APC regulation of ESRP1 and p120-catenin isoforms in colorectal cancer cells.

Maree Faux, Lauren King, Serena Kane, Christopher Love, Oliver Sieber, and Tony Burgess

Corresponding author(s): Maree Faux, The Walter and Eliza Hall Institute of Medical Research

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(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.)

RE: Manuscript #E20-05-0321
TITLE: "APC regulation of ESRP1 and p120-catenin isoforms in colorectal cancer cells."

Dear Maree,

Thank you for submitting your manuscript to MBoC. It has now been seen by two expert reviewers and their comments follow below.

As you will see, their opinion is somewhat split. Both agree that your study endeavours to address an important issue, namely deciphering the mechanisms that allow APC to regulate colon cancer plasticity. Reviewer 2, however, has concerns about the quality of data presented and the strength of the functional and/or phenotypic changes (for example, the effects of APC depletion on p120 isoform switching). Reviewer 1 is more supportive, but suggests that your findings might be more simply explained by restoration of canonical Wnt/b-catenin signalling; although Reviewer 1 is open-minded, this alternative explanation would make interpretation of your findings less novel. Both are also concerned that you infer p120 isoform switching based on electrophoretic migration alone, and this should be tested directly.

Underlying both the reviewers' comments is a concern - which I share - that the current manuscript doesn't provide substantive new insight (be it conceptual or mechanistic) to be appropriate for the general cell biology readership of MBoC. For this, I think that you would have to address the evidentiary issues that both reviewers have raised; and also provide something that extends what we already know. The latter might, for example, be focused on how APC regulates ESRPs and/or extension of their role in colon cancer (as reviewer 1 notes). Overall, I think that this would require additional experiments that are beyond what we would reasonably expect of a revision and it may be that your MS is better suited to a more specialized journal.

So, I have to formally decline your manuscript. I'm sorry that our decision can't be more positive on this occasion. (If you do feel that the issues that have been raised are ones that you wish to address, we are always open to discussion. But please address your correspondence through the MBoC office: mboc@ascb.org.)

Best wishes,

Alpha

Monitoring Editor
Molecular Biology of the Cell

Reviewer #1 (Remarks to the Author):

The Burgess lab showed previously that restoring WT APC expression in the APC deficient SW480 cell line rescues E-cadherin expression and epithelial morphology. Faux et. al. report here that APC also rescues expression of ESRP1 and ESRP2, triggers a shift in p120 isomers towards p120-3A. RNAseq analyses highlight cell-cell adhesion and EMT-relevant genes, including upregulation of

ESRP1 and ESRP2. The predicted downregulation of ESRP's 1 and 2 upon APC LOH are confirmed in human CRC and causality is supported by knockdown and addback studies. They suggest therefore that APC LOH is associated with downregulation of the ESRPs and increased expression of the mesenchymal p120 isoforms (p120-1A). The opposite occurs upon APC addback.

Comments:
1. In general, the paper is well written and easy to follow. Experiments are well designed and properly presented. I have only two somewhat substantive negative issues.
a. The discussion seems overly long and unnecessarily complicated by issues that can't or don't necessarily need to be addressed.
b. After reading the abstract, I was expecting some novel APC-mediated function that was independent of the Wnt pathway, but that didn't really materialize. Instead, it seems that one way or another, the major phenotypes are all still linked to the Wnt pathwayup, or at least not shown to be otherwise. For example, adding back APC restores ESRP expression, in line with the epithelial phenotype, but the phenotype is still most likely a function of the Wnt pathway (in reverse this time).
Also, interpretation of the data is somewhat skewed by preconceived notions as to how and when particular events take place. We can agree to disagree, but for what its worth, my take on the process in play is included below. Perhaps it will not seem that different, but maybe could allow for a simpler discussion.
In any event, it is now widely accepted that the cell of origin in CRC is the intestinal stem cell (ISC). The ISC already expresses high B-catenin on account of the high Wnt concentration in the niche, and it is believed to be significantly mesenchymal in character. Upon loss of APC, the ISC is hijacked by what is now a cell autonomous and constitutively active Wnt/B-catenin pathway. Characteristics of the ISC (e.g., indefinite self-renewal) come with the territory and contribute substantially to the transformed phenotype. The resulting cancer stem cell (CSC) has no need to undergo EMT because it is already at least part way there. Nor does It dedifferentiate, rather it simply fails to differentiate in the first place because opposing BMP gradiants cannot overcome the self-generated WNT signal. One would then expect these cells to favor the mesenchymal p120 isoforms and continue to proliferate, as the option to differentiate is no longer on the table. Reconstituting WT APC in SW480 cells simply removes the WNT signal and the cell returns to ground state - except for a bunch of mutations that likely are responsible for the fact that they don't simply die from lack of WNT. Since you are not adding Wnt, a more differentiated epithelial like phenotype is not unexpected.

