The Tumour Suppressor C/EBP delta Inhibits FBXW7 Expression and Promotes Mammary Tumour Metastasis

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1st Editorial Decision 17 May 2010

Thank you very much for submitting your research manuscript for consideration to The EMBO Journal editorial office. Three scientists provided their comments and assessment of the study that you will find enclosed below. All three referees appreciate the findings of C/EBPdelta as tumor suppressor that does however also contribute to tumor metastasis presumably by targeting FBXW7/mTOR signaling. Despite the general interest and positive remarks on your study, specific concerns that need to be addressed during revisions are expressed from all three scientists. Importantly, ref#2 requests some profiling data that could surface additional targets and thereby also substantiate the necessary discussion related to recent results from your lab published in PNAS. As the reports are very explicit and aimed at improving the overall quality of the study, we would be delighted to assess a thoroughly revised version of your paper in the near future. I also have to remind you that it is EMBO_J policy to allow a single round of major revisions only and that the decision on acceptance or rejection entirely depends on the content within the final version of your manuscript that will be assessed by at least some of the original referees!

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal
REFEREE REPORTS

Referee #1 (Remarks to the Author):

In the present study Balamurugan and coworkers present a novel role for the candidate tumor suppressor C/EBPδ in the integration of hypoxia and inflammation to promote tumor metastasis in a breast cancer model. Using a MMTV-c_neu transgenic mouse model as well as cell culture experiments they show that the inflammatory response protein C/EBPδ inhibits the expression of the tumor suppressor FBXW7 promoting mTOR/AKT/S6K1 signaling which favors the translation of hypoxia-inducible factor-1α (HIF-1α). The manuscript demonstrates for the first time a tumor suppressor function of C/EBPδ in a transgenic mouse model of breast cancer and furthermore shows an unexpected role of C/EBPδ in the promotion of tumor metastasis suggesting that C/EBPδ undergoes a switch from tumor suppressor activity in earlier stages of tumor development to a pro-metastatic activity at later stages. Overall, this is an interesting study but several problems remain.

Major aspects:

1. The presentation of the mouse data is somewhat confusing and should be explained in more detail especially in the case of the metastasis data. Here, the percentage of mice with metastasis is given which provides no information about the number of metastasis per mouse. In contrast, for the primary tumorigenesis only the numbers of tumors are taken into account. Since analyses for the numbers of tumors and metastasis were performed at different times (only dependent on the size of the largest palpable tumor) the results are very difficult to interpret. In our view, there is very little difference if any between WT, KO and Heterozygous. As a consequence the conclusions about "tumor initiation" by C/EBPδ and suppression of C/EBPδ remain doubtful.

2. The authors postulate that C/EBPδ directly inhibits FBXW7 gene expression. For this purpose they present endpoint ChIP-PCR assays but no direct transcriptional activity assays. The PCR appeared to lead to non-specific bands. Even more important, the in vivo binding data is not associated with any functional assay. To prove that C/EBPδ represses FBXW7 expression through this side, the authors should perform luciferase promoter assays in the presence and absence of C/EBPδ. Further, a mutation of the potential binding site would indicate whether this site is really important to C/EBPδ associated repression of FBXW7.

3. Control Blots for C/EBPδ expression in WT and KO cells are provided quite selectively and are not given for every Western Blot. As an example, in the initial Western Blot Figure 1D no C/EBPδ Blot is shown to prove that KO cells really do not express C/EBPδ anymore. When this is due to the changing usage of whole cells extracts or nuclear extracts and the different expression levels of C/EBPδ in the different cell lines than this should be at least clearly mentioned once. Nevertheless, for the initial Western Blot Figure 1 a C/EBPδ Blot should be included.

4. In the Results Section "Reduced HIF-1α expression correlates with impaired AKT signaling" the authors examine the relationship between phosphorylation of AKT, GSK-3β and the expression of HIF-1α in WT- and C/EBPδ-KO MEFs. They show that KO MEFs display a decrease of Gsk-3β phosphorylation that should lead to a decreased HIF-1α expression (Figure 4A). However, they do not provide HIF-1α Western Blots to prove this postulation. Likewise a C/EBPδ control blot for WT- and KO- MEFs is missing.

