THE EMBO JOURNAL

Manuscript EMBO-2011-77833

Transcription factor Dlx2 protects from TGFβ-induced cell cycle arrest and apoptosis

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Review timeline:

Submission date:	07 April 2011
Editorial Decision:	27 April 2011
Additional Author Correspondence	29 April 2011
Additional Editorial Correspondence	29 April 2011
Revision received:	26 July 2011
Editorial Decision:	08 August 2011
Accepted:	08 August 2011

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 27 April 2011

Thank you very much for submitting your paper on dual functions of Dlx2 in TGFbeta-mediated tumor progression for consideration to The EMBO Journal editorial office.

I received consistent comments from two scientists enabling a decision to also prevent unnecessary delay. As you will see form the enclosed comments both scientists emphasize the novelty of your findings. However, they also provide constructive comments to better support general significance of the proposed Dlx-2 switch. As their comments are really explicit and outline current limits that should be thoroughly addressed, there is not much need for me to repeat them here in length. Please do understand that we have to demand definitive molecular mechanism and thorough functional evaluation. I thus kindly ask you to take their comments serious and spend the necessary time for experimentation that should involve monitoring endogenous Dlx2 protein level and proliferation/apoptosis assays upon betacellulin and Dlx2-knockdown to validate causality

I also have to formerly remind you that it is EMBO Journal policy to allow a single round of revisions only and that the ultimate decision depends on the content and strength of a potential revised version.

Please do not hesitate to contact me in case of further questions (preferably via E-mail).

I am very much looking forward to your response and remain with best regards

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #2:

This manuscript addresses a critical question in TGFb regulation of cancer. How does TGFb suppress cell growth early in tumor development yet then shifts to a tumor promoting activity that promotes tumor progression, at later stages.
Using murine mammary gland cell lines treated with TGFb they profiled changes in gene expression over time of TGFb treatment. One gene, the homeobox gene Dlx-2, was found to be upregulated. They the go on to show, in NMuMG cells, that Dlx-2 counteracts the early TGFb-induced cell cycle arrest and apoptosis while also inducing later proliferation. It does so by first repressing transcription of TGFbRII, thereby dampening canonical TGFb signaling, and then activating transcription of the EGFR ligand betacellulin, to promote cell survival and proliferation. They finish by showing that manipulation of Dlx-2 level in B6 melanoma cells inhibits primary tumor growth and metastasis.
This is a potentially interesting and important finding that may explain how TGFb function during cancer development changes, or switches. But there remain a number of outstanding issues that need to be addressed. Foremost, at no time and in none of the experiments is endogenous Dlx-2 protein analyzed (an antibody). If they were to continue to treat NMuMG cells with TGFb for longer times does betacellulin level increase and cells begin to proliferate and if so do TGFbRII remain repressed? In other words can the proposed switch occur in a single cell line or does it require a tumorigenic cell line. The model would predict that both events occur in the same cell. In fact are similar results observed when tumorigenic breast cancer cell lines are used? Or is Dlx-2 level high in these cells at the start, precluding an analysis of early effects of TGFb?
Finally in the tumor experiment in vivo, in parental B6 cells does the level of Dlx-2 remain high in metastatic lesions? There is a report, in breast cancer, that Dlx-2 expression is lost in metastatic tumors. Indeed it would be nice to know just how much the level of Dlx-2 protein is increased in tumor cells and whether over-expressed Dlx-2 in NMuMG cells actually approximates Dlx-2 levels observed in tumors. Why did the authors not try to model their in vivo experiments around breast cancer, since all preliminary data was done in a breast cancer cell line? Are they certain that depletion of Dlx-2 actually inhibited metastasis or is it simply a reflection of decreased tumor burden due to Dlx-2 depletion? Maybe more direct assays of metastatic processes could be assessed - invasion through basement membrane, EMT functions, for example.

Specific Points

1. Figure 1. 1F - Three different shRNA against Dlx-2 are used (good!) but the effect of each upon cell proliferation is different despite the fact that each suppresses mRNA equally (Fig S1A). Why?

2. Figure 2. As discussed above what level of Dlx-2 protein over-expression have you achieved? Does this approximate the level seen in NMuMG cells treated with TGFb? Need to do Western blots.

