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## AP-2 promotes proliferation in breast tumour cells by direct repression of the CDKN1A gene

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### Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

15 May 2009

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Thank you for submitting your manuscript for consideration by the EMBO Journal. We sent your study to three referees, and I have now received the reports from two of them. The third referee has not returned his/her comments, and it is unclear whether he/she will do so. However, with the two reports we have in hand, we feel we can make a decision: to invite a revision of your manuscript.

As you will see, both referees show interest in your finding that AP-2gamma represses CDKN1A and are broadly supportive of publication in the EMBO Journal. However, both raise a number of technical concerns that would need to be addressed before we could consider publication. In addition, I would like to draw your attention to referee 2's comments regarding critical information being referred to as "data not shown". It is important that you include these data, either in the main figures or the supplemental information.

In the light of the referees' positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of both reviewers. Should the third report arrive, we will forward it to you, and may ask you to address any concerns raised by this reviewer. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript.

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

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Referee #2 (Remarks to the Author):

The authors show that TCFAP2c binds to the promoter of the p21/CDKN1A gene leading to a repression of p21. Loss of TCFAP2c leads to acquisition of active chromatin state at the CDKN1A locus and partial G1/S arrest. The data indicate, that gain of TCFAP2c expression is able to promote proliferation in breast epithelial cells.

This is an important finding, since there is quite a body of evidence that TCFAP2a acts in the opposite way by inducing p21 and hence acting as tumor suppressor.

However, the following comments need to be addressed:

- 1.) In the introduction of the manuscript the authors mention publications showing a correlation between elevated levels of TCFAP2c and reduced patients survival. However they neither mention nor discuss the presence of other data which do not see such a correlation ( e.g. Friedrichs et al Mod. Pathol. 2005). The authors should include a discussion on this topic.
- 2.) In the results part, page 5 they write: 'In order to validate the dataset further, 10 (4%) of the 254 most significantly regulated genes were analyzed by real-time PCR using the same RNA samples used for the arrays. All of the genes were up- or down-regulated to an extent consistent with the array data (data not shown).'
- Why was the same RNA sample used. This reflects a technical replicate and not a biological replicate. The experiment - transfection of siRNA must be repeated and the qRTs must be done on such an independently generated sample and the validated data shown as excel file. The reviewer would like to see the data not shown, at best presented as supplemental data (Excel File) in order to evaluate the significance of the validation.
- 3.) Page 6 (Silencing of AP2gamma). The authors write that the AP-2g silencing was not associated with an increase in genomic DNA fragmentation and therefore was also unlikely to lead directly to programmed cell death (data not shown). This is an important claim, here the data (and the method used) needs to be shown. Also, additional markers indicative for apoptosis (TUNEL, Caspases etc.) need to be performed in order to support this finding.
- 4.) Page 7 middle. The authors claim that the inability to establish sublines expressing AP2g-sh constructs confirms the proliferation deficiency. Again -data not shown- cell counting assays in silenced cultures are said to support this. Data of the cell counting assay need to be shown. The authors should set out to create incucible cell lines in order to definitely test this finding.
- 5.) At page 8, the authors claim, that the level of p53 was unaltered during the course of the experiment. However, there is a clear decrease in AP2g-siRNA sample number 3. The authors should comment on this.
- 6.) The authors show in fig 5 reporter assays which demonstrate the repression of CDKN1A by TCFAP2c. (5a)The amount of AP2g is given in relative numbers, here absolute concentrations are needed. Also repression and in activation is given in %, but it should be in 'fold activation' in order to see absolute numbers of repression and induction in these assays.

Referee #3 (Remarks to the Author):

In this work the relationship between TFAP2c and cell proliferation is examined in mammary tumor cell line MCF7 using RNAi knockdown strategy. Evidence is presented supporting a direct negative regulatory interaction of TFPA2c with a proximal cis-element in the promoter of the p21cip (cyclin dependent kinase inhibitor) gene. Thus elevated TFAP2c expression could enhance proliferation via this mechanism.