5. In Figure 5A, presenting that C/EBPδ augments endogenous mTOR- and HIF-1α- levels, the β-actin loading control is missing making it difficult to interpret the relatively small differences in mTOR expression. Same is true for the upper part of Figure 5C (Primary Tumor cells). Additionally, the mTOR protein band for KO cells looks quite uneven in this Figure, raising the question whether the postulated difference in band intensity is an artifact or not. This should be revisited by the authors.

6. In Figure 6D, PCR Amplicons after C/EBPδ-ChIP for treatment conditions with and without DFX are shown. In the DFX -untreated control unspecific bands appear for both of the C/EBPδ antibodies as well as for IgG. How do the authors explain these unspecific bands?

Minor aspects:
1. In the first Results Section "Loss of C/EBPδ increases mammary tumour multiplicity and decreases lung metastasis" the authors refer to the Kaplan Meier Plot of WT-, Heterozygous- and C/EBPδ KO- mice that does not show significant differences between the three groups. In this context the next sentence "Thus we infer that C/EBPδ inhibits tumour-initiating processes" is confusing and potentially not correct. Furthermore, the fact that the survival between the three groups was not changed significantly should be discussed in some detail.

2. In the Results Section "C/EBPδ is required for hypoxic HIF-1α accumulation and hypoxia adaption" the authors present that mammary glands of nulliparous healthy mice show low levels of C/EBP in the epithelial cells. However, in a study of Porter et al., (2001) C/EBP was among the genes abundantly expressed in normal mammary epithelial cells and a somewhat gradual decline from normal tissue to in situ and then to invasive carcinoma could be observed. The authors should comment on these opposite findings.

3. In the Figure Legends of Figure 1C (Immunhistochemistry of anti-C/EBPδ- and anti-hydroxyprobe-stained tissue) the authors should provide the magnification of the microscopic pictures.

4. In the Figure Legends the authors never indicate whether biological replicates of Western Blots were performed. This should be mentioned throughout the Figure Legends or in the Materials and Methods Section.

7. In their Paper "Akt1 Activation Can Augment Hypoxia-Inducible Factor-1A Expression by Increasing ProteinTranslation through a Mammalian Target of Rapamycin-Independent Pathway" Pore et al. (2001) show that glioblastoma cell lines including U251 don’t show reduced HIF-1α expression under hypoxic conditions and concurrent application of Rapamycin. This indicates that mTORC1 seems not to be involved in HIF-1α protein expression. The authors should discuss this in terms of their own findings for the U251 cell line.

Referee #2 (Remarks to the Author):

The manuscript by Balamurugan et al. describes the functions of the transcription factor C/EBPδ in mammary tumors development, both as a tumor suppressor and as a protein that favors metastasis. The Authors perform a molecular analysis that include activation of HIF-1α and mTOR signaling, finally pinpointing FBXW7 as a key target. The experiments are well performed and informative and the data well presented; the work has many merits, highlighting a role in breast cancer metastasis of the least studied member of the C/EBP family. Although the identification of FBXW7 as a gene targeted by C/EBPδ helps explaining the in vivo function of C/EBPδ in mammary cells, in my opinion it would be important to perform profiling analysis along tumor progression in the MMTV-cNeu model, in the C/EBPδ wt and KO mice configuration. This will most likely identify additional C/EBPδ targets that will help in the understanding of the molecular mechanisms of C/EBPδ, both as a tumor suppression and promoter of metastasis.

Referee #3 (Remarks to the Author):

Balamurugan et al showed that C/EBPdelta has tumor suppressor functions in a mammary gland tumor model and that C/EBPdelta also (paradoxically) promotes mammary tumor metastasis. The authors show that C/EBPdelta causes HIF1a stabilization (required for metastasis) via an AKT/mTOR translation control pathway by inhibition of FBXW7 expression that is involved in mTOR destabilization. This is an interesting manuscript that may help to elucidate functions of C/EBPδ in metastasis.