3. Figure 3. All ChIP experiments are done in cells over-expressing Dlx-2. Really should show endogenous Dlx-2 on TGFbRII promoter (and betacellulin promoter). Fig. 3D pSmad2 blots are not that impressive.

4. Figure 4E - the pEGFR blot could possibly be improved by doing a receptor IP - pEGFR Western blot (or the reverse).

5. Do repression of TGFbRII and induction of betacellulin expression ever overlap?

6. You may wish to discuss how Dlx-2 represses transcription in one instance while activating transcription in another. Could this be due to other TGFb-regulated signals?
Referee #3:

In the current manuscript the authors identify the transcription factor distal-less homeobox 2 Dlx2) as a critical regulator of transforming growth factor (TGF)-mediated malignant tumor progression and metastasis. They report that Dlx2 interferes with TGF -mediated cell cycle arrest and apoptosis in mammary epithelial cells via two distint mechanism of action. First, Dlx2 inhibits at the transcriptional level TGF receptor II gene expression leading to decreased canonical TGF signaling and reduction of p21. Second, Dlx2 triggers betacellulin expression, which is a ligand of EGFR, leading to increased phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling. The authors also show that Dlx2 promotes tumor growth and metastasis of melanoma cells. The authors conclude that Dlx2 is a novel important regulator that shifts TGF - mediated tumor suppression towards tumor promotion.

General comments:
The authors report novel and potentially important findings on the role of Dlx2 as a key regulator of TGF-mediated tumorigenesis. However, one major drawback of the current version resides in the fact that most experiments are performed in one cell line. To explore the general relevance of the reported findings, it is recommended to extend the experiments to additional cell lines. Furthermore, additional studies on expression of Dlx2 in primary tumor samples are recommended to be included in order to further test the relevance of this transcription factor for tumor biology.

Major points:

Figure 1:
In addition to analyzing mRNA expression levels of the Dlx2, also protein expression of Dlx2 should be examined. Furthermore, controls are missing showing that knockdown of the respective target genes, for example of Smad4 and Dlx2, was effective in the reported RNA interference experiments.

Figure 2:
In panel A scale bars are missing. In panel B statistical analysis should be included. For experiments shown in panels C and D controls showing increased expression of Dlx2 upon overexpression should be included.

Figure 4:
In panel A blots assessing total protein expression of PKB are missing. Since expression of Dlx2 results in increased phosphorylation of PKB and ERK, it would be interesting to investigate whether the combined inhibition of both PI3K and MAPK exerts a more potent inhibition of cell proliferation compared to inhibition of either pathway alone.

Figure 5:
To investigate whether betacellulin not only regulates proliferation by stimulating EGFR but also has an impact on cell death induction, additional experiments are recommended to assess the effect of betacellulin downregulation on apoptosis sensitivity.

Figure 6:
To extend the molecular studies also to the in vivo model, it is recommended to assess proliferation and cell death upon knockdown of Dlx2 also in the primary melanoma tumors as well as in the lung metastasis.

Additional Author Correspondence 29 April 2011

Many thanks for the reviews and the instructions. We are glad to see that the reviewers in principle appreciate our work. We on the other hand have gotten a clear message on a number of specific points; it is rare that three reviewers agree on several major points, and we will be more than happy to address these points within the technical possibilities.

We are now highly motivated and enthusiatic to respond to the reviewers' comments and to revise the manuscript accordingly. During our discussions we realized, however, that the amount of additional experimentation is substantial. We believe that we can address the majority of points, mainly the mechanistic questions on a biochemical and molecular biology level in additional cell types within the proposed 90 days time frame. However, additional mouse experimentation, in particular the inclusion of an additional in vivo model, will not be possible within this time frame.

Since we already used an in vivo model, orthotopic implantation of B16 melanoma cells to demonstrate the functional contribution of Dlx2 to tumor growth, we have samples to analyze the tumor and metastasis phenotypes in more detail, as requested. Together with the requested expression analysis of patient samples and samples from other mouse models, this will represent convincing data on the expression and functional contribution of Dlx2 to tumorigenesis and metastasis in vivo.

For the inclusion of additional functional studies in an in vivo model, such as a breast cancer model, we would need an extension of the time frame for revisions to a total of at least 5 months.

Please advise!

