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ZIP: a Novel Transcription Repressor, Represses EGFR Oncogene and Suppresses Breast Carcinogenesis

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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 07 May 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. As you will see referees 2 and 3 are very positive about the paper and would support publication in The EMBO Journal if it can be appropriately revised (referee 2). Referee 1 is more critical and feels that that functional link between ZIP, EGFR and breast cancer would need to be strengthened considerably before he/she could support publication here. Taking together all issues raised we have come to the conclusion that we would be able to consider a revised version of this manuscript in which the referees' criticisms need to be addressed (or responded to) in an adequate manner. In particular, it would be important strengthen the tumor link by some further experimentation along the lines suggested. Furthermore, we feel that the data in figure 7B and C need to be quantified (replicates and error bar should be added) and it would be helpful if you could add an alignment of potential ZIP homologues in different organisms (incl. Drosophila and C.elegans) and point out conserved domains.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will 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 COMMENTS

Referee #1 (Remarks to the Author):

Li & colleagues describe the identification and characterization of ZIP, a transcription factor (TF) that acts as a repressor that recruits the NuRD complex to target genes. ZIP was cloned from a mammary cDNA library and studied in great detail by the authors, who carried out a very thorough biochemical analysis on the TF.
-They show that ZIP acts as a repressor and is associated with HDAC activity, as shown, e.g., by in vitro HDAC activity assays in FLAG-ZIP IPs from HeLa cells.
-ZIP is complexed with the NuRD complex via an interaction with Mi-2.

-The ZIP DNA binding site was characterized by CASTing assays and genes with this potential site were identified in the EPD. 383 genes have putative ZIP binding sites in the 5' regulatory domain. (Supp File 1). EGFR was identified & studied in more detail.

-They show that ZIP binds an EGFR promoter sequence that has the putative ZIP binding site and that in cells, ZIP with the NuRD complex is recruited to the promoter of EGFR.

-In the final experiments they show the biological effects of lowering or increasing ZIP levels using the estrogen receptor positive (ER+) MCF7 breast cancer cells as a model. These cells are very well-characterized and known to express low levels of EGFR. They are also sensitive to estrogen withdrawal and accumulate in G1 phasse of the cell cycle.

-In Fig 5B they show that many mRNAs that have important roles in cancer are down-regulated when ZIP is overexpressed. The list includes not only EGFR but also PTEN, FGF5, PDGFB etc. I found additional potentially interesting targets on the list of 383 including c-Myc, IL-8 and some miRs. In my opinion, any one or a combination of multiple target genes could be important in the biological effects of ZIP

Fig 6F-they show that more ZIP is recruited to the EGFR promoter in serum starved conditions - 24 vs 12 hrs.

1. The statement on pg 17 of the ms " the observation is consistent with the functional connection between ZIP and EGFR and agrees to a growth promoting effect of EGFR" is an over-interpretation of the results.

2.- What happens to the level of ZIP in the serum-starved cells? Does the increase of ZIP on the EGFR promoter just reflect increasing ZIP expression? Is there an increase of ZIP on the c-Myc promoter, which is also known to be important in the proliferation of MCF7 cells? (see work from E. Musgrove lab).

Fig 7 - additional experiments to link ZIP and EGFR are shown.
3. -they need to show the increase and the decrease of ZIP in the ZIP expression experiment and the ZIP siRNA experiments, resp.

4. Panel B- the ctrl including EGFR alone needs to be added since simply by overexpressing EGFR in these cells they will certainly respond better to EGF.

5. The level of EGFR in each of the conditions needs to be shown. As the experiment is presented, there is no obvious connection between ZIP and EGFR and cell proliferation.

6. Panel C - the number of colonies in each condition need to be shown. Furthermore, the effects of EGFR expression in the vector ctrl experiment also need to be shown.

Panel D - the in vivo work is interesting, but an important control is missing. The results suggest

that high ZIP levels will slow down the tumor outgrowth kinetics, while ZIP KD makes the tumors grow faster. In the final group with ZIP KD and EGFR KD the tumors grow like the vector ctrl.

7. They need to do an experiment with the EGFR KD alone, in addition to the other combinations. How does EGFR KD affect the outgrowth of ctrl tumors? Do they have evidence that in the ZIP siRNA tumors there is higher EGFR expression? This needs to be examined.