Several points need attention:

1. A problem arises in conjunction with a recent publication of the same lab (PNAS, published ahead of print May 3, 2010, doi:10.1073/pnas.0913813107). In the PNAS publication the authors
showed that CEBPd targets cyclin D1 for degradation by activating expression of the Cdc27 subunit of the anaphase promoting complex. Cdc27 is involved in the degradation of several cell cycle regulators, including cyclin D1. In the PNAS publication the authors conclude that proteasomal down regulation of cyclin D1 through CEBPd activated Cdc27 was important to prevent tumor formation. Here, the authors show that CEBPd acts as a tumor suppressor and conclude that CEBPd affected mTOR / hypoxia adaptation is of major importance in metastasis. Accordingly, the authors aim to publish two related CEBPd dependent degradation mechanisms pathways that may show opposite effects on tumor formation metastasis (Cdc27 activation in the PNAS paper and FBXW7 inhibition in the EMBO paper). Although both studies are appreciated as independent findings (tumor formation metastasis), it is curious to see shared authorships on both publications, without any cross reference in the EMBO submission. Cyclin D1 is a major factor in breast carcinogenesis and it is puzzling to see that the authors avoid to experimentally address related issues and even omit discussing potential links between both studies (there is no reference to the PNAS publication in the EMBO J submission). Why do the authors skirt around these issues? The results (PNAS/EMBO J submission) also raise the questions how CEBPd can simultaneously act as a repressor activator (EMBO J/ PNAS, respectively) and one wonders whether/how other CEBPs (in particular CEBPb which had been described as important in ras induced carcinogenesis) are involved. These issues have to be addressed, at least in the discussion section.

2. The authors take us on an excursion of AKT/GSK phosphorylation / expression effects (Fig 4, 5) to arrive at FBXW7 (anti-onco) gene regulation and the function of FBXW7 on mTOR. Although the results might reflect the sequence of events that lead to the discovery of CEBPd dependent mTOR functions, the manuscript would profit (and become considerably shorter) from starting with mTOR/FBXW7 regulation, showing AKT/GSK etc. data as supplementary figures. One would also have liked to see the ubiquitination status of mTOR in WT-CEBPd/KO in relation to FBXW7 expression. Are such data available?

3. Most of the data describing the mechanism implying C/EBPdelta in hypoxia have been obtained from MEF, tumor cell lines, or primary tumor cells in vitro/ex vivo. Showing that FBXW7 is absent reduced and mTOR is enhanced in C/EBPdelta metastatic tumors (at least in the hypoxic areas) would strengthen this connection. Have the authors examined whether Notch is involved (FBXW7-g-secretase connection)?

4. Some results are difficult to appreciate by the data provided. Quantification of the blot shown in Fig5A (“Exogenous expression of C/EBPdelta augmented endogenous mTOR”) is required for this conclusion. Fig 5B. shows that mTOR is responsible for HIFalpha expression because of the effect of rapamycin but it does not show that C/EBPdelta is inducing HIFalpha expression through mTOR at that point as mentioned page 11, 1st paragraph. The authors should modify their conclusions accordingly.

5. Fig 3A. What is the difference between the mode of action of DFX and CocI2 in inducing HIFalpha expression? In WT MEF both CoCl2 and DFX induced HIFalpha expression but only DFX could do it in KO MEF. Why?

6. Throughout the manuscript and in Fig7, translational effects of mTOR are attributed to S6K regulation. However, mTOR also regulates eIF4E activity through phosphorylation of 4EBPs. How do the authors know that the mTOR effect is mediated through S6K and not through eIF4E?

Minor points:

Fig 1E and F: Results (glycolytic adaptation and cell viability by MTT assays) are mean +/- SEM of 3 independent experiments. Was the experiment done 3 times on the same preparation of primary cells or on 3 different preparations of primary tumors? It is important to clarify this point.
Thank you for the review of our manuscript. We were pleased that the three reviewers judged the report as interesting and generally well done. We sincerely thank the reviewers for their thoughtful evaluation and constructive criticism, and the chance to improve our report with valuable new data and clarifications.

We are now submitting a revised manuscript. In response to the reviewers, we have performed many additional experiments and they further support our previous conclusions. To facilitate the reviewers’ task, the relevant modifications are indicated in blue within the manuscript and the point-by-point response below. In addition, a list of the modifications to the figures is provided.

