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Iro/Irx transcription factors negatively regulate Dpp/TGF- β pathway activity during intestinal tumorigenesis

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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.)

Editor: Nonia Pariente

1st Editorial Decision 25 March 2014

Thank you for your patience while your study has been under peer-review at EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although the referees find the topic of interest, they raise serious concerns and question the conclusiveness and physiological relevance of the findings, on which EMBO reports places particular emphasis.

As the reports are below, I will not detail them here. However, the human and mouse part of the study would clearly need to be considerably strengthened by the use of additional colon cancer derived and colon epithelial systems to support your model, and include functional assays in these systems. In addition, analysis of Irx3 and Irx5 expression in human tumors and normal tissue should be provided, and the various technical as well as conceptual concerns addressed.

In all, the three reports seem reasonable and the issues raised would need to be fully addressed for a revision to be successful here. Please note that it is our policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

Revised manuscripts must be submitted within three months of a request for revision unless

previously discussed with the editor; they will otherwise be treated as new submissions. Revised manuscript length must be a maximum of 28,500 characters (including spaces). When submitting your revised manuscript, please also include editable TIFF or EPS-formatted figure files, a separate PDF file of any Supplementary information (in its final format) and a letter detailing your responses to the referees.

Do not hesitate to get in touch with me if I can be of any assistance during the revision process.

I look forward to receiving a revised version of your study.

REFEREE REPORTS:

Referee #1:

The paper by Martorell et al uses a model of colorectal cancer in drosophila midgut to elucidate a mechanism for somatic loss of TGF-β responsiveness in cases with no identifiable alterations in the pathway. The paper utilizes a system recently published by the same authors (Martorell et al 2014. doi: 10.1371/journal.pone.0088413) whereby mutant APC/Ras clones are induced in the midgut by means of the MARCM technique. These clones are shown to developed into aggressive tumour-like outgrowths within 4 weeks. In this current study, the authors show that Dpp signaling is down regulated in these lesions due to up-regulation of Mirror, a negative transcriptional regulator and member of the Iro/Irx protein complex.
The authors go onto show that cancer cell responsiveness to TGF-β (the mammalian Dpp counterpart) can be regulated by the Iro/Irx proteins Irx3 and Irx5.

The experiments in this paper are clear and logical and the authors provide good evidence for an alternative mechanism to explain somatic loss of TGF-β in colorectal cancer. However, there are some elements of the manuscript that should be clarified.

Main points.

Why do not all clones induced with APC/Ras develop into tumour-like growths? This suggests that the majority of APC/Ras clones are selected against. Moreover, although not addressed in this manuscript, normal clone size distributions would suggest that WT clones would be seen to reduce in number, but increase stochastically in size over time. The authors do not observe this.

When assessing proliferation of APC/Ras clones, control clones showed less proliferation than Mirr_RNAi or Tkv* clones (Fig 2c/d/e). The authors suggest that this rules out a blocking effect of Dpp on cell division. However although no quantification of this proliferation is made, it appears that Dpp induces increased proliferation within clones. This might have the opposing effect on tumour growth than expected. Moreover, the data showing that Mirr_RNAi and Tkv* clones contained an increase in EC cells suggests this increase in proliferation is driving cells not only to proliferate but also to differentiate.

Although the authors have uncovered a novel mechanism that potentially regulates TGF-β, it is not clear from the data that this explains cases where somatic mutations in the pathway are absent. Although the authors explain the role of this regulation in the CRC pathway, their model suggests it occurs prior to TGF-β pathway mutations. Could the authors show cases of colorectal carcinoma that lack TGF-β pathway mutation to up-regulate Irx3/5?

Minor points.

Why aren't the additional components of the Iro/Irx complex overexpressed in APC/Ras clones?

Can the authors add the quantification of clones induced using Mirr_RNAi_TkvDN and the TkvDN control to table 1?

The authors state that Mirr_RNAi clones contain the same level of Stat92E as WT clones. However the q-PCR data shows a 2-fold increase in levels (Fig 2g). This is also true in Fig 1a, where APC-

Ras_MirrRNAi is 4 fold that of the control.

The authors use cell lines (Hela and MDA231) to investigate Irx3 and Irx5 affects on SMAD3 using shRNAs. However these experiments were performed on non-CRC cell lines. Can the experiments be repeated on the same CRC human cell line (SW837) as used in the reporter assay experiments?

Figure 1K shows increased Mirror protein expression in APC/Ras clones. However, there is no control comparison and therefore the results are inconclusive. Could the authors perform a western blot on sorted GFP cells to show the increase between WT and APC/Ras clones?

Figure 4 shows a model whereby Mirror/Irx regulates Dpp/TGF-β pathway during early Adenoma formation. They authors show that this leads to a 60% reduction in TGF-β responsiveness and that in tumours where mutations in the pathway are seen this reduces again by 40%. It is not clear where the percentages for this model are taken from.