Maybe this is different, maybe not. Either way, I think the authors should try to clean up the discussion. At least try to remove speculation about things that are not central to the point of the paper.

2. I don't have much else to add. The role of the ESRP's in controlling p120 isoform expression is not controversial, and the reversion to an epithelial phenotype upon addback of APC has been described previously. Application of the ESRPs in the context of GI cancer is interesting. I could object to the notion that the isoforms you point to are actually p120-1A and p120-3A as you would probably have to confirm by RNAseq but it doesn't really change anything.

Reviewer #2 (Remarks to the Author):

This report focuses on the interplay between an important tumor suppressor gene, adenomatous

polyposis coli (APC) and posttranscriptional splicing of a key cell-cell adhesion protein, p120-catenin. Authors report that colon cancer cells bearing a truncated version of APC express similar amount of two p120 isoforms, a mesenchymal-type p120 A1 and an epithelial-type p120 A3. Expression of full length APC in these cells results in p120 isoform switch toward the epithelial A3 protein. Furthermore, they suggest that APC-dependent expressional regulation of the epithelial splice regulatory protein 1 (ESPR1) is responsible for the described APC-driven switch in p120 isoforms. Overall, the study has scientific merit, since it addresses molecular mechanisms that could mediate epithelial-to-mesenchymal transition in colorectal cancer. However, this reviewer feels that the present manuscript does not have enough exciting mechanistic or even phenomenological data to understand functional roles of the p120 isoform switching and ESPRs in colorectal cancer.

Comments:

1. The authors should present additional evidence to support their conclusion that two p120 catenin molecular forms with different electrophoretic motility are indeed two splice variants of this protein. Perhaps, including RT-PCR analysis of p120 1A expression using isoform-specific primers would strengthen their conclusion.
2. There is no data included to show actual overexpression of wild-type APC in SW480 stable cell lines, generated to overexpress full-length APC. In Figure 3 immunoblots, the band of the full-length APC is barely visible.
3. The effects of APC knockdown on the p120 isoform switch is very unimpressive. Even when the majority of APC (both truncated and full length seems to be depleted) the difference in p120 1A and 3A isoform levels still persists in different experimental groups. This is difficult to reconcile with direct APC-related regulation of p120 splicing.
4. Likewise, the effects of ESPR1 and ESPR2 knockdown on p120 isoform switch are not impressive. Even if depletion of these splicing factors increases amount of the 1A isoform in APC-expressing cells, the effects are modest and do not reverse the APC-dependent shift from the 1A to the 3A expression. Perhaps, performing co-knockdown of ESPR1 and ESPR2 could increase the magnitude of these effects.
5. Depletion of ESPR1, or ESPR2 has some effects on p120 isoform switch only in APC-overexpressed, but not parental cancer cells. Does this mean that these splicing factors fail to regulate p120 catenin in APC truncated cells? What about their role in regulating p120 catenin expression in normal epithelial and mesenchymal type cells?
6. I wonder if loss of ESPR1 and ESPR2 could reverse the mesenchymal-to-epithelial transition caused by APC overexpression.
7. What is the effect of Wnt inhibitor on expression of two p120 catenin isoforms?