Further, we want to point out that our recent article by Pawar et al. (2010) had not been accepted for publication at the time of the initial submission of this manuscript. We are pleased that we are now able to include this citation, which indeed facilitates the interpretation of the results presented here.

Because the revised manuscript was longer than permitted, we moved more of the Methods to the Supplementary Information and introduced minor modifications throughout the body of the text and the figure legends. Although these changes are not specifically marked, we were careful to not change the meaning of any text.

Dr. Hsin-Hwa Tsai has been added as an author based on her contributions to FBXW7 promoter cloning and analysis.

On behalf of all authors, I thank you for this chance to revise and improve our manuscript and hope that it will now be acceptable for publication in The EMBO Journal.

Referee #1

Major aspects:

1. The presentation of the mouse data is somewhat confusing and should be explained in more detail especially in the case of the metastasis data. Here, the percentage of mice with metastasis is given which provides no information about the number of metastasis per mouse.

Re: We have expanded our description of the mouse data (page 5-6) and included quantification of the metastatic load (no difference between KO and WT metastasis-bearing mice).

In contrast, for the primary tumorigenesis only the numbers of tumors are taken into account. Since analyses for the numbers of tumors and metastasis were performed at different times (only dependent on the size of the largest palpable tumor) the results are very difficult to interpret.

Re: The data from number of tumors and lung metastasis are in fact from the same time point (end point). We have clarified this in the figure legend (Fig.1B).

In our view, there is very little difference if any between WT, KO and Heterozygous. As a consequence the conclusions about “tumor initiation” by C/EBPd; and suppression of C/EBPd remain doubtful.

Re: We hypothesize that C/EBPd has an effect on tumor initiation, i.e. increased number of tumors are initiated but do not grow faster, because there is no acceleration of the end point. We have modified the text to clarify this reasoning and adjusted the conclusion (page 5).

2. The authors postulate that C/EBPd directly inhibits FBXW7 gene expression. For this purpose they present endpoint ChIP-PCR assays but no direct transcriptional activity assays. The PCR appeared to lead to non-specific bands.
Re: We have optimized the ChIP assay for FBXW7 and have replaced the data with a new experiment, which does not show the unspecific band (Fig.5E).

Even more important, the in vivo binding data is not associated with any functional assay. To prove that C/EBPd represses FBXW7 expression through this side, the authors should perform luciferase promoter assays in the presence and absence of C/EBPd. Further, a mutation of the potential binding site would indicate whether this site is really important to C/EBPd associated repression of FBXW7.

Re: As suggested by the reviewer the FBXW7 promoter was cloned into a reporter construct and the C/EBP binding site was mutated. As seen in the new panel Fig. S6D, C/EBPd inhibits the reporter activity and mutation of the C/EBP binding site alleviates this effect. These data support the conclusion that C/EBPd regulates the FBXW7 promoter directly. Given that we had expected a requirement of the chromatin context for promoter inhibition by C/EBPd, we thank the reviewer for requesting this experiment, which will give us a tool for further analyses.

3. Control Blots for C/EBPd expression in WT and KO cells are provided quite selectively and are not given for every Western Blot. As an example, in the initial Western Blot Figure 1D no C/EBPd Blot is shown to prove that KO cells really do not express C/EBPd anymore. When this is due to the changing usage of whole cells extracts or nuclear extracts and the different expression levels of C/EBPd in the different cell lines than this should be at least clearly mentioned once. Nevertheless, for the initial Western Blot Figure 1 a C/EBPd Blot should be included.

Re: As the reviewer correctly assumed, the “selective” demonstration of C/EBPd expression controls was indeed in part due to the use of different types of extracts. C/EBPd is not detectable in whole cell extracts of cells used in this study except from immortalized MEFs, MCF10A, and U251. We apologize that we did not clarify this point. We added this information to the legend of Figure 1D. We agree that it would be illustrative to show the lack of C/EBPd in KO tissues in the context of the first figure. Therefore, we added data from nuclear extracts to Figure 1D. Furthermore, we added the citation for the KO mice (Sterneck et al., 1998) in the first paragraph of the Results (page 5).