Referee #2:

Overview:
This study is an assessment of molecular alterations that may play a role in colorectal cancer tumor formation. The authors use a Drosophila model system of intestinal neoplasms to identify a novel mechanism that appears to play a role in neoplasm transformation. The studies of the model system are well done and provide support for a model in which Apc and Kras induced tumors are induced to progress via increased Mirror expression. They provide evidence that increased Mirror is a mechanism for impairing Dpp signaling, which then induces the progression of "tumor-like growths" in cells that have mutated Apc and Ras. The authors then assess the role of increased Irx3 and Irx5 expression in human colon neoplasms, as they are orthologs of Mirror. They show that increased Irx3 and Irx5 correlate with decreased Smad3 expression and that knockdown of these genes can increase activity of a TGF-ß luciferase reporter. The strengths of the study are the novelty of the finding, well-designed studies in the Drosophila system, and potential relevance to human colorectal cancer. Weaknesses of the system are the studies in the human cell lines and inconsistency of the studies in primary human tissues and mouse models with regards to their model. The authors have found (or provide evidence by citing prior publications) that Irx3 and Irx5 are overexpressed in human and mouse colon adenomas. However, ApcMin mouse adenomas do not progress to cancer and only 10% of human adenomas will progress to cancer. Thus, these results do not support their model that the increased expression of Irx3 and Irx5 promote the progression of adenomas. In addition, 2/3 of the cell lines used are not colon derived and only one colon cancer cell line system is used, which also limits the relevance of their results to human colorectal cancer. Although the authors do show that the Irx proteins do affect TGF-ß signaling in the SW837 cell line, they do not provide any functional evidence that these effects alter tumor cell behavior, and studies in only one cell line are not robust enough to demonstrate that the results are generalizable to colon cancers in general. These limitations significantly limit the relevance of their results to human cancer

Specific Comments:
1) The use of multiple colon derived adenoma and immortalized colon epithelial systems is needed to provide more robust support for their model.
2) The expression of Irx3 and Irx5 in human colorectal cancer as well as human colon adenomas and normal colon epithelium is needed to assess the correlation of expression of these proteins with cancer progression. The quoted studies actually argue against their model.
3) The expression of Irx in human tissue samples should be correlated with common TGF-ß signaling target genes, p21, PAI1, MYC, etc. to demonstrate that Irx may affect TGF-ß signaling in human tissues. These genes should also be assessed in the manipulated cell lines.
4) Functional studies in human colon adenoma cell lines that assess transformation are needed.

Referee #3:

In this study Martorell et al use a drosophila model for colorectal cancer and mammalian cell culture experiments to propose a model in which the combined activation of EGFR-Ras and Wnt signalling suppressed the TGFb pathway via activation of Mirror/IrX.
This model is interesting as it could explain the control on the TGFb pathway without direct mutations of pathway component (SMAD, etc..).
Nevertheless, there are some issues listed below that should be addressed to justify publication:

1- The model proposes a synergy from two oncogenic pathways (EGFR/Ras and WNT) to regulate mirror and then tgf-b pathway. Therefore, the introduction is very poor in presenting what is know about EGFR and WNt pathway activity in the intestine, in particular in drosophila as is where the model is developed. Previous work from the Egdar and Sansom laboratories should be mentioned and discussed.
2- As per authors model, in Drosophila they need Ras-APC double hit to see mirror up regulation. In the mouse, they see IRX up in APC mutants only. Please discuss this discrepancy. In the human adenoma, is it know if there are APC and Ras mutation in the samples used?
3- Experiments form panels 3d and 3e were done using a CRC cell line. Why did experiments in 3C used cell lines form breast cancer instead?
4- 2k: please provide a control image of PDM1 staining in esg-gfp control tissue.
5- 1a, b: please provide data with Ras only clones for expression of mirror and TGF-b pathway components. This are important controls for a model in which Mirrow regulation requires the input of both pathways.
6- 1, please provide an staining for mirror in control clones in a similar gut segment.
7- I am confuses about the statement that ras/APC clones are mainly composed of undifferentiated cells. The undifferentiated cells in this tissue are the ISCs so delta should be unregulated but is down regulated in Ras/APC. The authors should remove the claim or provide data with an EB marker, the only other undifferentiated cell type.

1st Revision - authors' response 28 July 2014

Point-by-point responses to the referees:

Referee #1:

The paper by Martorell et al uses a model of colorectal cancer in drosophila midgut to elucidate a mechanism for somatic loss of TGF-β responsiveness in cases with no identifiable alterations in the pathway. The paper utilizes a system recently published by the same authors (Martorell et al 2014. doi: 10.1371/journal.pone.0088413) whereby mutant APC/Ras clones are induced in the midgut by means of the MARCM technique. These clones are shown to developed into aggressive tumour-like outgrowths within 4 weeks. In this current study, the authors show that Dpp signaling is down regulated in these lesions due to up-regulation of Mirror, a negative transcriptional regulator and member of the Iro/Irx protein complex. The authors go onto show that cancer cell responsiveness to TGF-β (the mammalian Dpp counterpart) can be regulated by the Iro/Irx proteins Irx3 and Irx5. The experiments in this paper are clear and logical and the authors provide good evidence for an alternative mechanism to explain somatic loss of TGF-β in colorectal cancer. However, there are some elements of the manuscript that should be clarified.

Main points

1) Why do not all clones induced with APC/Ras develop into tumour-like growths? This suggests that the majority of APC/Ras clones are selected against. Moreover, although not addressed in this manuscript, normal clone size distributions would suggest that WT clones would be seen to reduce in number, but increase stochastically in size over time. The authors do not observe this.

We agree with the referee, the fact that not all Apc-Ras clones develop into tumor-like overgrowths suggest that the majority of these clones are selected against. As we described in our previous work, most of the Ras and Apc-Ras clones disappear four weeks after clone induction, a mechanism that does not depend on apoptosis (please see Martorell et al., 2014, doi: 10.1371/journal.pone.0088413 for details). These results lead us to hypothesize the existence of a Ras^{V12}-driven tumor suppression mechanism in the adult *Drosophila* midgut, although the nature of this mechanism remains to be elucidated. In our previous work we suggested that Ras^{V12} expression might be sufficient to induce the delamination of the cells when surrounded by wild-type epithelia, as it has been shown in cultured cells (Hogan et al., 2009, doi: 10.1038/ncb1853). Another possibility would be that Ras^{V12} is able to induce cellular senescence (Longo, 2004, doi: 10.1126/sageke.2004.39.pe36). In any case, only the clones Apc-Ras able to overcome this tumor suppression mechanism would be able to overgrow and form tumors. Here we are precisely using the clones that overcome this tumor suppression mechanism to describe a role of Mirr regulating the response to the Dpp signaling pathway. We have improved the text in an attempt to clarify this issue.