Many thanks and best regards

Additional Editorial Correspondence 29 April 2011

Thanks for your query that I am happy to answer promptly. I do fully agree that thorough establishment of the mechanistic underpinnings would be in your case much more insightful than expanding to a (presumably better suited) second mouse model. Trying to be realistic and pragmatic at this point, I kindly ask you to focus your efforts on the former for the current study.

Yours sincerely,

Editor
The EMBO Journal

1st Revision - Authors' Response 26 July 2011

We appreciate the reviewers' comments and constructive criticisms on our manuscript. We have used the past 3 months to address most of the points raised by the reviewers. In particular, we have performed a number of additional experiments to better characterize the functional contribution of Dlx2 to overcoming TGFβ-mediated growth arrest and apoptosis.
In particular, we have expanded parts of the functional studies on Dlx2 to a murine breast cancer cell line and to the B16 melanoma cell line to document the generality of Dlx2 directly regulating the expression of the *TGFβRII* and the *betacellulin* genes.
Moreover, we have now tested and employed a number of antibodies against Dlx2 and employed them for Dlx2 protein expression level analysis by immunoblotting, subcellular localization by immunofluorescence stainings, and in vivo expression by immunohistochemistry and immunofluorescence. Notably, this analysis demonstrates that Dlx2 is localized to the nucleus of TGFβ-treated cells, supporting its critical function in transcriptional control.
Finally, we have expanded our studies on the signaling pathways induced by upregulated betacellulin expression and EGFR signaling. While the pharmacological inhibition of PI3K, MEK and EGFR signaling lead to a significant inhibition of Dlx2-mediated cell proliferation and survival, shRNA-mediated ablation of Dlx2 expression was not sufficient to repress these signaling pathways. Conversely, forced expression of Dlx2 significantly increased Erk1/2 phosphorylation, while phosphorylation of PKB was not markedly affected. Based on our recent report that the upregulated expression of NCAM in TGFβ-treated NMuMG cells (and other cells undergoing EMT) stimulates FGF receptor signaling and with it Erk1/2 activation (Lehembre F, Yilmaz M, Wicki A, Schomber T, Strittmatter K, Ziegler D, Kren A, Went P, Derksen PW, Berns A, Jonkers J, Christofori G (2008) NCAM-induced focal adhesion assembly: a functional switch upon loss of E-cadherin. *EMBO J*, 27:

2603–2615), we conclude that other pathways are also stimulating this pathway and that the loss of Dlx2 cannot replicate the complete repression of PI3K or MEK signaling by pharmacological inhibitors.
Based on the time constraints, it has not been possible to include additional animal experimentation or another animal model of cancer, in particular breast cancer, to demonstrate the in vivo function of Dlx2 during tumor progression. The decision not to include additional animal experimentation has been discussed and agreed on with the editor of EMBO J. A large number of minor additional experiments and changes in Figures and Text have been performed as detailed in the point-by-point reply below.
In summary, the results from these additional experiments are providing a substantial amount of new information on the functional role of Dlx2 in tumor progression. As a consequence, the manuscript has been substantially revised: the text, in particular the presentation and interpretation of the results, has been revised to accommodate the new data. Many new panels have been added to the original figures (7 figures) and the supplemental figures (5 figures), almost all of which have been substantially revised.