4. In the Results Section "Reduced HIF-1a expression correlates with impaired AKT signaling" the authors examine the relationship between phosphorylation of AKT, GSK-3b and the expression of HIF-1a in WT- and C/EBPd-KO MEFs. They show that KO MEFs display a decrease of GSK-3b phosphorylation that should lead to a decreased HIF-1a expression (Figure 4A). However, they do not provide HIF-1a Western Blots to prove this postulation. Likewise a C/EBPd control blot for WT- and KO-MEFs is missing.

Re: In Figure 3A (previous Figure 4A) serum stimulation was used as an established means to see robust activation of AKT and downstream events in whole cells extracts. We have added C/EBPd expression data to this panel. However, better detection of HIF-1a requires hypoxia or otherwise stabilizing conditions such as DFX. This experiment was a step toward the data in panel B where we do correlate this pathway with HIF-1a expression.

5. In Figure 5A, presenting that C/EBPd augments endogenous mTOR- and HIF-1a levels, the b-actin loading control is missing making it difficult to interpret the relatively small differences in mTOR expression. Same is true for the upper part of Figure 5C (Primary Tumor cells). Additionally, the mTOR protein band for KO cells looks quite uneven in this Figure, raising the question whether the postulated difference in band intensity is an artifact or not. This should be revised by the authors.

Re: As suggested by the reviewer we replaced HIF-1b data with b-actin as loading control (now Figure 4A). In addition we included quantification of the mTOR/actin expression levels. We also revised Figure 4C (previously 5C) with a new set of data where the protein detection is more even and included the loading control actin.

6. In Figure 6D, PCR Amplicons after C/EBPd-ChIP for treatment conditions with and without DFX are shown. In the DFX -untreated control unspecific bands appear for both of the C/EBPd antibodies as well as for IgG. How do the authors explain these unspecific bands?

Re: We have optimized the reagents and assay conditions and replaced the data. The results are equivalent but the unspecific band is no longer present (now Fig.5E).
Minor aspects:

1. In the first Results Section "Loss of C/EBPd increases mammary tumour multiplicity and decreases lung metastasis" the authors refer to the Kaplan Meier Plot of WT-, Heterozygous- and C/EBPd KO- mice that does not show significant differences between the three groups. In this context the next sentence "Thus we infer that C/EBPd inhibits tumour-initiating processes" is confusing and potentially not correct. Furthermore, the fact that the survival between the three groups was not changed significantly should be discussed in some detail.

Re: We have expanded the description and interpretation of the results according to the suggestion (page 5).

2. In the Results Section "C/EBPd is required for hypoxic HIF-1a accumulation and hypoxia adaption" the authors present that mammary glands of nulliparous healthy mice show low levels of C/EBPd in the epithelial cells. However, in a study of Porter et al., (2001) C/EBPd was among the genes abundantly expressed in normal mammary epithelial cells and a somewhat gradual decline from normal tissue to in situ and then to invasive carcinoma could be observed. The authors should comment on these opposite findings.

Re: The data by Porter et al (2001) are based on RNA in human tissue, while our data represent protein expression in mouse. While there may be regulation of expression at the level of protein, we believe that the main difference is the structure of the tissue. The mature mouse mammary gland lacks the lobular structures of the normal human breast tissue, which exhibits most of the C/EBPd expression (our unpublished data). To address this question we have more clearly indicated the differences in analysis in the revised manuscript (page 6).

3. In the Figure Legends of Figure 1C (Immunohistochemistry of anti-C/EBPd and anti-hydroxyprobe -stained tissue) the authors should provide the magnification of the microscopic pictures.

Re: The original magnification (400x) is now mentioned in the figure legend (Figure 1C).

4. In the Figure Legends the authors never indicate whether biological replicates of Western Blots were performed. This should be mentioned throughout the Figure Legends or in the Materials and Methods Section.

Re: As suggested by the reviewer, we now mention in Materials and Methods that all Western data are representative of biological replicates.