The idea that TFAP2 factors can control proliferation and differentiation is not new, but this paper

does make a significant novel contribution by providing a reasonable molecular pathway for the proliferation aspect, and the relevance to breast cancer biology is an added plus. The data adequately support the conclusions.

(1) My only substantive criticism has to do with Figure 6, the analysis of deletion mutants of the p21cip gene: Given the knowledge that a putative TFAP2 binding site exists at -105 (which, I gather from the citation, is in press from the authors' lab), carrying out a deletion series like this seems kind of silly. Why not just mutate the site?

(2) An EMSA experiment would be a nice addition, but not essential in view of the ChIP data.

(3) I would like to see some additional discussion of how TFAP2c might function as a repressor. Another sentence or two about the TFAP2c knockout phenotype vis a vis proliferation would also be nice.

(4) I had to Google KU-70. Better to define it somewhere.

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Additional correspondence - editor

19 May 2009

I have now received the comments from the third referee on your manuscript, which are attached below. As you will see, he/she finds your manuscript potentially interesting, but feels that it is currently at a rather preliminary stage. While this report does not change our decision to invite a revision of your manuscript, I would ask that you respond to this reviewer's criticisms, as well as to the comments of the other two referees, in your revised version.

I am sorry for the delay with this third report, and I look forward to receiving your revision.

Best wishes,

Editor  
The EMBO Journal

Referee #1:

Remarks to the Author:

Manuscript by Williams et al. describes the role of Ap2gamma in gene expression in breast cancer. Authors find that silencing Ap2gamma by siRNA in MCF-7 breast cancer cells causes cell cycle arrest in G1, which is paralleled by gain in p21Cip1 expression. The loss of Ap2gamma binding to the p21 promoter is accompanied by loss of binding of p300 and HDAC2, and gain of Histone 4 acetylation, suggesting that Ap2gamma silencing causes changes favoring p21 gene transcription.

Although this is an interesting study, it appears to be quite preliminary in the present form.

Major comments:

1. First major critique is absence of cDNA microarray validation: the gene expression profiling has been done only once (no biological repeats or repeats with different siRNAs), and only in one breast cancer cell line (non-aggressive MCF7). The fold change differences reported are 2-fold and lower, which is below the cut-off fold change of 2 generally accepted for Affimetrix cDNA microarray experiments. Therefore, the validity of the cDNA microarray data is unclear.
2. In addition to the above described biological validation, differential expression of known Ap2 target genes also had to be validated by qPCR. Instead, it is presented in Fig. 1 as raw cDNA microarray data. In addition, authors state that they validated 10 genes by qPCR, but they never showed any data.
3. The information in Fig 2 is not very informative since it comes from a non-

validated gene array experiment, in which all differences in gene expression are below the cut-off of 2-fold. Furthermore, no qPCR validation for any genes has been demonstrated. Finally, it is unclear whether genes in a specific functional group are positive or negative regulators of the corresponding process. Overall, this functional analysis is preliminary.

4. p21cip1 is a well known Ap2 target gene, as demonstrated by El-Deiry and group (1993), and confirmed by many other investigators. Interesting is the opposite manner in which Ap2alpha and Ap2gamma regulate p21cip1. However, authors do not attempt to answer the question "how?". Furthermore, little is done to investigate the mechanism of Ap2alpha-induced p21 suppression by itself. The few ChIP experiments shown do not provide clear insight as to how Ap2gamma suppresses p21 gene transcription: while H4 acetylation and loss of HDAC2 recruitment observed in parallel with an increase in p21 expression are somewhat intuitive, the loss of p300 in parallel with an increase in histone acetylation suggests p300's irrelevance. The binding site of Ap2gamma should have been identified by mutational analysis. The phosphorylation of p53, which is stabilized judging from its levels, has not been verified. Overall, the conducted studies are a good beginning and have to be built upon.

Minor points:

1. Representative cell cycle analysis diagrams should be present for cell cycle studies.
2. Original ChIP gel pictures should be presented for all ChIP experiments. Negative IgG controls should be included in the gels. Amplification of DNA fragments containing no binding sites for the immunoprecipitated transcription factors should be included.
3. page 5, top paragraph. Although authors mention some co-factors analysis, none of the proteins featured in Supplemental Figure 1b are transcriptional co-factors of Ap2.