We also would like to point out that in humans it is estimated that only 10% of adenomas will develop into carcinomas, suggesting the possibility that only those adenomas that are able to overcome tumor suppression mechanisms may be able to develop further into carcinomas.

Finally, we refer to our previous manuscript (Martorell et al., 2014, doi: 10.1371/journal.pone.0088413) for a detailed description of the behaviour of wild-type clones.

2) When assessing proliferation of APC/Ras clones, control clones showed less proliferation than Mirr_RNAi or Tkv* clones (Fig 2c/d/e). The authors suggest that this rules out a blocking effect of Dpp on cell division. However although no quantification of this proliferation is made, it appears that Dpp induces increased proliferation within clones. This might have the opposing effect on tumour growth than expected. Moreover, the data showing that Mirr_RNAi and Tkv* clones contained an increase in EC cells suggests this increase in proliferation is driving cells not only to proliferate but also to differentiate.

Our results show that Apc-Ras clones in which the Dpp pathway activity is enforced, either by the expression of a dominant active form of the Dpp receptor Tkv or by decrease of Mirr expression by means of a Mirr RNAi, do not overgrow, despite both Wg/Wnt and EGFR/Ras pathways are activated. We hypothesized that this phenotype might be due to an effect of Dpp

blocking cell division and/or forcing cell differentiation to the undifferentiated, dividing cells that form Apc-Ras clones (please see the response to question #7 of Referee 3 for more details about these cells). Stainings with the mitotic marker PH3 suggested that cell division was not blocked, as the average number of $PH3^{+}$ cells in Apc-Ras-Tkv* or Apc-Ras-$Mirr^{RNAi}$ clones was higher that in wild type clones of similar size (Fig 2c,d,e). Moreover, expression of either Tkv* or Mirr RNAi in a wild type background did not induce cell proliferation (Fig 2k,i). On the other side, as the referee points out, the increased number of ECs in Apc-Ras-Tkv* and Apc-Ras-$Mirr^{RNAi}$ clones suggested that Dpp could be driving cells to differentiate. Confirming this hypothesis, we provide evidence that:

1. ISC marker Dl, EE marker Pros, EC marker Myo31DF (Fig 2f) and the differentiation inducer Stat92E (Fig. 2g) expression levels are restored to wild-type levels in Apc-Ras-$Mirr^{RNAi}$ clones.
2. Tkv^{Q253} or $Mirr^{RNAi}$ overexpression in normal progenitor cells results in the ectopic expression of the EC marker Pdm1 (Fig. 2k,l).

Together, these results suggest that Dpp pathway activity might restrict tumor growth by inducing cell differentiation, imposing an EC or EE fate in cells that would otherwise develop into a mass of undifferentiated, proliferative cells. We have changed the wording of this section to clarify this issue.

3) Although the authors have uncovered a novel mechanism that potentially regulates TGF-β, it is not clear from the data that this explains cases where somatic mutations in the pathway are absent. Although the authors explain the role of this regulation in the CRC pathway, their model suggests it occurs prior to TGF-β pathway mutations. Could the authors show cases of colorectal carcinoma that lack TGF-β pathway mutation to up-regulate Irx3/5?

We now provide additional evidences that IRX suppresses the anti-tumoral effect of TGF-beta in early stage CRC:

1. We include data showing that IRX is upregulated in adenomas (Fig S2a). Mutations in TGF-β pathway components occur predominantly at later stages of tumorigenesis (Fearon, 2011, doi: 10.1146/annurev-pathol-011110-130235) implying that most of these benign tumors display a wilt-type TGF-β response. More importantly, our analysis indicates that levels of IRX correlate inversely with gene expression signature of response to TGF-β in human adenomas (Fig 3c).
2. We have reconstituted TGF-β pathway in CRC cell lines that carry inactivating mutations in TGF-β pathway components. We demonstrate that TGF-β induces a cytostatic response in these cells, which is dampened by IRX5.

3. Furthermore, in this revised version we incorporate a cell competition assay in which we demonstrate that high levels of IRX expression confer a growth advantage in presence of TGF-β, whereas in the absence of TGF-β, control cells overcompete IRX5 expressing cells. We speculate that in this context, carcinoma cells that acquire mutations in the TGF-β pathway would then have selective pressure to reduce the level of IRX expression.

Minor points:

1) Why aren't the additional components of the Iro/Irx complex overexpressed in APC/Ras clones?

We do not know why the other two components of the Iro complex are not over-expressed in Apc/Ras clones. Ara and Caup share a 44% identity in their protein-coding sequences and display a similar expression pattern. In contrast, Mirr is more divergent both in sequence identity (33%-34%) and in expression pattern (Gomez-Skarmeta et al., 1996, DOI: 10.1016/S0092-8674(00)81085-5), and performs functions largely different from Ara and Caup, which usually act redundantly (Bilioni et al., 2005, DOI 10.1073/pnas.0502480102). We believe that Mirr expression in Apc-Ras clones may fall into one of these differential expression scenarios of the Iro-complex members.

2) Can the authors add the quantification of clones induced using Mirr_RNAi_TkvDN and the TkvDN control to table 1?

As requested, we have added the quantifications of Apc-Ras-Tkv^{DN}-Mirr^{RNAi} clones and Apc-Ras-Tkv^{DN} control clones to Table 1. Moreover, a box-plot graph of the clone area (GFP^{+}) per anterior midgut area has been added to supplementary Fig. S1b.