Point-by-point reply

Reviewer #1:
1. In cancer cell lines, Dlx2 is presumably activating betacellulin and repressing TGFBRII; The authors should knock down Dlx2 and show modulation of TGFBRII receptor expression.
We appreciate this important comment. We have now analyzed TGFβRII mRNA expression upon shRNA-mediated depletion of Dlx2 expression in NMuMG cells. Indeed, the results show that TGFβRII expression is increased in Dlx2-depleted cells upon TGFβ treatment. The results are now shown in Suppl. Figure S2A.
2. With respect to Dlx2 regulation of Betacellulin expression, the authors should perform luciferase reporter assays with the promoters of betacellulin and also conduct CHIP for endogenous DLX2 at the betacellulin and TGFBRII promoters.
We have been unable to retrieve the published promoter-reporter constructs for betacellulin. In fact, we failed to get in contact with the authors of the relevant publication by their available coordinates. Our chromatin-immunoprecipiation experiments also revealed that the potential binding site for Dlx2 may not even lie within the promoter region previously used in the publication and that the promoter region of betacellulin is more complex than anticipated. In the shortness of time, we thus were not able to perform the proposed promoter-reporter experiments on betacellulin (we already did on TGFβRII). However, we have expanded on the specific binding of Dlx2 to the *betacellulin* gene promoter by chromatin immunoprecipitation experiments:
To exclude potential artifacts introduced by overexpression of transcription factors, we have now complemented the ChIP experiments done with antibodies against HA-tagged Dlx2 with experiments using antibodies against endogenous Dlx2.
We now demonstrate that endogenous Dlx2 directly binds the promoters of the *TGFβRII* and the *betacellulin* genes in NMuMG and B16 cells (shown in Figures 3C and 5F and Suppl. Figures S2D and S4C) and in Py2T murine breast cancer cells (not shown).
3. Promoter-luciferase reporter assays should be done for TGBRII and betacellulin in the setting of DLX2 KD (2 hairpins) or control KD.
As mentioned in point 2, we were not able to establish a promoter-reporter construct for betacellulin. While a functional promoter-reporter construct for TGFβRII has been at hand, we have preferred to demonstrate the direct binding of endogenous Dlx2 to the promoter of the *TGFβRII* and *betacellulin* genes by chromatin-immunoprecipitation (see also point 2). Finally, we have included the analysis of TGFβRII expression upon shRNA-mediated ablation of Dlx2 expression, now shown in Suppl. Figure S2A.
4. In Fig. 4, the authors use pharmacology to implicate the MAPK and PI3K pathways as downstream mediators of the survival phenotype of Dlx2 overexpression. A better experiment would have been for the authors to add TGF-b, +/- DLX2 KD to see if these pathways are modulated in a tgf/dlx2 dependent manner. For example, does DLX2 KD increase Psmad2 or p21, or decrease MYC. Additionally, It would be preferred to see KD experiments for MAPK or PI3K components confirming these phenotypes.
We thank the reviewer for this comment. We have now expanded and revised the signaling effector analysis in gain and loss of function approaches. Yet, due to the complexity of the figure, we have split the figures and show the most important data in a new Figure 4 and the data without major changes in a new Suppl. Figure S3.

Since the pharmacological inhibitors not only repress Dlx2-mediated signaling but also signaling mediated by other inducers activated during TGFβ treatment of NMuMG cells, their effect is much stronger than the mere ablation of Dlx2 expression. In fact, depletion of Dlx2 does not have a major effect on the overall activation of PKB or Erk1/2 (new Suppl. Figure S3). Moreover, we have previously reported that the upregulated expression of NCAM in TGFβ-treated NMuMG cells (and other cells undergoing EMT) stimulates FGF receptor signaling and with it Erk1/2 activation (Lehembre F, Yilmaz M, Wicki A, Schomber T, Strittmatter K, Ziegler D, Kren A, Went P, Derksen PW, Berns A, Jonkers J, Christofori G (2008) NCAM-induced focal adhesion assembly: a functional switch upon loss of Ecadherin. *EMBO J*, 27: 2603–2615). From these insights, we conclude that other pathways are also stimulating PI3K or MEK signaling and that the loss of Dlx2 cannot replicate the complete repression of PI3K or MEK signaling by pharmacological inhibitors. Along these lines, while inhibition of EGFR signaling clearly induces apoptosis and growth arrest of the cells, it does not completely abrogate their growth, again arguing for additional signaling pathways being active.

5. When showing phenotypes for Betacellulin, 2 hairpins are required.

We agree with the reviewer. In new Figure 5B, 5C, and 5E and Suppl. Figure S4A we included in total 3 different siRNA sequences against BTC. In the rescue experiment shown in Figure 5D we used a mix of these three siRNAs.

6. Regarding the metastasis phenotype in figure 6c, how much of the effect is due to proliferation/survival as opposed to metastatic colonization? Could the authors do an orthotopic assay to show that when the control and dlx2 KD tumors are the same size in the primary tumor, the metastatic burden is lower in the Dlx2 KD cohort?