Additional correspondence - author

20 May 2009

- Many thanks for sending through the reviews last week and today.

In general the comments from reviewers 2 and 3 and the first points from reviewer 1 overlap and I accept the need for the data requested and we will provide this in the revised m/s within the time frame you stipulate.

I would like to clarify with you however to what extent you feel we have to fulfill the request within point 4 of this latest review to describe the mechanism of how AP-2gamma works as a repressor - to be honest this is a whole extra paper and would take much more than 3 months to address.

This reviewer (#1) seems to think we have done a very preliminary piece of work but I am not certain how thoroughly they have looked at the m/s. Just to mention two examples:

- Point 1 - in fact the microarray was done with 3 biological repeats with 3 different siRNAs and very carefully analysed in a statistically robust manner (detailed in the supplementary material) rather than just using old fashioned fold change;
- Point 4 - the ChIP experiments were not done with gels but more accurate qPCR using the controls they ask for to subtract background and the p53 levels are NOT stabilised but remain at control cell levels as we show (Fig. 4).

Given the comments of the other reviewers I am happy to provide more discussion of the mechanisms, as suggested by Referee 3 (point 3) but I would appreciate greatly your feedback on this.

Thanks for your message earlier in the week, and apologies for having taken a few days to get back to you.

I can sympathise to some extent with your feeling that referee 1 is a little dismissive of the amount of work you have done, and understand that fully elucidating the molecular mechanism by which AP-2gamma acts as a repressor likely goes beyond the scope of this paper.

However, referee 1 specifically states: "Interesting is the opposite manner in which Ap2alpha and Ap2gamma regulate p21cip1. However, the authors do not attempt to answer the question "how"?". In fact, this was one of the things that we (the editorial team) found most intriguing about your study: the apparent opposing effects of AP-2alpha and gamma on CDKN1A. I feel that you should be able to address this question to some extent. For example, you show that AP-2gamma knockdown leads to decreased p300 occupancy at the promoter and increased H4ac levels. Does AP-2alpha have the opposite effect? Do the two transcription factors compete for binding on the promoter?

It may also be that you can address some of these concerns by further discussion: are there other cases where AP-2gamma and alpha have opposing roles? Is AP-2gamma known to act as a repressor in other situations?

As I said above, we do not expect that you should be able to provide a complete molecular answer to the problem, but I do feel that some additional experiments and discussion along these lines would be valuable.

I should also let you know that, if you feel you will need longer than the three months normally allowed for a revision, we can extend this deadline (to up to six months). Please let me know if you think you will need additional time, and that should not be too much of a problem.

I hope these comments are useful, and please do not hesitate to get in touch if you have any further questions.

Best wishes,

Editor  
The EMBO Journal

The changes we have made in response to the issues raised by each reviewer are as follows:

Referee 1

Major point 1:

i We would like to stress that our array expression profiling was in fact done not once but three times using a separate series of transfections each time. Furthermore, additional biological replicates, in the form of three distinct siRNA sequences against AP-2 $\gamma$ , were built into the experimental design.

i Validation of 11 genes from the array, including some known AP-2 target genes, using RNA from cells transfected with a fourth AP-2 $\gamma$  siRNA is now included as Supplementary Figure

S2. Please also see the response to Referee 2, point 2.

i We wished to determine the effect of AP-2 $\gamma$  in ER positive breast cancer so used MCF-7 cells as a model. We have however also explored the role of AP-2 $\gamma$  in proliferation in additional cell lines (see text p7 and Figure 10).

i Fold change was the first method used to identify differentially expressed genes in microarray experiments but it has been criticised as the cut-off threshold is arbitrary. We have therefore followed Hsiao et al (2004) and others and used classical statistical techniques to generate an adjusted p-value for each probeset and used a p-value of 0.01 to denote significance. The fold change indicated in the array data in Supplementary Table I was used to represent the magnitude and direction of the differential expression on the arrays, but there is an underestimation of fold change expression commonly observed using microarrays (Yuan et al, 2002). Indeed, some of the genes in our validation set, including TFAP2C itself, show greater fold change when analysed by qPCR (Supplementary Figure S2). This is mentioned in the text (p5).