Indeed, an array analysis on all these tumors might yield very interesting targets. But it is not possible with the data presented to conclude that EGFR is the ZIP target responsible for these effects.

As mentioned above - this is an ER driven mammary cancer model. Since c-Myc is a well described target gene of ER it is possible that ZIP is influencing Myc levels and this is responsible for the changes in outgrowth kinetics.

Minor
They mention that ZIP is mapped to chromosome 20q13.3. There are many studies on chromosomal regions that are amplified or deleted in human tumors. Is the locus with the ZIP gene altered in human tumors?

Referee #2 (Remarks to the Author):

Comments to Li et al.,
In this manuscript the authors characterize a novel transcriptional repressor, ZIP, that is involved in the regulation of the EGFR gene. They characterize a DNA binding domain of ZIP and show that ZIP interacts with the well-characterized transcriptional co-repressor complex NuRD. The ZIP mediated repression is inhibited by TSA, which is indicative of an HDAC activity being involved in the repression. They show a direct binding of ZIP and Mi-2 to the EGFR promoter, which seems to be important for repression from this promoter in a transient assay. ZIP over-expression leads to a decreased tumor growth, which is supported by the observation that a lower level of ZIP expression is associated with malignant breast carcinomas. The authors provide a complete analysis of the function of a novel zinc-finger containing transcription factor. All experiments are well controlled and are of high interest for the scientific community. I only have a few minor comments that would improve the manuscript and support the conclusions of the paper.
DETAILED CRITIZISMS:
Fig.1: Throughout the whole paper the rational of why the authors selected ZIP as an object to study its function remains unclear. The authors could explain a bit better of why they choose ZIP.
Fig.2: In panel D as well as in Fig.4 Panel C they used a (E)GFP fusion protein to study localization of ZIP. Why did they not use an anti-ZIP antibody to investigate the sub-cellular localization? In both assays ZIP clearly localizes to very distinct foci, which they do not discuss in the main text. Is this an artifact due to over-expression? The resolution is not sufficient to draw a conclusion of whether or not ZIP co localizes with members of the NuRD complex. The NURD components clearly do not show such enrichment to the ZIP foci.
Fig.4 panel E: The co-elution of endogenous ZIP with components of the NuRD complex is not convincing. Most of the ZIP protein is in fact not co-eluting with NuRD.
Fig.6: The ChIP experiments shown in panels B-D lack several controls and require a more detailed description. It is for example unclear which primers the authors use to map ZIP binding to the EGFR promoter. Are they spanning the ZIP binding site described in panel A? Also an unrelated region that is not bound by ZIP should be used as a negative control to measure non-specific binding of chromatin to the antibody resins. Although the reduced levels of H3ac look quite impressive, it is unclear whether this is due to an increased recruitment of an HDAC enzyme or due to a loss of histones? Here a control ChIP using anti-H3 (C-term) antibodies would be helpful. Also a PCR using a control promoter, which's H3 acetylation status is not affected by ZIP over expression would be helpful. This could exclude the possibility that ZIP over expression leads to a general up regulation of components of the NuRD complex, which in turn might lead to a decreased histone acetylation level. Have the authors tested this possibility?

Referee #3 (Remarks to the Author):

This is an impressive report going from the identification of a cDNA sequence encoding a zinc finger protein, to the elucidation of at least one of its functions and the underlying molecular mechanisms.
Many pertinent, thoughtful and complementary approaches have been used to gradually shed light on the activity of this novel factor.
Finally, the findings reported here may have important implications in the understanding of EGFR overexpression in breast cancer.

1st Revision - authors' response 07 June 2009

Response to refereesí comments-

Referee #1:

Major points:

1. The statement on pg 17 of the ms "the observation is consistent with the functional connection between ZIP and EGFR and agrees to a growth promoting effect of EGFR" is an over-interpretation of the results.

Authors: we have deleted this sentence in the revision.

2. What happens to the level of ZIP in the serum-starved cells? Does the increase of ZIP on the EGFR promoter just reflect increasing ZIP expression? Is there an increase of ZIP on the c-Myc promoter, which is also known to be important in the proliferation of MCF7 cells? (see work from E. Musgrove lab).

Authors: we examined the protein level of ZIP in the serum-starved cells and did not observe an increase in the level of ZIP protein in these cells. The data have been added to the revision as Figure 6F (right panel). In addition, we examined the recruitment of ZIP on c-Myc promoter and did not found the occupancy of ZIP on the c-Myc promoter (Figure 6B).