This is an excellent suggestion to discern tumor growth from the actual metastatic dissemination. However, due to the limited time and in agreement with the editor of EMBO J we did not perform additional animal experimentation. Instead, we have determined the extent of apoptosis and proliferation in primary tumors formed by shcontrol and shDlx2 B16 melanoma cells. The results are now shown in Figure 6E and Suppl. Figure S5C and indicate that the ablation of Dlx2 function results in increased apoptosis, although statistically not significant, and no change in proliferation rates. From the data on primary tumor size and metastatic nodule size (as counted by microscopically detectable nodules) as well as the increased rate of apoptosis in Dlx2-depleted cells, we conclude that the difference in primary tumor growth and in metastatic outgrowth is due to increases in apoptosis and not to the actual metastatic dissemination of B16 melanoma cells.

Reviewer #2

If they were to continue to treat NMuMG cells with TGFb for longer times does betacellulin level increase and cells begin to proliferate and if so do TGFbRII remain repressed?

Upon longterm treatment of NMuMG cells with TGFβ for longer than 10 days betacellulin levels rather decline from the highest levels observed at day 6 to 8. As described in the manuscript, the critical phase of TGFβ-mediated growth arrest and apoptosis occurs between days 4 to 7 of TGFβ treatment. After emerging from this crisis, long term treated NMuMG cells proliferate normally and undergo full EMT. In a separate project we are investigating the epigenetic changes occurring during TGFβ-induced EMT in NMuMG cells. Notably, major changes in DNA methylation and histone modifications together with additional changes in gene expression occur at later stages of TGFβ treatment, indicating that the initial resistance against TGFβ, at least in part provided by Dlx2, is stabilized by additional epigenetic adaptations that still need to be identified. These studies are out of the scope of this manuscript and, thus, are not specifically discussed. On the other hand, TGFβRII mRNA levels stay low in longterm TGFβ-treated NMuMG cells (data not shown).

*In other words can the proposed switch occur in a single cell line or does it require a tumorigenic cell line. In fact are similar results observed when tumorigenic breast cancer cell lines are used? Or is Dlx-2 level high in these cells at the start, precluding an analysis of early effects of TGF*b*?*

In the revised version of the manuscript we have now included the analysis of Dlx2 expression in a tumorigenic murine breast cancer cell line that has been established from a tumor of a MMTV-PyMT transgenic mouse (Py2T) and that undergoes full EMT upon TGFβ treatment. In contrast to NMuMG cells, in Py2T cells Dlx2 is already expressed at detectable levels in the absence of TGFβ, yet, as is the case in NMuMG cells, Dlx2 expression is increased upon TGFβ treatment (Suppl. Fig. S1A). In a similar manner, B16 melanoma cells already express Dlx2 and respond to TGFβ with increased levels of Dlx2 mRNA and protein (Figure 6A and Suppl. Figure S1A, respectively). In contrast to NMuMG cells, Py2T and B16 cells do not suffer as much from TGFβ-induced growth arrest and apoptosis in vitro and, thus, these cells are nor suitable to study the early effects of TGFβ.

However, shRNAmediated reduction of Dlx2 expression in B16 melanoma cells significantly reduced primary tumor growth and metastasis formation, at least in part due to a moderate increase in apoptosis (Figure 6).

Finally in the tumor experiment in vivo, in parental B6 cells does the level of Dlx-2 remain high in metastatic lesions?

We have now performed immunohistochemical and immunofluorescence analysis of Dlx2 expression in primary tumors and metastasis formed by shcontrol and shDlx2-transfected B16 cells. Both, shcontrol and shDlx2 cells express nuclear Dlx2, with lower levels of Dlx2 expression in shDlx2 cells, as determined by these microscopic methods (Suppl. Figure S5B). Based on the rather incomplete knockdown of Dlx2 expression (approximately 50% by mRNA expression; Suppl. Figure S5A), it is not possible to conclude that only Dlx2 expressing cells can form metastasis. Genetic ablation of all Dlx2 expression would be required to clearly come to this conclusion.

There is a report, in breast cancer, that Dlx-2 expression is lost in metastatic tumors. Indeed it would be nice to know just how much the level of Dlx-2 protein is increased in tumor cells and whether over-expressed Dlx-2 in NMuMG cells actually approximates Dlx-2 levels observed in tumors.

We have now performed a direct comparative immunoblotting analysis of lx2 expression in NMuMG cells, Py2T murine breast cancer cells established from a tumor of a MMTV-PyMT transgenic mouse, and B16 melanoma cells (Suppl. Figure S1A). This analysis shows that in the tumorigenic cell lines Dlx2 levels are already high in the absence of TGFβ, and that upon TGFβ treatment the tumorigenic cell lines express levels of Dlx2 comparable to NMuMG cells forced to express Dlx2.