Major points 2 and 3:

i Validation of 11 genes from the array is now included as Supplementary Figure S2.

i The comment about our original Figure 2 expresses one of the known limitations of gene ontology, namely that it is only as good as the annotation behind it. Here the analysis clearly suggests a cell cycle involvement but we do not claim that this does anything more than highlight to us which pathways to investigate in the context of AP-2 $\gamma$  silencing. So as to give more prominence to the functional data we present in the rest of the manuscript, this figure has been moved to Supplementary Figure S3 and this section of the text has been shortened in the resubmission.

Major point 4:

i The MCF-7 line only expresses significant levels of AP-2 $\gamma$ . We have recently examined other breast lines which express both family members and now show by ChIP that arresting the growth of these lines using anti-oestrogen does indeed lead to not only decreased occupancy by AP-2 but increased AP-2 $\alpha$  binding at the p21 locus. These data are presented as an additional Figure 10.

i While we agree that showing increased histone acetylation and loss of HDAC2 in silenced cells are only the first steps towards a molecular understanding how AP-2 $\gamma$  represses p21, they are necessary steps along the path. Our studies to date indicate that a series of other cofactors are involved and the repression process is therefore complex and thus fully elucidating the mechanism is beyond the scope of the current work.

i As p300 is a known cofactor of AP-2 $\gamma$  factors, we investigated whether it also left the p21 locus with AP-2 in silenced cells. A role for p300 in repression has been suggested in the literature and this is now explored more in the Discussion. See also response to Referee 3, point 3.

i It was mentioned in the original m/s that an AP-2 binding site within the CDKN1A promoter had already been identified but this information is more prominently mentioned in the new version (Figure 6). See also response to Referee 3, points 1 and 2.

i We find no evidence for p53 stabilisation during AP-2 $\gamma$  silencing. As shown in Figure 4C, the levels of p53 remain low and constant under both control and silencing conditions. We investigated this further by comparing silenced cells with those treated with the DNA damaging agent, cisplatin and we include this data in Referees Fig. 1 in the referee-only supplementary material. In part B of this figure the levels of p53 are clearly increased at higher doses of cisplatin and far exceed those seen in any lane in part A (same as Figure 4C but reproduced here to allow easier comparison), indicating that the p53 levels in AP-2 $\gamma$  silenced cells are very low compared to a situation where stabilisation is known to occur.

Minor points

i Representative cell cycle analysis diagrams for each condition have been added as Supplementary Figure S4, as requested.

i In order to be able to quantitate effectively, all our ChIP experiments were done using sybr green quantitative PCR and we have not used gels at all. The results are expressed as fold enrichment over the controls and all our antibodies were checked for negligible background when using fragments with no binding sites (Figure 7B, SAT2 columns).

i An extra panel has been added to Supplementary Figure S1 showing that AP-2 $\gamma$  silencing does not also lead to a non-specific reduction in levels of its known cofactors, Cited 2 and 4 and

p300/CBP.

Referee 2

Point 1:

As the reviewer points out, there is indeed a large amount of conflicting data about the expression and role of AP-2 factors in breast cancer. We have discussed this extensively in our recent J. Pathology paper, including citing the reference mentioned. An independent, in-depth review of the field (Pellikainen JM & Kosma VM, 2007, Int J Cancer 120: 2061-2067) has suggested that much of the controversy stems largely from the use of different antibodies with varying specificity and avidity. Rather than repeat this extensive discussion in the current manuscript, we have altered the introduction (p2) to emphasise that this is a controversial area. We have also altered the start of the discussion to emphasise that the current study is an extension of our own previous work. We have also cited here other work from the same group mentioned by the reviewer as they have provided important *in vivo* evidence concerning the effect AP-2 $\gamma$  has on epithelial cell proliferation.