3) The authors state that Mirr_RNAi clones contain the same level of Stat92E as WT clones. However the q-PCR data shows a 2-fold increase in levels (Fig 2g). This is also true in Fig 1a, where APC-Ras_MirrRNAi is 4 fold that of the control.

The referee is right in pointing this out. What we meant is that the levels of Stat92E and Med are restored to at least the wild type levels in Apc-Ras-MirrRNAi. We have clarified this issue in the main text.

4) The authors use cell lines (Hela and MDA231) to investigate Irx3 and Irx5 affects on SMAD3 using shRNAs. However these experiments were performed on non-CRC cell lines. Can the experiments be repeated on the same CRC human cell line (SW837) as used in the reporter assay experiments?

We would like to perform this experiment, but we found out that SW837 cells did not express detectable levels of IRX3 and IRX5, precluding the possibility to use shRNAs for these genes and analyze its effect on SMAD3 expression. That was the reason that prompt us to use other cell types that express detectable levels of IRX3 and IRX5. However, as we realize that this piece of data may be confusing and it does not provide any relevant point, we have moved it to Supplementary Fig S2b.

5) Figure 1K shows increased Mirror protein expression in APC/Ras clones. However, there is no control comparison and therefore the results are inconclusive. Could the authors perform a western blot on sorted GFP cells to show the increase between WT and APC/Ras clones?

Certainly, a western blot on sorted GFP^+ cells would show the increase of Mirr expression between wild type and Apc-Ras clones. We believe, however, that this piece of information is already supported by the qRT-PCR analysis (Fig. 1b) and by Mirr staining (Fig 1k) and, in our opinion, it does not justify the cost of performing a new sorting experiment. Of note, we have added a control staining for Mirr in wild-type clones in the supplementary Fig S1a.

6) Figure 4 shows a model whereby Mirror/Irx regulates Dpp/TGF-β pathway during early Adenoma formation. They authors show that this leads to a 60% reduction in TGF-β responsiveness and that in tumours where mutations in the pathway are seen this reduces again by 40%. It is not clear where the percentages for this model are taken from.

The percentages were taken from the reduction in the TGF-β reporter response shown in SW837 cells when transfected with human IRX3 (Fig. 3a). In any case, these percentages were meant to indicate that there is only a reduction in the response to TGF-β, not an absolute effect.

Referee #2:

This study is an assessment of molecular alterations that may play a role in colorectal cancer tumor formation. The authors use a Drosophila model system of intestinal neoplasms to identify a novel mechanism that appears to play a role in neoplasm transformation. The studies of the model system are well done and provide support for a model in which Apc and Kras induced tumors are induced to progress via increased Mirror expression. They provide evidence that increased Mirror is a mechanism for impairing Dpp signaling, which then induces the progression of "tumor-like growths" in cells that have mutated Apc and Ras. The authors then assess the role of increased Irx3 and Irx5 expression in human colon neoplasms, as they are orthologs of Mirror. They show that increased Irx3 and Irx5 correlate with decreased Smad3 expression and that knockdown of these genes can increase activity of a TGF-ß luciferase reporter. The strengths of the study are the novelty of the finding, well-designed studies in the Drosophila system, and potential relevance to human colorectal cancer.

Weaknesses of the system are the studies in the human cell lines and inconsistency of the studies in primary human tissues and mouse models with regards to their model.

The authors have found (or provide evidence by citing prior publications) that Irx3 and Irx5 are overexpressed in human and mouse colon adenomas. However, ApcMin mouse adenomas do not progress to cancer and only 10% of human adenomas will progress to cancer. Thus, these results do not support their model that the increased expression of Irx3 and Irx5 promote the progression of adenomas.

We thank the reviewer for this appreciation and agree with him/her that our model does not predict that the increased expression of Irx3 and Irx5 promote the progression of adenomas (i.e. IRX3/5 do not increase malignancy, including as invasive features, desmoplastic reaction, metastasis, etc.… which characterize CRCs as compared to adenomas). Following this criticism we have corrected any reference to tumor progression in this revised version. Instead, we propose that the expression of Irx3 or 5 provides a growth advantage to adenoma cells in the TGF-β rich environment characteristic of early stage tumorigenesis (without necessarily influencing malignancy). Mutations in TGF-β pathway components occur in late stages of tumor progression, yet adenoma cells develop mechanisms that enable them to expand in the presence of TGF-β. Indeed, we have found that IRX3 expression is elevated in human and mouse adenomas. In this revised version, we provide extensive data that support our model (please read below).

In addition, 2/3 of the cell lines used are not colon derived and only one colon cancer cell line system is used, which also limits the relevance of their results to human colorectal cancer. Although the authors do show that the Irx proteins do affect TGF-ß signaling in the SW837 cell line, they do not provide any functional evidence that these effects alter tumor cell behavior, and studies in only one cell line are not robust enough to demonstrate that the results are generalizable to colon cancers in general. These limitations significantly limit the relevance of their results to human cancer

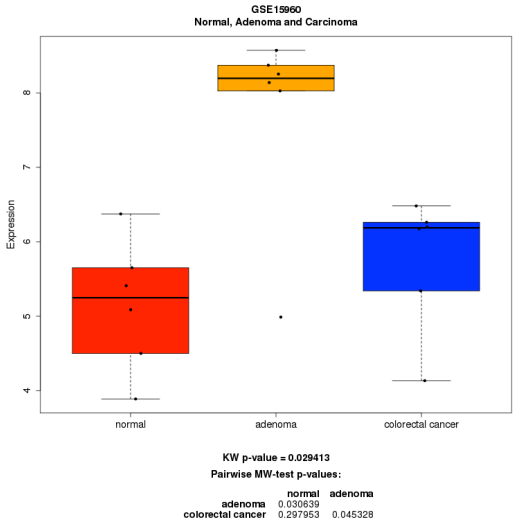
Our experiments were limited by the fact that SW837 cells are the only CRC cell line that is responsive to TGF-β at the transcriptional level, although the cytostatic effect of TGF-β is lost in these cells, making them unable to be used in functional assays. To address this criticism, we have now engineered a non-TGF-β responsive CRC line, LS174T cells, to be able to respond to TGF-β by the introduction of a wild-type TGFBR2 receptor. We now show that these cells respond to TGF-β through a potent cytostatic effect that include a canonical cell cycle arrest by downregulation of c-MYC and upregulation of P15-CDKN2B. We demonstrate that this cytostatic response is reduced by expression of IRX5.