Why did the authors not try to model their in vivo experiments around breast cancer, since all preliminary data was done in a breast cancer cell line?

We agree with the reviewer that the use of a mouse model of breast cancer would support a better flow of the manuscript and appropriately connect the in vitro observations with the matching in vivo experimentation. However, we chose to employ the B16 melanoma model for in vivo experimentation, in order to provide more general insights into Dlx2 functions in various cancer types. Our data mining analysis revealed a profound correlation of Dlx2 expression and tumor progression in melanoma patients. B16 cells also showed an upregulation of Dlx2 expression upon TGFβ treatment and they can be used in an orthotopic, syngeneic transplantation mouse model where a fully functional tumor microenvironment, including the immune system, potentially provides TGFβ to induce Dlx2 expression in tumor cells. Based on the time constraints and also after discussion with the editor of EMBO J, we have decided not to include another animal model of cancer to demonstrate the in vivo function of Dlx2 during tumor progression. However, we have expanded on the functional analysis of Dlx2 in the Py2T and the B16 tumor cell lines to better connect the functional results with the in vivo experimentation.

Are they certain that depletion of Dlx-2 actually inhibited metastasis or is it simply a reflection of decreased tumor burden due to Dlx-2 depletion?

From the data on primary tumor size and metastatic nodule size (as counted by microscopically detectable nodules) as well as the increased rate of apoptosis in Dlx2-depleted tumors (Figure 6E) and no change in proliferation rate (Suppl. Figure S5C), we conclude that the difference in primary tumor growth and in metastatic outgrowth is due to increased apoptosis and not to the actual metastatic dissemination of B16 melanoma cells.

Maybe more direct assays of metastatic processes could be assessed – invasion through basement membrane, EMT functions, for example.

Within a major study of transcriptional control during EMT, we have also assessed the potential contribution of Dlx2 to EMT. While forced expression of Dlx2 lead to a slight upregulation of EMT markers, it was not sufficient to induce full EMT. Moreover, loss of Dlx2 function had no effect on cell migration, cell invasion or any markers of EMT, indicating that Dlx2 is not an important factor for the morphogenic process of EMT but is rather critical for cells to overcome TGFβ-induced growth arrest and apoptosis. These studies are outside the scope of this manuscript, which has a focus on Dlx2's functions in overcoming TGFβ-induced growth arrest.

1. Figure 1. 1F - Three different shRNA against Dlx-2 are used (good!) but the effect of each upon cell proliferation is different despite the fact that each suppresses mRNA equally (Fig S1A). Why?

We have further analyzed the knock-down efficiencies and observe a trend in correlation between the reduction in Dlx2 expression and the biological effects of the three shRNAs (new Suppl. Figure S1C).