3. They need to show the increase and the decrease of ZIP in the ZIP expression experiment and the ZIP siRNA experiments, resp.

Authors: These data have been added to the revision in Figure 7D.

4. Panel B- the ctrl including EGFR alone needs to be added since simply by overexpressing EGFR in these cells they will certainly respond better to EGF.

Authors: we included EGFR alone in initial experiments but did not show it because, as the referee rightfully pointed out, its effect is expected. To comply with the criticism, we have added the data from EGFR alone to the revision in Figure 7B.

5. The level of EGFR in each of the conditions needs to be shown. As the experiment is presented, there is no obvious connection between ZIP and EGFR and cell proliferation.

Authors: The level of EGFR in cells under each of the conditions has been examined by Western blotting. The data have been added to the revision in Figure 7D.

6. Panel C - the number of colonies in each condition need to be shown. Furthermore, the effects of EGFR expression in the vector ctrl experiment also need to be shown.

Authors: The colony counts have been added to the revision in Figure 7C. The data showing the effect of EGFR expression alone have also been added in Figure 7C.

7. They need to do an experiment with the EGFR KD alone, in addition to the other combinations. How does EGFR KD affect the outgrowth of ctrl tumors? Do they have evidence that in the ZIP siRNA tumors there is higher EGFR expression? This needs to be examined. As mentioned above - this is an ER driven mammary cancer model. Since c-Myc is a well described target gene of ER it is possible that ZIP is influencing Myc levels and this is responsible for the changes in outgrowth kinetics.

Authors: as we explained in point #4, EGFR KD alone was included in initial experiment and the data have now been provided in Figure 7E. In addition, we have examined the levels of EGFR and ZIP in all of these tumors, and the data indicate that the expression of EGFR is indeed higher in the ZIP siRNA tumors (Fig. 7E, right panel). Measurements of c-Myc expression by Western blotting indicate that c-Myc levels were not influenced by either ZIP overexpression or ZIP knockdown (Fig. 7D, right panel).

Minor points:

1. They mention that ZIP is mapped to chromosome 20q13.3. There are many studies on chromosomal regions that are amplified or deleted in human tumors. Is the locus with the ZIP gene altered in human tumors?

Authors: we have searched the database at NCBI and found that so far there is no indication that the locus with the ZIP gene is altered in human tumors. But we will look into this issue in future investigations.

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Response to refereesí comments-

Referee #2:

Major points:

1. Fig. 1: Throughout the whole paper the rational of why the authors selected ZIP as an object to study its function remains unclear. The authors could explain a bit better of why they choose ZIP.

Authors: One of the primary research focuses in our lab is transcription regulation. The initial structural analysis of ZIP prompted us to investigate its potential role in transcriptional regulation. We have added to the revision the relevant information to explain this rationale (page 7).

2. Fig. 2: In panel D as well as in Fig.4 Panel C they used a (E)GFP fusion protein to study localization of ZIP. Why did they not use an anti-ZIP antibody to investigate the sub-cellular localization? In both assays ZIP clearly localizes to very distinct foci, which they do not discuss in the main text. Is this an artifact due to over-expression? The resolution is not sufficient to draw a conclusion of whether or not ZIP co localizes with members of the NuRD complex. The NURD components clearly do not show such enrichment to the ZIP foci.

Authors: ZIP is an uncharacterized protein and the antibodies against ZIP were generated only in the late phase of our study. The staining of endogenous ZIP with these antibodies yielded no satisfying images; possibly due to the low abundance of ZIP protein in cells. Generally, the level of a transcription repressor in cells is pretty low, and ZIP is no exception. As we agree with the referee that the co-localization data are not very helpful, in light of the refereeís criticism, we have removed these images from the revision. We are purifying the antibodies and preparing monoclonal antibody. Hopefully, this issue could be addressed in the future.

3. Fig. 4 panel E: The co-elution of endogenous ZIP with components of the NuRD complex is not convincing. Most of the ZIP protein is in fact not co-eluting with NuRD.

Authors: As we stated in the manuscript, in addition to transcriptional repression activity through recruiting NuRD complex, ZIP might be also involved in other cellular processes. However, we agree with the referee on the point that the elution profile of ZIP is not completely overlapped with the NuRD complex, especially in chromatographic fractions with low molecular masses. The exact reason for that is not clear at the moment. We have modified the text and tuned down our argument in interpreting this data (page 11).