RE: Manuscript #E20-05-0321

TITLE: “APC regulation of ESRP1 and p120-catenin isoforms in colorectal cancer cells”

Dear Professor Yap,

Thank you for agreeing to consider a revised manuscript. We appreciate your advice about our manuscript and have attempted to take on board, all of the issues raised by you and the reviewers. We have included new data, revised the manuscript and shortened the Discussion. We believe that our experiments show the link between APC and ESRP: the master epithelial splicing regulator. This link provides substantive new insights into a previously unexplored biology for APC.

We believe the manuscript is stronger for the revisions and we would like to express our appreciation to the reviewers for the time they spent on our manuscript.

Yours sincerely,

Maree Faux and Tony Burgess

In response to the reviewers:
The issues raised by the reviewers are displayed in green and our responses follow directly.

We have made substantial changes to the manuscript and have included new data in our revised manuscript. We have revised all sections of the manuscript. There are now 7 Figures and 5 supplemental figures.

Summary of changes to the Figures

Figure 1: Unchanged
Figure 2: qRT-PCR using isoform specific primers included in Figure 2D
Figure 3: Revised Figure A B C to show APC depletion at 72 h and quantitation of β-catenin included in Figure 4D.
Figure 4: confocal microscopy of p120-catenin and E-cadherin in ESRP1 depleted cells in Figure 4G.
Figure 5: immunoblot of a panel of cell lines probed with antibodies to p120-catenin, β-catenin, E-cadherin and β-tubulin included in Figure 5A with transcript data from human tumours shown in Figure 5B-D (previously Figure 5A-C)
Figure 6: Wnt inhibition A-C (previously Figure 5D-F)
Figure 7: Schematic model for APC regulation of p120-catenin isoform splicing (previously Figure 5G, H).

Figure S1: related to Figure 2: Details of isoform specific probes
Figure S2: related to Figure 3: Depletion of APC at 72 h and includes quantitation of the ratio of isoforms 1A:3A
Figure S3: related to Figure 4: Depletion of ESRP1, ESRP2 and ESRP1+2
Figure S4: related to Figure 4: Depletion of ZEB1
Figure S5: related to Figure 6: Distribution of p120-catenin isoforms following Wnt inhibition

Response to the reviewers’ comments:

Reviewer 1:

1a. The discussion seems overly long and unnecessarily complicated by issues that cant or don't necessarily need to be addressed.

Response: We have revised the Discussion completely. It is considerably shorter with more focus on the key findings relevant to our manuscript.

1b. …the major phenotypes are all still linked to the Wnt pathway, or at least not shown to be otherwise.

Response: We have revised the manuscript and the discussion, in particular, to make clearer what we believe our data means in the context of the current Wnt signalling APC signalling paradigms.

2. The isoforms are actually p120-1A and p120-3A?

Response:
We now include qRT-PCR analysis of p120-catenin 1A and 3A expression in SW480 and SW480+APC cells to confirm that the two p120-catenin molecular forms are indeed two splice variants of p120-catenin (Figure 2D). Primers that span *CTNND1* exon boundaries 1-2, 2-3 and 3-4 will only detect isoform 1A. We show that with four taqman primer sets (spanning exon1-2, 1-2, 2-3 and 3-4), *CTNND1* is significantly higher in SW480 compared to SW480+APC cells whereas primers that span *CTNND1* exon boundaries 4-5 and 16-17, which detect both isoform 1A and 3A, show similar levels of *CTNND1* expression. We also include primers spanning exon14-15 that detect isoforms 1B, 2B and 3B and show no difference in expression between the cell lines. *CTNND1* primers are detailed in the 'Materials and Methods' and Supplemental Figure 1S. We have added the following text to the revised manuscript:

"p120-catenin 1A and 3A isoform expression was also analysed with q-PCR using isoform specific primers (Figure 2D, Supplemental Material Fig S1). *CTNND1* mRNA expression was significantly higher with the primers that spanned the exon boundaries for *CTNND1* 1-2, 2-3 and 3-4 in SW480 compared to SW480+APC, i.e. consistent with the expression of p120-catenin isoform 1A in SW480 but not SW480+APC cells (Figure 2D). In contrast, there was no difference in *CTTND1* expression with the primers which detect both 1A and 3A isoforms, i.e. those spanning exons 4-5and 14-15 (Figure 2D)."