2. Figure 2. As discussed above what level of Dlx-2 protein over-expression have you achieved?

Does this approximate the level seen in NMuMG cells treated with TGFb? Need to do Western blots.
Has been done, see above. Now shown in Figure 1B and Suppl. Figure S1A.
3. Figure 3. All ChIP experiments are done in cells over-expressing Dlx-2. Really should show endogenous Dlx-2 on TGFbRII promoter (and betacellulin promoter). Fig. 3D pSmad2 blots are not that impressive.
We agree with the reviewer on the potential artifacts introduced by overexpression of transcription factors. We have now expanded the ChIP experiments done with antibodies against HA-tagged Dlx2 by experiments employing antibodies against endogenous Dlx2. We now demonstrate that endogenous Dlx2 directly binds the promoters of the *TGF*β*RII* and the b*etacellulin* genes in NMuMG and B16 cells (shown in Figures 3C and 5F and Suppl. Figures S2D and S4C) and in Py2T murine breast cancer cells (not shown). We apologize for the quality of the p-Smad2 immunoblot, yet despite several attempts we were not able to improve on it (the levels of p-Smad2 seem to be rather low in NMuMG cells).
4. Figure 4E - the pEGFR blot could possibly be improved by doing a receptor IP - pEGFR Western blot (or the reverse).
We appreciate the reviewer's suggestion, yet we would like to propose that this figure is of sufficient quality to demonstrate the increased phosphorylation of EGFR upon Dlx2 expression.
5. Do repression of TGFbRII and induction of betacellulin expression ever overlap?
We interpret this question as to whether the changes in the expression of the two genes occur at the same time, i.e. with the upregulation of Dlx2 expression. Our results indicate that within the resolution of the time courses performed (days 0, 2, 4, 6, 8, 10) the two genes are regulated at comparable time windows. On the other hand, differences in regulation of the two genes may be based on the differential regulation of various cofactors involved in gene activation and repression, respectively, and thus the positive and negative regulation of genes by one transcription factor may not generally coincide.
6. You may wish to discuss how Dlx-2 represses transcription in one instance while activating transcription in another. Could this be due to other TGFb-regulated signals?
This is a well-taken suggestion, also for future investigations. As discussed in point 5, most if not all transcription factors use a number of cofactors to exert their activating or repressive function. Naturally, a number of these factors may be regulated by TGFβ signaling. While ongoing chromatin-immunoprecipitation-deep sequencing and gene expression profiling experiments in our laboratory have revealed a large number of genes directly activated or repressed by Dlx2, we do not know the nature of the additional cofactors of Dlx2 at this point and time. This is clearly highly speculative and a focus of current investigations, and we would prefer not to include these speculations into the current manuscript.

Reviewer #3:
To explore the general relevance of the reported findings, it is recommended to extend the experiments to additional cell lines.
We thank the reviewer for this important comment. In the revised version of the manuscript we have now included the analysis of Dlx2 expression in a tumorigenic murine breast cancer cell line that has been established from a tumor of a MMTV-PyMT transgenic mouse (Py2T) and that undergoes full EMT upon TGFβ treatment. In contrast to NMuMG cells, in Py2T cells Dlx2 is already expressed at detectable levels in the absence of TGFβ. As is the case in NMuMG cells, Dlx2 expression is increased upon TGFβ treatment (Suppl. Fig. S1A). In a similar manner, B16 melanoma cells already express Dlx2 and respond to TGFβ with increased levels of Dlx2 mRNA and protein (Figure 6A and Suppl. Figure S1A, respectively). In contrast to NMuMG cells, Py2T and B16 cells do not suffer as much from TGFβ-induced growth arrest and apoptosis in vitro and, thus, these cells are nor suitable to study the early effects of TGFβ. However, shRNA-mediated reduction of Dlx2 expression in B16 melanoma cells significantly reduced primary tumor growth and metastasis formation, at least in part due to a mild increase in apoptosis (new Figure 6E). We also expanded our studies on the transcriptional activities of Dlx2 to additional cellular systems by showing Dlx2' s ability to bind directly the promoters of the *betacellulin* and *TGF*β*RII* genes in NMuMG and B16 cells (shown in Figures 3C and 5F and Suppl. Figures S2D and S4C) and in Py2T murine breast cancer cells (not shown).
Furthermore, additional studies on expression of Dlx2 in primary tumor samples are recommended to be included in order to further test the relevance of this transcription factor for tumor biology.
We agree with the reviewer that a comprehensive and meaningful study on the expression of Dlx2 in patient samples is warranted. We have further validated our data mining results, as included in the manuscript in Table I, yet these analyses rely on mRNA expression data, and protein expression