Point 2:

Since the array data were derived using three separate sets of transfections each using three distinct siRNA sequences (biological replicates) against AP-2 $\gamma$ , we had thought that our validation was robust. However, we have now done a further set of transfections using a fourth, more recently validated siRNA sequence plus the non-silencing control and used these RNA samples to validate by qPCR a total of 11 up- and down-regulated genes plus AP-2 $\gamma$  itself. These data are included in graphical format as Supplementary Figure S2 and the associated Excel file is submitted as Referees Fig 2 in the referee-only supplementary material.

Point 3:

As reported in the original manuscript, we initially used FACS to examine the proportion of cells with sub-G1 DNA content to investigate if increased apoptosis might be occurring in the AP-2 $\gamma$  silenced cells. We found that the percentage of cells with subG1 DNA content 72 or 96 hours after transfection was always very low in both the silenced and control cells, suggesting that the rate of apoptosis was low. In addition, we did not observe any morphological changes in silenced cells to suggest an increased rate of apoptosis. In contrast, the increase in the proportion of cells with G1 DNA content, coupled with the decreased expression of cell cycle-associated genes on the arrays, were clearly very much more significant. DNA fragmentation (subG1) is admittedly a late event in apoptosis, therefore we have also now looked for cells in early stages of apoptosis by assaying for Annexin V binding in silenced and control cells. These data are presented in Supplementary Figure S6 which demonstrates that rates of apoptosis in two AP-2 $\gamma$  inducible lines (see point 4 below) remained very low with or without the addition of doxycycline for up to 96 hours. The experimental set-up was checked with a positive control using staurosporine-treated cells which, as expected, showed significant induction of apoptosis. Therefore, using two distinct measures of cell death (sub-G1 and Annexin V staining), we cannot find any indication that silencing of AP-2 $\gamma$  induces apoptosis in our experiments.

Point 4:

We have very recently successfully established MCF-7 derived cell lines with doxycycline-inducible silencing for AP-2 $\gamma$  and these are now described in the manuscript and an extra figure (Figure 3), showing the proliferation deficiency in these lines once induced, is now included as requested.

Point 5:

There may be slight variations in p53 level between experiments but the point we wish to make is that p53 levels definitely do not increase significantly in the silenced cells through stabilisation and therefore p53 cannot be responsible for the induction of p21<sup>cip</sup> that we observe. See also answer to Referee 1, point 4.

Point 6:

The absolute amounts (in  $\mu$ g) of AP-2  $\gamma$  expression plasmid added to the transfection experiments in

Figure 5A are indeed given, and are stated in the figure legend. For Figure 5B, our understanding is that fold activation, i.e. reporter alone values set at 1, would be essentially the same as we have portrayed with reporter alone set at 100% and the activation by alpha and repression by gamma reflected by increase/decrease compared to the control value of 100%.

Referee 3

Point 1:

The referee is correct and we do intend to publish our identification of an AP-2 binding site at -106 in the p21 promoter separately ñ this work will be resubmitted shortly. As mentioned by Referee 1, an AP-2 site was identified close to this position some years ago but we find that both AP-2 $\alpha$  and AP-2 $\gamma$  actually bind slightly upstream of this site. We considered that it would be very distracting to the main message of the current manuscript to try to include too much discussion of this complexity here which is why we used the deletion series. We take the Referee's point, however, and we have moved the original figure to supplementary Figure S9 and include here a new Figure 6 showing reporter activity from the wild-type construct compared to two different mutants which prevent AP-2 binding. The loss of AP-2 $\gamma$  binding in MCF-7 cells leads to increased reporter activity due to the relief of repression.