Reviewer #2:

This report focuses on the interplay between an important tumor suppressor gene, adenomatous polyposis coli (APC) and posttranscriptional splicing of a key cell-cell adhesion protein, p120-catenin. Authors report that colon cancer cells bearing a truncated version of APC express similar amount of two p120 isoforms, a mesenchymal-type p120 A1 and an epithelial-type p120 A3. Expression of full length APC in these cells results in p120 isoform switch toward the epithelial A3 protein. Furthermore, they suggest that APC-dependent expressional regulation of the epithelial splice regulatory protein 1 (ESPR1) is responsible for the described APC-driven switch in p120 isoforms. Overall, the study has scientific merit, since it addresses molecular mechanisms that could mediate epithelial-to-mesenchymal transition in colorectal cancer. However, this reviewer feels that the present manuscript does not have enough exciting mechanistic or even phenomenological data to understand functional roles of the p120 isoform switching and ESPRs in colorectal cancer.
Comments:

1. The authors should present additional evidence to support their conclusion that two p120

catenin molecular forms with different electrophoretic motility are indeed two splice variants of this protein. Perhaps, including RT-PCR analysis of p120 1A expression using isoform-specific primers would strengthen their conclusion.

Response:
See Response to Reviewer 1 point 2.

2. There is no data included to show actual overexpression of wild-type APC in SW480 stable cell lines, generated to overexpress full-length APC. In Figure 3 immunoblots, the band of the full-length APC is barely visible.

Response:
The overexpression of wild-type APC in SW480 stable cells is reported in Faux et al 2004 J Cell Science 117:427-439). Here we showed expression of the wild-type APC mRNA by RT-PCR and expression of the myc-epitope tagged APC by immunoblot analysis (Faux et al., 2004). We also showed that full-length APC was functional as SW480+APC cells show reduced β-catenin and Tcf/LEF transcriptional signaling and has recently been confirmed by altered Wnt target gene expression by RNAseq analysis (King et al., 2016). The RNAseq data presented in Figure 1D shows that APC expression is increased in SW480+APC compared to parental SW480 cells (log2 fold change, $P<0.05$). We observed in our original paper that full-length APC expression levels were low and that the low expression levels may have allowed the cells to survive, as previous studies have shown that over-expression of APC leads to apoptosis (Morin et al 1996 PNAS, Groden et al., 1995 Cancer Res). In Figure 3 immunoblots, the full-length APC band is weak, especially compared with the strong signal for the truncated APC protein. However, this is a reproducible signal for the full-length APC protein and demonstrates that the protein is expressed, albeit weakly. The transfer of larger molecular weight proteins is less efficient than smaller proteins and combined with the low level of expression of full-length APC results in a low level of signal.
We have included the following sentence in the manuscript text in relation to the immunoblot of APC in Figure 3A:

"Only low levels of full-length APC are present in the SW480+APC cells (Figure 3A and (Faux et al., 2004))."

3. The effects of APC knockdown on the p120 isoform switch is very unimpressive. Even when the majority of APC (both truncated and full length seems to be depleted) the difference in p120 1A and 3A isoform levels still persists in different experimental groups. This is difficult to reconcile with direct APC-related regulation of p120 splicing.