1) The use of multiple colon derived adenoma and immortalized colon epithelial systems is needed to provide more robust support for their model.

We failed to genetically manipulate primary adenoma and CRC cells, which precludes the experiments suggested by this reviewer. We hope that the abovementioned experiments using cell lines provide sufficient support for our conclusions.

2) The expression of Irx3 and Irx5 in human colorectal cancer as well as human colon adenomas and normal colon epithelium is needed to assess the correlation of expression of these proteins with cancer progression. The quoted studies actually argue against their model.

In our model we propose that IRX3/IRX5 expression may provide a growth advantage to adenoma cells in the TGF-β rich environment characteristic of early stage tumorigenesis. In later stages, once mutations in components of the TGF-β pathway occur, IRX3/IRX5 expressing cells may not have any competitive advantage and, therefore, we did not expect a high IRX expression in carcinoma samples. Accordingly, we have found that high IRX expression correlates with adenomas, but not with carcinomas (Figure A)



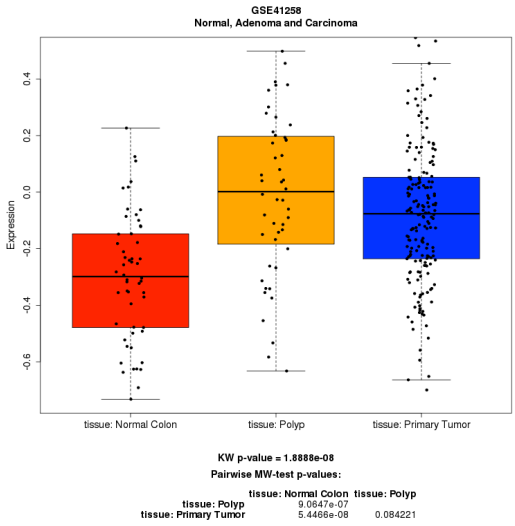


Figure A. Box-plot showing IRX expression during the transition from normal mucosa to carcinoma in two different gene sets.

Remarkably, we have performed a cell competition assay where we show that cells overexpressing IRX5 overcompete control cells in presence of TGF-β (Fig 4b) but, in absence of TGF-β, IRX5 expressing cells are overcompeted by control cells (Fig. 4a), suggesting that IRX5 expression may be deleterious for cells when they do not respond to TGF-β. We speculate that in this context, carcinoma cells that acquire mutations in the TGF-β pathway would then have selective pressure to reduce the level of IRX expression.

3) The expression of Irx in human tissue samples should be correlated with common TGF-ß signaling target genes, p21, PAI1, MYC, etc. to demonstrate that Irx may affect TGF-ß signaling in human tissues. These genes should also be assessed in the manipulated cell lines.

To address this criticism, we have now used LS174T cells with a reconstituted TGFBR2 to derive TGF-β response gene expression signatures. These signatures contain many well-established TGF-β response genes, including those mentioned by the reviewer. We used these signatures as surrogates of the response to TGF-β in colonic tumors. Using cohorts of adenoma samples we demonstrate that average expression of the TGF-β response signature in adenomas correlate inversely to that of IRX genes. These data has greatly strengthened the notion that IRX modulates the response to TGF-β in adenomas.

4) Functional studies in human colon adenoma cell lines that assess transformation are needed.

As mentioned above, we now show that IRX5 dampens the cytostatic response induced by TGF-β in CRC cells. In addition, we performed competition experiments mixing control and IRX5 expressing cells. These experiments show that IRX5 expression confers a competition advantage in the presence of TGF-β. We believe that these new data strengthen the notion that IRX5 enables the expansion of adenoma cells in TGF-β rich context in the mammalian intestine.

Referee #3:

In this study Martorell et al use a drosophila model for colorectal cancer and mammalian cell culture experiments to propose a model in which the combined activation of EGFR-Ras and Wnt signalling suppressed the TGFb pathway via activation of Mirror/IrX. This model is interesting as it could explain the control on the TGFb pathway without direct mutations of pathway component (SMAD, etc..).

Nevertheless, there are some issues listed below that should be addressed to justify publication:

1- The model proposes a synergy from two oncogenic pathways (EGFR/Ras and WNT) to regulate mirror and then tgf-b pathway. Therefore, the introduction is very poor in presenting what is know

about EGFR and WNt pathway activity in the intestine, in particular in drosophila as is where the model is developed. Previous work from the Egdar and Sansom laboratories should be mentioned and discussed.

We agree with the referee and we have added a brief reference to the role of EGF/Ras and Wnt pathways in the adult *Drosophila* midgut. Regretfully, restrictions in the length of the manuscript do not allow us to describe and discuss properly the previous work done on these pathways.

2- As per authors model, in Drosophila they need Ras-APC double hit to see mirror up regulation. In the mouse, they see IRX up in APC mutants only. Please discuss this discrepancy. In the human adenoma, is it know if there are APC and Ras mutation in the samples used?