4. Fig. 6: The ChIP experiments shown in panels B-D lack several controls and require a more detailed description. It is for example unclear which primers the authors use to map ZIP binding to the EGFR promoter. Are they spanning the ZIP binding site described in panel A? Also an unrelated region that is not bound by ZIP should be used as a negative control to measure non-specific binding of chromatin to the antibody resins. Although the reduced levels of H3ac look quite impressive, it is unclear whether this is due to an increased recruitment of an HDAC enzyme or due to a loss of histones? Here a control ChIP using anti-H3 (C-term) antibodies would be helpful. Also a PCR using a control promoter, which's H3 acetylation status is not affected by ZIP overexpression would be helpful. This could exclude the possibility that ZIP over expression leads to a general up regulation of components of the NuRD complex, which in turn might lead to a decreased histone acetylation level. Have the authors tested this possibility?

Authors: To comply with the criticism, we have performed the suggested experiments and added the data to the revision. The primers used in ChIP assays were illustrated in Figure 6B: primer pair "a" cover the ZIP binding element and primer pairs "b" and "c" cover unrelated regions. In addition, c-Myc primers were used as a negative control. Primer pair "a" was used in Fig. 6C, 6D, 6E, and 6F. To address the refereeís concern about the loss of histones, ChIP assays were performed using anti-H3 and anti-H4 antibodies. The data indicate that there was no detectable loss of histones in that region (Fig. 6E). We also measured H3 acetylation status of c-Myc promoter and found that it is not affected by ZIP overexpression (Fig. 6E). ZIP overexpression did not result in elevated expression of HDAC1 and HDAC2. The data have been added to the revision in Figure 6E.

2nd Editorial Decision 29 June 2009

Thank you for sending us your revised manuscript. Our original referees 1 and 2 have now seen it again, and you will be pleased to learn that in their view you have addressed their criticisms in a satisfactory manner, and that the paper will be publishable in The EMBO Journal. Still, referee 1 feels that there are a few issues that need to be addressed (see below) before we can ultimately accept your manuscript. I would therefore like to ask you to deal with the issues raised. Please let us have a suitably amended manuscript as soon as possible.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The revised version of the ms by Li & colleagues is very good. With the exception of a few comments & corrections I find it now acceptable for publication.

1. on pg 18 when discussing Fig 7, the panel referred to as 7E left panel, should be 7F.

2. for the data on soft agar growth in panel 7C - was EGF added to the medium?

3. in the introduction on pg 4 they refer to amplification in the EGFR gene "...were restricted to region of the regulatory sequences and associated with EGFR expression in epithelial breast tumors..."
This statement is not clear. Is this only a short <~2Kb region of the gene that is amplified in some breast tumors? In other tumor types, e.g., glioblastomas, the entire gene is amplified. This statement needs more clarification.

4. p27 of the methods - tumor xenografts. The mice used are certainly female BALB/c nude mice - not WT mice. This should be corrected.

Referee #2 (Remarks to the Author):

The authors addressed all the points I have raised.

2nd Revision - authors' response 29 June 2009

Response to referees #1ís comments
1. on pg 18 when discussing Fig 7, the panel referred to as 7E left panel, should be 7F.
Authors: That has been corrected.
2. for the data on soft agar growth in panel 7C - was EGF added to the medium?
Authors: The cells were grown in medium supplemented with standard fetal bovine serum which contains EGF.
3. in the introduction on pg 4 they refer to amplification in the EGFR gene "...were restricted to region of the regulatory sequences and associated with EGFR expression in epithelial breast tumors..." This statement is not clear. Is this only a short <~2Kb region of the gene that is amplified in some breast tumors? In other tumor types, e.g., glioblastomas, the entire gene is amplified. This statement needs more clarification.
Authors: as stated in Brandt et alís review in Clinical Cancer Research (2006; 12: 7252-7260), "Using microsatellite analysis, data from our studies showed that amplifications in the egfr gene were restricted to region of the regulatory sequence in the 5í-end of intron 1ÖÖ". The statement has been modified according to this reference.
4. p27 of the methods - tumor xenografts. The mice used are certainly female BALB/c nude mice - not WT mice. This should be corrected.
Authors: That has been corrected.