Response:
The knockdown of APC produces a robust and reproducible shift in the proportion of the 1A isoform at 72 h. We had originally included the data from 48 and 72 h post siRNA transfection. While there is a visible increase in the 1A isoform at 48 h, the increase is more evident at 72 h and is highly statistically significant. We note that there is little detectable isoform 1A in SW480+APC cells in the mock control treated cells and that this is increased 2.7 +/- 0.2 -fold ($P=0.0004$, unpaired *t*-test, n=3 independent experiments) following APC siRNA treatment. The corresponding increase in β-catenin, which is expected following APC siRNA treatment, is also evident but not significant at 48 h and is statistically significant at 72 h ($P=0.045$, unpaired *t*-test, n=2 independent experiments). The fold increase in β-catenin at 72 h post APC siRNA transfection compared to mock is 2.6 +/- 0.7, similar to the fold increase in p120-catenin isoform 1A. There is no dispute that APC directly regulates β-catenin levels which show equivalent changes as we now show for p120-catenin isoform 1A. We have now removed the data from the

48 h treatment and include the 72 h treatment in Figure 3A which shows the result more clearly. We have now included the quantitation for β-catenin in Figure 3D. We also present the ratio of isoforms 1A:3A which is significantly increased in SW480+APC cells from 0.07+/-0.01 to 0.21+/-0.04 (P=0.016, unpaired *t*-test) in Supplemental Figure S2B. This shows a 3.16+/-0.59 -fold increase in the ratio of 1A:3A. Depletion of APC therefore results in a detectable shift in isoform balance. While this may not be impressive, the difference is significant and demonstrates that APC regulates p120-catenin isoform switching. Our experiments show that this occurs via regulation of the epithelial splice regulatory protein, ESRP1. We do not believe that we would expect to see a complete reversal of the p120-catenin isoform balance under the experimental conditions of the transient siRNA transfection. Indeed, as we point out above, we see a similar fold-change in β-catenin levels. We have revised the wording in the text 'to test if APC regulates p120-catenin isoform levels' and removed 'directly' and modified the manuscript text to reflect the changes to the figures:

"APC promotes a switch to an epithelial p120-catenin isoform
Expression of full-length APC in SW480 cells results in altered Wnt and cell adhesion transcriptional signatures and is accompanied by redistribution of adhesion- and tight- junction proteins. To test if APC regulates p120-catenin isoform levels, we depleted APC using siRNA. The truncated APC protein, present in SW480, SW480 control and SW480+APC cells, and full-length APC, present only in SW480+APC cells, are efficiently depleted at 72 h post transfection (Figure 3A; Figure S2 A). Only low levels of full-length APC are present in the SW480+APC cells (Figure 3A and (Faux et al., 2004)). siRNA-mediated depletion of APC perturbs p120-catenin isoform expression whereby isoform 1 is increased upon APC depletion (2.7±0.2 fold over mock control) (Figure 3B, D). siRNA-mediated depletion of truncated APC protein also results in a modest but significant increase in p120-catenin isoform 1 in both SW480 parental and SW480 control cells (Figure 3), which suggests a residual function for the truncated APC protein that is likely to be Wnt-independent. While the p120-catenin isoform 3A does not change markedly upon APC depletion, the ratio of p120-catenin isoforms 1A:3A is increased significantly in SW480+APC cells (3.16±0.59 fold increase) in APC siRNA compared to mock control treated cells (Figure S2 B). Depletion of APC results in a similar fold increase in β-catenin levels (2.6±0.7 fold) and a reduction of E-cadherin in SW480+APC cells (Figure 3C; Figure S2 C). The increase in p120-catenin isoform 1 following depletion of APC demonstrates that full-length APC controls p120-catenin isoform levels in SW480 cells."

Revised Figure 3: APC depletion by APC siRNA
A. APC immunoblot at 72 h
B. P120-catenin immunoblot at 72 h
C. β-catenin immunoblot at 72 h
D. Quantification of 1A, 3A and β-catenin

Revised Supplemental Figure S2:
A. APC depletion at 72 h: quantification
B. p120-catenin ratio 1A:3A
C. E-cadherin blots and quantification

4. Likewise, the effects of ESPR1 and ESPR2 knockdown on p120 isoform switch are not impressive. Even if depletion of these splicing factors increases amount of the 1A isoform in APC-expressing cells, the effects are modest and do not reverse the APC-dependent shift from the 1A to the 3A expression. Perhaps, performing co-knockdown of ESPR1 and ESPR2 could increase the magnitude of these effects.