As the referee points out, in mouse adenomas (mutant for Apc) there is an up-regulation of IRX expression (Fig. S2a), suggesting that in vertebrate systems IRX expression could be independent of Ras activity. However, in order to grow the mouse adenoma samples it is necessary to add EGF to the culture media, otherwise the adenomas do not grow. Therefore, we cannot rule out the requirement of EFGR/Ras activity to induce the expression of Irx genes. Further work is required to clarify the genetic regulation of Irx genes in vertebrate models.

3- Experiments form panels 3d and 3e were done using a CRC cell line. Why did experiments in 3C used cell lines form breast cancer instead?

SW837 CRC cells do not express detectable levels of IRX3/5, therefore the use of shRNAs in these cells would be not be informative. That was the reason that prompt us to use other cell types that express detectable levels of IRX3 and IRX5. However, as we realize that this piece of data may be confusing and it does not provide any relevant point, we have moved it to Supplementary Fig S2b. However in this revised version we include several additional experiments using human CRC cell lines:

1. We have reconstituted TGF-β pathway in CRC cell lines that carry inactivating mutations in TGF-βpathway components. We demonstrate that TGF-β induces a cytostatic response in these cells, which is dampened by IRX5.
2. Furthermore, in this revised version we incorporate cell competition assays which demonstrate that high levels of IRX expression confer a growth advantage in presence of TGF-β whereas in the absence of TGF-β, control cells overcompete IRX5 expressing cells. We speculate that in this context, carcinoma cells that acquire mutations in the TGF-β pathway would then have selective pressure to reduce the level of IRX expression.
3. Additionally, we include data showing that IRX is upregulated in adenomas (Fig. S2a). Mutations in TGF-β pathway components occur predominantly at later stages of

tumorigenesis (Fearon, 2011, doi: 10.1146/annurev-pathol-011110-130235) implying that most of these benign tumors display a wild-type TGF-β response. More importantly, our analysis indicates that levels of IRX correlate inversely with gene expression signature of response to TGF-β in human adenomas (Fig. 3c).

4- 2k: please provide a control image of PDM1 staining in esg-gfp control tissue.

We have incorporated a staining for Pdm1 in control clones in the supplementary Fig S1c.

5- 1a, b: please provide data with Ras only clones for expression of mirror and TGF-b pathway components. This are important controls for a model in which Mirrow regulation requires the input of both pathways.

As described in our previous paper, Ras clones mostly disappeared four weeks after clone induction (see figure B, below), precluding the possibility to sort enough GFP^+ cells to perform qRT-PCRs analysis for Mirr and TGF-β pathway components.

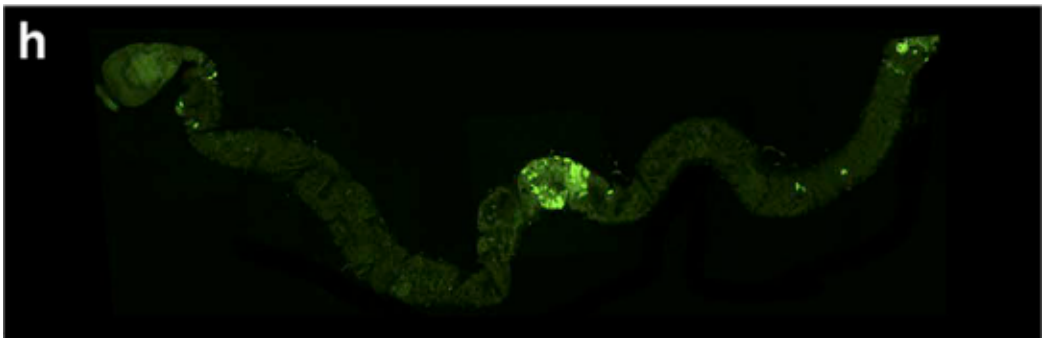


Figure B. Ras clones four weeks after clone induction. Notice that very few GFP^+ cells remain in the anterior and posterior midgut. Most GFP^+ cells are located in the gastric region, which does not form tumor-like overgrowths in Apc-Ras conditions, suggesting that these cells do not respond to the genetic programs imposed by Wg and EGFR pathways. Figure 1h from Martorell et al 2014 (doi: 10.1371/journal.pone.0088413).

The referee is right, as it could be considered that Ras activity is enough to induce the expression of Mirr, but that in the absence of Wg activity this is not enough to form overgrowths and survive in the adult midgut. As Ras clones do not survive to that stage, we do not have the means to rule out this possibility. We have however performed a transcriptional profile analysis of control, Apc, Ras and Apc-Ras clones one week after clone induction, where the clones are still present and distributed along the gut in all four genetic conditions (manuscript in preparation). Our data shows that Mirr is only up-regulated in Apc-Ras clones, but not in Apc nor Ras clones (Table A, below), arguing in favour of a model where, at least in *Drosophila*, both EGFR/Ras and Wnt activities are required to induce the expression of Mirr.

	Apc	Ras	Apc-Ras
Mirror	1,53	1,01	5,83

Table A. Fold change of Mirror expression in one week old Apc, Ras and Apc-Ras clones compared to control clones.

6- 1, please provide an staining for mirror in control clones in a similar gut segment.

We have incorporated a staining for Mirror in control clones in the supplementary Fig S1a

7- I am confuses about the statement that ras/APC clones are mainly composed of undifferentiated cells. The undifferentiated cells in this tissue are the ISCs so delta should be unregulated but is down regulated in Ras/APC. The authors should remove the claim or provide data with an EB marker, the only other undifferentiated cell type.