data would be more meaningful.
Unfortunately, testing essentially all available antibodies, in collaboration with local pathologists we thus far were unable to establish a robust staining procedure for Dlx2 on tissue microarrays or other clinical specimen with available information on clinical outcome. A thorough study to evaluate the diagnostic or prognostic value of Dlx2 for tumor progression of specific cancer types will require a major stand-alone project, which seems outside the scope of this manuscript.
Figure 1:
In addition to analyzing mRNA expression levels of the Dlx2, also protein expression of Dlx2 should be examined. Furthermore, controls are missing showing that knockdown of the respective target genes, for example of Smad4 and Dlx2, was effective in the reported RNA interference experiments.
We appreciate this important comment. Regulation of endogenous regulation of Dlx2 on protein levels is now shown in Figure 1B. The efficiency of the knockdown of Dlx2 is now shown in Suppl. Figure S1B and C. The shSmad4 cell line has been previously characterized and published and was kindly provided to us by the authors (Deckers M, van Dinther M, Buijs J, Que I, Lowik C, van der Pluijm G, ten Dijke P (2006) The tumor suppressor Smad4 is required for transforming growth factor betainduced epithelial to mesenchymal transition and bone metastasis of breast cancer cells. *Cancer Res*, 66: 2202–2209).
Figure 2:
In panel A scale bars are missing. In panel B statistical analysis should be included. For experiments shown in panels C and D controls showing increased expression of Dlx2 upon overexpression should be included.
Scale bars have been included in Figure 2A, statistical analysis has been performed for Figure 2B (mentioned in the legend to the figure), and the extent of Dlx2 overexpression in the Dlx2-NMuMG cell pool has been analyzed by immunoblotting and is shown in Figure 1B and Suppl. Fig. S1A).
Figure 4:
In panel A blots assessing total protein expression of PKB are missing. Since expression of Dlx2 results in increased phosphorylation of PKB and ERK, it would be interesting to investigate whether the combined inhibition of both PI3K and MAPK 8 *exerts a more potent inhibition of cell proliferation compared to inhibition of either pathway alone.*
We thank the reviewer for spotting this oversight. We have now expanded the signaling effector analysis in gain and loss of function approaches. Yet, due to the complexity of the figure, we have split the figures and show the most important data in a new Figure 4 and the data without major changes in a new Suppl. Figure S3. Our experience with a number of transformed and non-transformed cell lines tells us that a combination of the inhibition of PI3K and MEK activation will kill the cells. Since the pharmacological inhibitors not only repress Dlx2-mediated signaling but also signaling mediated by other inducers activated during TGFβ treatment of NMuMG cells, their effect is much stronger than the ablation of Dlx2 alone (which does not have a major effect on the activation of PKB or Erk1/2; new Suppl. Figure S3). For example, we have previously reported that the upregulated expression of NCAM in TGFβ-treated NMuMG cells (and other cells undergoing EMT) stimulates FGF receptor signaling and with it Erk1/2 activation (Lehembre F, Yilmaz M, Wicki A,Schomber T, Strittmatter K, Ziegler D, Kren A, Went P, Derksen PW, Berns A, Jonkers J, Christofori G (2008) NCAM-induced focal adhesion assembly: a functional switch upon loss of E-cadherin. *EMBO J*, 27: 2603–2615). From these insights, we conclude that other pathways are also stimulating PI3K or MEK signaling and that the loss of Dlx2 cannot replicate the complete repression of PI3K or MEK signaling by pharmacological inhibitors. Along these lines, while inhibition of EGFR signaling clearly induces apoptosis and growth arrest of the cells, it does not completely abrogate their growth, again arguing for additional signaling pathways being active.
Figure 5:
To investigate whether betacellulin not only regulates proliferation by stimulating EGFR but also has an impact on cell death induction, additional experiments are recommended to assess the effect of betacellulin downregulation on apoptosis sensitivity.
In the revised manuscript, we have quantified the rates of apoptosis upon ablation of betacellulin expression. These results are now shown in Figure 5E.
Figure 6:
To extend the molecular studies also to the in vivo model, it is recommended to assess proliferation and cell death upon knockdown of Dlx2 also in the primary melanoma tumors as well as in the lung metastasis.
We have determined the extent of apoptosis and proliferation in primary tumors formed by shcontrol and shDlx2 B16 melanoma cells. The results are now shown in Figure 6E and Suppl. Figure S5C, respectively, and indicate that the ablation of Dlx2 function results in increased apoptosis, although

statistically not significant, and no change in proliferation rates.

2nd Editorial Decision 08 August 2011

I did receive final remarks on your study that I am happy to attach to this message. Before final and official acceptance, we would be grateful to receive an 'author contribution' and COI-statement following the acknowledgements in your study.

Please provide these as soon as possible to enable efficient production.

The editorial office will subsequently be in touch related to necessary paperwork.

Please allow me to congratulate on this occasion to a fine study.

Yours sincerely

Editor
The EMBO Journal

REFEFREE REPORTS:

Referee #2:

In this revised manuscript the authors have adequately addressed or discussed previous concerns/questions.

Referee #3:

Since the authors adequately addressed all concerns in the revised manuscript, the paper is now recommended for publication.