Point 2:

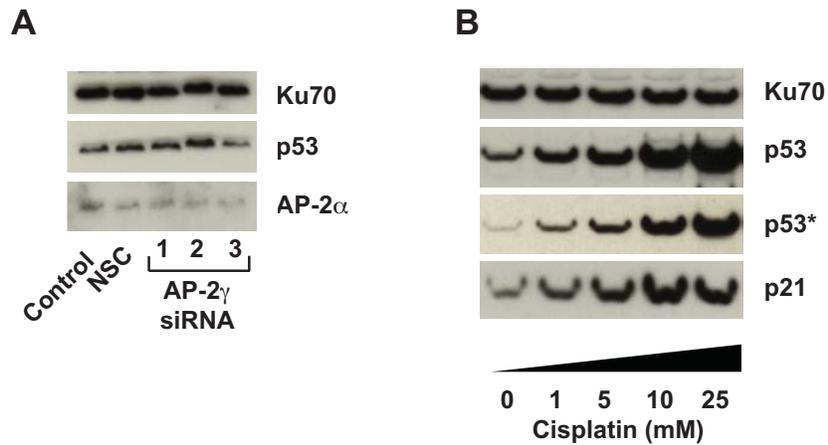
We will include EMSA data when we resubmit the separate manuscript detailing our identification of the AP-2 site at -106.

Point 3:

Additional discussion concerning AP-2 repressor function is now included in the Discussion (p14), as requested. Multiple examples of AP-2 factors repressing expression of various genes occur in the literature but very few suggest a molecular mechanism whereby repression is effected. A correlation with the mouse knock-out is also mentioned at the start of the discussion.

Point 4:

A definition of Ku-70 has now been added to the legend to Figure 2 as this is where this protein is first mentioned in the main manuscript.



**Referees Figure 1**

**Comparison of p53 levels in MCF7 cells silenced for AP-2 $\gamma$  or incubated with cisplatin.**

**A)** Cells were transfected with control or AP-2 $\gamma$  silencing siRNAs, passaged at 48 hours and harvested at 72 hours. (see also Fig. 4 in main m/s).

**B)** MCF-7 cells were treated with cisplatin (conc as shown) and harvested 24 hours later.

Whole Cell extracts (5 $\mu$ g/lane) were separated by SDS/PAGE and blotted to membrane and probed with primary antibodies against Ku-70, p21, AP-2 $\alpha$  and p53 as indicated. \*Represents films that have undergone a very brief exposure (~2 seconds) to better estimate p53 levels.

DOWNREGULATED GENES		
TFAP2C	NSC	g-si
	0.9406321	0.17708378
	1.06311488	0.19965081
	0.9962531	0.187352
KRT81	NSC	γ-si
	0.95397562	0.19286245
	1.04824483	0.22026829
	0.9977796	0.2052
APOD	NSC	γ-si
	0.86365747	0.14458576
	1.15786648	0.19712922
	0.9784762	0.164763
SPDEF	NSC	γ-si
	0.9377486	0.4703048
	1.066384	0.570814
	0.9958676	0.513265
KIAA1324	NSC	γ-si
	0.9559077	0.7309024
	1.046126	0.8186648
	0.9979662	0.771053
S100A7	NSC	γ-si
	0.9035106	0.0436851
	1.079558	0.0959359
	0.979788	0.054593

UPREGULATED GENES		
MALL	NSC	γ-si
	0.60575461	6.98257113
	1.65083349	8.3903532
	0.743412	7.586394
SPRR3	NSC	γ-si
	0.79052293	5.80327654
	1.26498544	6.0972209
	0.9444917	5.944801
PERP	NSC	γ-si
	0.60020578	3.98528862
	1.66609514	4.52383232
	0.7336992	4.228972
INHBA	NSC	γ-si
	0.92392379	6.28472948
	1.08234036	7.23034573
	0.993736	6.707856
TAGLN	NSC	γ-si
	0.7516491	6.872949
	1.330408	8.158796
	0.9179431	7.433224
IGFBP3	NSC	γ-si
	0.9537451	1.719414
	1.048498	1.889925
	0.9977568	1.798626

Decision letter

27 August 2009

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Many thanks for submitting the revised version of your manuscript. It has now been seen again by referees 1 and 2. Both referees are satisfied with the revision and find that your manuscript is now suitable for publication; neither have any specific comments. I am therefore pleased to be able to tell you that we can now accept your manuscript - you will receive the formal acceptance message shortly.

Best wishes,

Editor