We have changed the wording to clarify this issue. Tumors often trans-differentiate into cell types not normally present in a tissue. During the characterization of Apc-Ras clones described in our previous paper, we observed that Apc-Ras clones were mostly composed by a new cell type not present in normal tissue (please see Martorell et al 2014, doi: 10.1371/journal.pone.0088413 for details). Briefly, we observed that most cells within Apc-Ras clones were undifferentiated (negative for the EE marker Prospero and the EC marker Pdm1), but they were not EBs (negative for the EB marker Su(H)-mCherry) nor ISCs (negative for the ISC marker Dl). However, they had the capacity to divide, as some of them were positive for PH3 staining. Therefore, we concluded that Apc-Ras clones are mostly composed by a new, undifferentiated cell population able to divide.

Correspondence - editor 20 August 2014

I have now received a complete set of reviews from the referees, which I include below for your information. Referee 3 was unavailable, so referee 2 has assessed the responses to his/her concerns. As you see, although appreciating the efforts made during revision, referee 2 considers that his/her main point regarding the strength of the data obtained with human and mouse lines, which was also highlighted by me as an important issue to address during revision, has been insufficiently addressed. Although acknowledging the difficulties in working with primary cell lines, s/he points out a few available immortalized and adenoma lines that could be used to strengthen the work.

We could thus consider opening an exceptional second round of revision in this case. However, I would be interested to know what you would find feasible to do within a 1.5 month period to address the outstanding concerns. To ensure that the decision-making process is not delayed, please respond within 24-48 hours.

I look forward to your response.

REFEREE REPORTS:

Referee #1:

The paper by Martorell et al has been improved with additional data figures and amended text.
The authors have provided detailed responses to previous comments made by this reviewer.
I recommend the manuscript for publication in EMBO reports.

Referee #2:

The authors have adequately responded to the points raised by reviewers #2 and #3 except for the following:
1) The authors were not successful in using primary adenoma or primary colon epithelial cell lines. This is not unexpected given that primary cell lines are technically very difficult to use. There are a number of available cell lines that have already been established from adenomas (Vaco-330, Vaco-235, AAC1, LT7) and cell lines from the conditionally immortalized Immorto mouse colon (YAMC, Whitehead et al, 1994) that could be used. Studies with these cell lines would substantially strengthen this manuscript.

Correspondence - authors 22 August 2014

Thanks for your response. After a careful reading of your e-mail and the comments of reviewer#2, we believe the request for functional experiments including new human cell lines is not reasonable at this point. We base this opinion on the two following considerations:

1. Our work unveils a new, previously unknown mechanism of regulation of tumor progression by using a Drosophila CRC model, which has been specifically engineered to uncover the molecular systems involved in colon tumorigenesis. Our data stand by itself by reliably showing that a) Drosophila's Dpp/TGF-β signalling pathway has a tumor suppressor activity, which is silenced in adult Apc-Ras intestinal tumors, and b) Dpp/TGF-β activity is reduced by the Iro/Irx complex protein Mirror, which transcriptionally down-regulates some of the key elements of the Dpp pathway in the Drosophila tumors. These findings identify for the first time the Irx/TGF-β pathway as a potential mechanism involved in the modulation of the adenoma to carcinoma transition, thus granting further work in a vertebrate CRC setting. Of course, we acknowledge the relevance of establishing the conservation of this mechanism beyond the Drosophila CRC model. But we have already performed a major effort during the revision to provide extensive evidence supporting a role for this mechanism in vertebrates, including data showing that IRX is upregulated in adenomas and that levels of IRX correlate inversely with gene expression signature of response to TGF-β in human adenomas. More importantly, all the functional

experiments using CRC cells lines also go in line with a role of IRX as a regulator of TGF-beta signaling in human CRC. Particularly, we have reconstituted TGF-β pathway in CRC cell lines that carry inactivating mutations in TGF-β pathway components and we demonstrate that TGF-β induces a cytostatic response in these cells, which is dampened by IRX5. Also, we have performed cell competition assays in which we demonstrate that high levels of IRX expression confer a growth advantage in presence of TGF-β, whereas in the absence of TGF-β, control cells overcompete IRX5 expressing cells.In summary, our work is sustained by the findings in Drosophila and, although we agree in that the analyses in vertebrates could be developed in more detail, we strongly believe that extending this part is beyond the scope of the current manuscript.

2. We think reviewer#2 request of using Vaco-330, Vaco-235, AAC1, LT7 or YAMC cell lines is not reasonable. We (Eduard Batlle lab) are one of the leading labs in CRC research and over more than 15 years we have never used the cell lines proposed by the reviewer. They are not mainstream and in fact they are not available in any public repository, which implies that their obtention is subjected to the signing of the corresponding MT agreements (provided that somebody may have still have them available). Second, these cells have been poorly characterized in the literature. This constitutes an important obstacle, since we would need to first analyze their sensitivity to TGF-β before performing the requested experiments. Therefore, the experiments requested by reviewer#2 are virtually impossible to perform in the suggested time frame.

The work should have be considered as a whole but unfortunately reviewer#2 has not taken into consideration all the compelling evidence obtained though the analysis of the Drosophila tumors. We are convinced that at this stage the combined evidences obtained in Drosophila and vertebrates provides solid ground for the proposed model. We are skeptical that the addition of experiments using new cell lines as suggested by reviewer#2 would modify the conclusions or relevance. In any case, the technical and logistical limitations imposed by the use of these cell lines as explained above represent an important drawback. We respectfully hope that in light of these arguments you can reconsider the necessity of performing these additional experiments.

Thank you very much for your time and attention.

2nd Editorial Decision 27 August 2014

Thank you for your patience while we have assessed your response to the last round of referee reports. We have, on balance, decided not to request the experiments indicated by referee 2 and I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

- The length of your manuscript exceeds that of a normal EMBO reports article, and I have seen that you have quite a detailed material and methods section. Although basic Materials and Methods required for understanding the experiments performed must remain in the main text, we recommend

to include additional detailed information as Supplementary Material.