5. In their Paper "Akt1 Activation Can Augment Hypoxia-Inducible Factor-1A Expression by Increasing Protein Translation through a Mammalian Target of Rapamycin-Independent Pathway" Pore et al. (2001) show that glioblastoma cell lines including U251 don’t show reduced HIF-1a expression under hypoxic conditions and concurrent application of Rapamycin. This indicates that mTORC1 seems not to be involved in HIF-1a protein expression. The authors should discuss this in terms of their own findings for the U251 cell line.

Re: We thank the reviewer for pointing out this apparent discrepancy. As a result, we reproduced the data in the aforementioned publication and show that differences in results are mostly due to different treatment conditions, although we still see mTORC1 involvement in both cases (see page 11-12 and new Supplementary Figure S5B).

Referee #2 (Remarks to the Author):

Although the identification of FBXW7 as a gene targeted by C/EBPd helps explaining the in vivo
function of C/EBPd in mammary cells, in my opinion it would be important to perform profiling analysis along tumor progression in the MMTV-cNeu model, in the C/EBPd wt and KO mice configuration. This will most likely identify additional C/EBPd targets that will help in the understanding of the molecular mechanisms of C/EBPd, both as a tumor suppression and promoter of metastasis.

We concur with the reviewer that such gene expression profiling analysis could provide valuable information about the C/EBPd null tumor model. However, we would like to suggest that “understanding of the molecular mechanisms of C/EBPd, both as a tumor suppression and promoter of metastasis” is beyond the scope of a single manuscript. For example, many laboratories are studying since many years the dual functions of TGF-beta in tumorigenesis. Therefore, we propose to focus here on the more novel discovery of a metastasis-promoting function of C/EBPd. Following the reviewer’s suggestion we performed microarray analysis of advanced tumors from WT and KO mice (n=6). However, we did not find any genes that were more than 1.5-fold differentially expressed, except one snRNA (1.7-fold), which may in part be due to pseudogene(s). Therefore, we chose not to show these data. The result is not too surprising given that the highest expression of C/EBPd is restricted to hypoxic regions, which become diluted in bulk tumor preparations. In addition, the activity of C/EBPd may also be regulated and further regionally restrict its functions. Also, expression profiling is only the first step toward target identification.

To provide evidence for additional pro-metastatic target genes of C/EBPd, we have performed RNA and ChIP analysis of the CXCR4 gene. Although this is also a known HIF-1 target, the complete absence of CXCR4 in KO tumor cells (Fig.1D) and independent data by our collaborator Dr. Wang from analysis of MEF, suggested that this is also a direct target of C/EBPd. Indeed, our data confirm that C/EBPd contributes directly to CXCR4 gene activation (Fig. S2, and page 7) and therefore present an additional pro-metastatic target gene.

Concerning the tumor suppressor-like function of C/EBPd, we can now cite two recently accepted manuscripts from our lab, which present two plausible mechanisms. These papers were not accepted at the time of our first submission of this manuscript, and are now discussed on pages 16-17. We plan to further analyze the mechanism of the tumor suppressor activity of C/EBPd in vivo through a next generation mouse model with cell-type specific deletion of the C/EBPd gene.

Referee #3 (Remarks to the Author):

Several points need attention:

