, a role for impaired C/EBPα function in tumourigenesis was strengthened by the observation that C/EBPα re-expression is able to inhibit tumourigenesis both *in vivo* and *in vitro*.[15,16]

In our study, we observed downregulation of C/EBPα in about 30% of GC cases. In an earlier study, we have described a frameshift mutation of C/EBPα in a GC. This mutation was deleterious and absent from adjacent non-neoplastic tissue.[17] These results in the GC model are in keeping with the aforedescribed role of C/EBPα in tumourigenesis, whereby loss of C/EBPα would be associated to loss of differentiation and sustained proliferation of tumor cells. On top of C/EBPα loss of expression, we have shown earlier that C/EBPβ is over-expressed in cells retaining a proliferative phenotype such as those seen in dysplastic and cancer lesions. C/EBPβ is able to counteract, either by heterodimerization or repression of expression, the differentiating activity of C/EBPα. Altogether, either aberrant over-expression of C/EBPβ or loss of expression of C/EBPα are present in the majority of GC cases. Hence, these results suggest that changes in expression/function of both C/EBPα and C/EBPβ may be pieces of the same puzzle rather than independent events in gastric carcinogenesis. This possibility, together with other putative mechanisms of post-translational or protein–protein interaction, would help explaining why expression of C/EBPα is still seen in about 70% of GC cases.

In other cancer models, loss of C/EBPα has been linked with oncogenic Ras activation.[16] In GC, activating RAS mutations do occur in a subset of microsatellite unstable tumors.[13] By using specific inhibitors for p38 and ERK1/2, both downstream effectors of Ras signaling, we were able to show that inhibition of C/EBPα expression was dependent on the activation of this pathway. Moreover, inhibition of p38 and ERK1/2 increased TFF1 expression and strongly reduced MKN28 cell proliferation and Cyclin D1 levels, in a set of alterations most likely linked with the observed increase in C/EBPα expression.

In summary, we show that in normal gastric mucosa, C/EBPα is expressed mainly in the differentiated foveolar epithelium where it co-localizes with TFF1. We show that C/EBPα is downregulated in a considerable percentage of GC. We additionally show that C/EBPα re-expression in a C/EBPα-negative cell line leads to a reduction in proliferation that is accompanied by an increase in P27 and reduction of cyclin D1 levels. In parallel, we show an increase in the expression of TFF1 in C/EBPα-transfected cells. Finally, we show that treatment of a C/EBPα expressing cell line with MAPK inhibitors leads to increased C/EBPα and TFF1 expression, and a concomitant reduction on cell proliferation and Cyclin D1 expression. Overall, these results substantiate the role of the C/EBP transcription factor family in homeostasis of the gastric epithelium and in the process of gastric carcinogenesis.

**DISCLOSURE/CONFLICT OF INTEREST**
The authors declare no conflict of interest.

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