- Statistical information is missing in several figure legends. Please note that wherever error bars are presented, information regarding the number of independent times an experiment was performed, the type of error calculated and what the bars represent (mean, median,...) must be stated. In addition, please note that "n" should be 3 or more to calculate errors and perform other statistical analyses. Figures 1A, 1B, 1I, 1J, 2F, 2G, 3A and Supp Fig 1B need additional information in the legend.
In addition, the legends to Supp Fig 2 D and E seem to have switched and n=2 in SF2D, which is not a sufficient sample to perform the data analysis provided.

- Please provide figures 3 and 4, as well as the supplementary figures, in higher resolution.

- As a standard procedure, we edit the title and abstract of accepted studies to make them more accessible and appealing to a general readership (please find the edited versions below my signature). In this case, I think the title needs to be more general and independent of the model system used (especially as you also analyze human cells). I have also edited the abstract to include some of the data you incorporated during revision, and which strengthens the role of Irx in inhibiting TGF-beta in vertebrates. Please read the edited version carefully and let me know if you do not agree with any of the changes.

- During our standard check against published articles, the last sentence of the results and discussion section was highlighted as too similar to one in your previous study "Conserved Mechanisms of Tumorigenesis in the Drosophila Adult Midgut". In order to avoid possible unpleasant problems post-publication, please rephrase this sentence.

- Every EMBO reports paper now includes a 'Synopsis' to further enhance its discoverability. Synopses are displayed on the html version and they are freely accessible to all readers. The synopsis includes a short standfirst text -I have added my proposal for this text below- as well as 2-3 one sentence bullet points that summarise the paper. These should be complementary to the abstract -i.e. not repeat the same text. This is a good place to include, as appropriate, key acronyms and quantitative and organism (yeast, mammalian cells, etc) information. This synopsis will be accompanied by a graphic that is being worked on by our in-house team based on your figure 4C. Could you supply the bullet points to accompany the standfirst? Do let me know if you would like to modify the standfirst blurb:

"Iro/Irx transcription factors are shown to suppress the TGF-β pathway, reducing its tumor suppressor activity, in flies and human cells. They could thus enable tumor growth in the presence of TGF-β, before of mutations in this pathway occur.

2-3 bullet points"

- We now encourage the publication of original source data -particularly for electrophoretic gels and blots, but also for graphs- with the aim of making primary data more accessible and transparent to the reader. If you agree, you would need to provide one PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures and an Excel sheet or similar with the data behind the graphs. The files should be labeled with the appropriate figure/panel number, and the gels should have molecular weight markers; further annotation could be useful but is not essential. The source files will be published online with the article as supplementary "Source Data" files and should be uploaded when you submit your final version. If you have any questions regarding this please contact me.

After all remaining corrections have been attended to, you will receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

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Edited title and abstract

Irx transcription factors negatively regulate TGF-β pathway activity during midgut tumorigenesis

"Activating mutations in Wnt and EGFR/Ras signaling pathways are common in colorectal cancer (CRC). Remarkably, clonal co-activation of these pathways in the adult Drosophila midgut induces "tumor-like" overgrowths. Here we show that, in these clones and in CRC cell lines, Dpp/TGF-β acts as a tumor suppressor. Moreover, we discover that the Iroquois/Irx-family-protein Mirror down-regulates the transcription of core components of the Dpp pathway, reducing its tumor suppressor activity. We also show that this genetic interaction is conserved in human CRC cells, where the Iro/Irx proteins Irx3/Irx5 diminish the response to TGF-β. Irx3/Irx5 are upregulated in human adenomas and their levels correlate inversely with the gene expression signature of response to TGF-β. In addition, Irx5 expression confers a growth advantage in the presence of TGF-β, but is selected against in its absence. Together, our results identify a set of Iro/Irx proteins as conserved negative regulators of Dpp/TGF-β activity. We propose that during the characteristic adenoma-to-carcinoma transition of human CRC, the activity of Irx proteins could reduce the sensitivity to the cytostatic effect of TGF-β, conferring a growth advantage to tumor cells prior to the acquisition of mutations in TGF-β pathway components. "

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I recommend the manuscript for publication in EMBO reports.

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2nd Revision - authors' response 01 September 2014

I am writing to submit the final version of our manuscript. In this version we have addressed all the minor issues/corrections that you pointed out in your last letter. In detail,

- We have moved most of the Methods section to Supplementary information.

- We have added the statistical information that was missed in some figure legends. Regarding the legend of Supp Fig2D, it corresponded to a graph that was eliminated in the final version of the figure but we forgot to eliminate it from the legend.

- We have increased the resolution of Figures 3, 4 and all supplementary figures.

- Following your suggestion, we have changed the title to: "Iro/Irx transcription factors negatively regulate Dpp/TGF-β pathway activity during intestinal tumorigenesis".

- We have also changed the last sentence of the results and discussion section.

Regarding the synopsis, as I already stated in our previous communication, we agree with the image provided by the graphics editor and with the standfirst text you provided. The bullet points we propose are:

1) Mirror is a new genetic regulator of Dpp pathway activity in *Drosophila* midgut tumors.

2) As predicted by the *Drosophila* model, IRX expression in human colorectal cancer cells confers a growth advantage in presence of TGF-β.

3) In human adenomas, IRX correlate inversely with a gene expression signature of response to TGF-β.

Finally we are submitting the original source data. We have prepared an excel file with the data behind the graphs and a PDF file with the western blot shown in Supplementary Figure 1B.

Thank you very much for your help during all this process.

3rd Editorial Decision 04 September 2014

I am very pleased to accept your manuscript for publication in EMBO reports. Thank you for your contribution to our journal. Your article will, barring any delays with the production process post-acceptance, be included in our December issue in print and appear in 2-3 weeks online ahead of print.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