1. A problem arises in conjunction with a recent publication of the same lab (PNAS, published ahead of print May 3, 2010, doi:10.1073/pnas.0913813107). In the PNAS publication the authors showed that CEBPd targets cyclin D1 for degradation by activating expression of the Cdc27 subunit of the anaphase promoting complex. Cdc27 is involved in the degradation of several cell cycle regulators, including cyclin D1. In the PNAS publication the authors conclude that proteasomal down regulation of cyclin D1 through CEBPd activated Cdc27 was important to prevent tumor formation. Here, the authors show that CEBPd acts as a tumor suppressor and conclude that CEBPd affected mTOR / hypoxia adaptation is of major importance in metastasis. Accordingly, the authors aim to publish two related CEBPd dependent degradation mechanisms/pathways that may show opposite effects on tumor formation/metastasis (Cdc27 activation in the PNAS paper and FBXW7 inhibition in the EMBO paper). Although both studies are appreciated as independent findings (tumor formation/metastasis), it is curious to see shared authorships on both publications, without any cross reference in the EMBO submission. Cyclin D1 is a major factor in breast carcinogenesis and it is puzzling to see that the authors avoid to experimentally address related issues and even omit discussing potential links between both studies (there is no reference to the PNAS publication in the EMBO J submission). Why do the authors skirt around these issues? The results (PNAS/EMBO J submission) also raise the questions how CEBPd can simultaneously act as a repressor/activator (EMBO J/ PNAS, respectively) and one wonders whether/how other CEBPs (in particular CEBPb which had been described as important in ras induced carcinogenesis) are involved. These issues have to be addressed, at least in the discussion section.
Re: We are indeed excited that we can now cite our recent publication by Pawar et al. in this manuscript. By no means did we intend to “skirt around the issue”, although we realize that this impression could be obtained. Only, the Pawar et al. paper was not accepted at the time we submitted this study to EMBO J. Therefore, we felt that it was premature to cite it. We have indeed addressed Cdc27 and cyclin D1 expression in MMTV-Neu tumors, but we did not find these modified by the C/EBPd genotype. This result and some possible explanations are now discussed on pages 16-17. The opposite effect of C/EBPd on two different degradation pathways is very fascinating and their crosstalk will be the subject of future studies in our lab.

Following the reviewers comment, we have tested the effect of C/EBPa and C/EBPb, and found that they do not alter FBXW7 protein levels (Fig. S6A, and page 13).

2. The authors take us on an excursion of AKT/GSK phosphorylation/expression effects (Fig 4, 5) to arrive at FBXW7 (anti-onco) gene regulation and the function of FBXW7 on mTOR. Although the results might reflect the sequence of events that lead to the discovery of CEBPd dependent mTOR functions, the manuscript would profit (and become considerably shorter) from starting with mTOR/FBXW7 regulation, showing AKT/GSK etc. data as supplementary figures. One would also have liked to see the ubiquitination status of mTOR in WT-CEBPd/KO in relation to FBXW7 expression. Are such data available?

Re: The reviewer’s suggestion to restructure the manuscript is a valid one. However, at this stage, we prefer to refrain from such major action, because of the risk of creating new issues with the presentation of the data. We have, however, moved Figure 3 to the Supplementary Information.

According to the reviewer’s suggestion we have added FBXW7 expression levels to the mTOR ubiquitiniation data (now Fig. 4E), which confirm their inverse relationship.

3. Most of the data describing the mechanism implying C/EBPdelta in hypoxia have been obtained from MEF, tumor cell lines, or primary tumor cells in vitro/ex vivo. Showing that FBXW7 is absent/reduced and mTOR is enhanced in C/EBPdelta metastatic tumors (at least in the hypoxic areas) would strengthen this connection. Have the authors examined whether Notch is involved (FBXW7-g-secretase connection)?

Re: We attempted mTOR and FBXW7 immunohistochemistry. However, we did not identify quantitative changes in expression across tumor sections against the background stain. Furthermore, the two-fold differences between genotypes seen by biochemistry in pure cell populations (e.g. Fig 4A and 5A) would be almost impossible to detect by immunohistochemistry. However, as a surrogate we present data on S6 phosphorylation as a downstream effectors’ activity. Although S6 phosphorylation is downregulated in hypoxic areas, consistent with a general reduction in translational activity in those areas (Kaper et al., 2006), we indeed see reduced levels of S6 phosphorylation in C/EBPd null tumors. Representative examples of nine tumors analyzed per genotype is now shown in Figure 3C as an in vivo demonstration of reduced translational pathway activity in C/EBPd KO tumors.

We have indeed observed differences in NICD levels as predicted. However, we would like to reserve these data for a subsequent publication in the context of another project.

4. Some results are difficult to appreciate by the data provided. Quantification of the blot shown in Fig 5A ("Exogenous expression of C/EBPdelta augmented endogenous mTOR") is required for this conclusion. Fig 5B shows that mTOR is responsible for HIFalpha expression because of the effect of rapamycin but it does not show that C/EBPdelta is inducing HIFalpha expression through mTOR at that point as mentioned page 11, 1st paragraph. The authors should modify their conclusions accordingly.

Re: As suggested by the reviewer, the mTOR signal was quantified and normalized to the loading control b-actin (now Fig. 4A). The conclusion from Fig.4B (previously Fig. 5B) “that C/EBPd is inducing HIF-1a expression through mTOR” is based on the result that the observed rescue of the KO phenotype by exogenous C/EBPd was abrogated by rapamycin.

5. Fig 3A. What is the difference between the mode of action of DFX and Coc12 in inducing HIFalpha expression? In WT MEF both CoC12 and DFX induced HIFalpha expression but only DFX could do it in KO MEF. Why?
Re: While DFX stabilizes HIF-1α by depleting the iron required for PHD activity, CoCl2 inhibits PHDs by oxidizing ascorbic acid, which is one of the cofactor required for PHD activity. Therefore these drugs have different modes of action. In immortalized KO MEF, CoCl2 is less efficient in stabilizing HIF-1α compared to WT MEF. We do not know the reason for this observation. Given that DFX was used for all other experiments in this study, we decided to delete the CoCl2 data from Figure 3A (now Fig.S3A).

6. Throughout the manuscript and in Fig7, translational effects of mTOR are attributed to S6K regulation. However, mTOR also regulates eIF4E activity through phosphorylation of 4EBPs. How do the authors know that the mTOR effect is mediated through S6K and not through eIF4E?

Re: Based on this suggestion we assessed 4E-BP1 phosphorylation at Ser65, which suggests that C/EBPδ knockout cells have reduced levels of 4E-BP1 inhibition that may contribute to reduced HIF-1α expression (Fig.3A). This data corroborates the role of C/EBPδ in promoting translation through AKT/mTOR. As a result of these new data, we also amended the concluding schematic (Fig. 6).

Minor points:

Fig 1E and F: Results (glycolytic adaptation and cell viability by MTT assays) are mean +/- SEM of 3 independent experiments. Was the experiment done 3 times on the same preparation of primary cells or on 3 different preparations of primary tumors? It is important to clarify this point.

Re: The experiment was done with three different preparations of primary tumor cells harvested from 3 different tumors. We have clarified this point in the revised figure legends.

Main Figures:

Figure 1 (D) added C/EBPδ and H2AX expression data (nuclear extracts).

Figure 2: no changes.

Figure 3: Moved to the Supplement as Figure S3.

Figure 4 (now Figure 3): (A) added data on the expression of phospho4E-BP1, total 4E-BP1, C/EBPδ, and b-actin. (C) new data showing immunohistochemistry of pS6\textsuperscript{S235/236} and hypoxyprobe in WT and KO mammary tumors.

Figure 5 (now Figure 4): (A) HIF-1b was replaced by β-actin loading control and quantification of the mTOR/actin signal is provided. (C) Data from primary tumor cells (top panel) were replaced with a repeat experiment along with the loading control β-actin (better Western quality). (E) FBXW7 expression data were added.

Figure 6 (now Figure 5): (E) FBXW7 ChIP data were replaced with a repeat experiment (improved qPCR protocol).

Figure 7 (now Figure 6): 4E-BP1 was added to the schematic.

Supplementary Figures

Figure S1: no changes
Figure S2: moved to Figure S3 as panel C.
The current Figure S2 provides new data on CXCR4 regulation by C/EBPd (ChIP and mRNA levels).

Figure S3 is now Figure S4
The current Figure S3 was previously main Figure 3. The data on CoCl2 treatment were removed from panel A.

Figure S4 is now Figure S5A and includes a new panel (B) with new data on the effect of rapamycin on HIF-1α protein levels in U251 and U87MG cells

Figure S5 (now Figure S6)
(A) New data assessing the effect of C/EBPa and C/EBPb on FBXW7 protein levels.
(D) New data on regulation of FBXW7 promoter-reporter by C/EBPd are shown. The previous data in panel S5D are now Figure S7.

Additional Correspondence 20 October 2010

I did receive comments from one of the original referees that is essentially satisfied with the revisions provided.

The editorial office will thus be soon in touch with an official acceptance letter and related to essential paperwork.

I like to congratulate to your study.

Editor
The EMBO Journal