Response:

The effects of ESRP1 depletion result in a robust and reproducible increase in the p120-catenin isoform 1A in APC-expressing cells (4.1+/-0.5 -fold, P=0.0027, one-tailed unpaired *t*-test) whereas the effects of ESRP2 depletion are more modest (2.0+/-0.4 -fold, P=0.05). The effects do not reverse the shift from 1A to 3A upon APC expression. As with the transient APC siRNA transfection, we would not expect to see a complete reversal upon transient depletion of ESRPs. We note that Horiguchi et al. (2012 Oncogene) report that ESRPs attenuate malignant phenotypes of cancer cells as ESRP overexpression failed to affect the expression of mesenchymal marker proteins and that Shipiro et al (2012 PLOS Genetics) show that overexpression of ESRP or depletion of another splicing factor RBFOX2 resulted in changes in cell morphology towards a partial epithelial phenotype.
We have revised the manuscript text to indicate this:

"Depletion of ESRP1 did not reverse the shift from 1A to 3A isoforms, but did result in a greater than 4-fold increase in the p120-catenin isoform 1 in SW480+APC cells (Figure 4E, F)"

We have now performed the co-knockdown of ESRP1 and ESRP2 to test whether depletion of both epithelial splice regulators would increase the magnitude of ESRP depletion. These results are presented in Supplemental Figure S3. There is a clear increase in isoform 1A in ESRP1, ESPR2 and ESRP1+2 depletion compared to mock, but the combination did not increase isoform 1A further. The ratio of isoforms 1A:3A is presented and demonstrates that ESRP1 and ESRP1+2 show a significant increase (P=0.003 and P=0.01, respectively) whereas depletion of ESRP2 did not quite reach significance of $P<0.05$ (P=0.07). This data suggests that ESRP1 is more likely to regulate p120-catenin isoform switching in SW480+APC cells and is consistent with transcript expression data that shows ESRP1 is more highly expressed than ESRP2. We note that Warzecha et al (2010 EMBO J) refer to sustained and prolonged knockdown using shRNAs which effectively reduced the levels of each protein. Warzecha et al (2010) reported that sustained ESRP1 knockdown was associated with some changes in splicing, but there was little or no change in splicing upon knockdown of ESRP2 alone and that combined knockdown generally achieved the largest change and suggested that ESRP1 is the more robust splicing regulator.

We explored the connection with ZEB1 and splicing regulation by depleting ZEB1 and probing for changes in ESRP1/2 and p120-catenin. SW480 cells express relatively high levels of ZEB1 and expression is reduced significantly in SW480+APC cells (shown in Figure 1D). We see little expression of ESRP1/2 in SW480 cells but upon ZEB1 depletion, ESRP2 is clearly visible, with no change in ESRP1. ZEB1 depletion, however, did not appear to affect p120-catenin isoform distribution (as shown in Figure S4).

We also include new data showing immunofluorescent staining of p120-catenin and E-cadherin in SW480+APC cells treated with ESRP1 siRNA to characterise phenotypic changes in cell contact staining. We show that that depletion of ESRP1 by siRNA results in reduced cell-cell contacts and an increase in p120-catenin in the nucleus (Figure 4G). As SW480 parental cells display minimal cell contact staining and a stronger nuclear signal, the staining in the ESRP1 depleted cells indicates a partial switch to a more mesenchymal phenotype. These data are consistent with a reduced epithelial phenotype in ESRP-depleted cells which is reflected in the increase in isoform 1A.

We have revised the manuscript text to incorporate these changes:

"Depletion of ESRP1 did not reverse the shift from 1A to 3A isoforms, but did result in a greater than 4-fold increase in the p120-catenin isoform 1 in SW480+APC cells (Figure 4E, F). p120-catenin isoform 1 was also increased when ESRP2 was depleted (Figure 4E, F). We tested the combination of ESRP1 and ESRP2 siRNAs to determine whether depletion of both

ESRPs would further increase the levels of isoform 1A. However, co-knockdown of ESRP1 and 2 resulted in a similar increase to the knockdown of ESRP1 alone (Figure S3), suggesting that ESRP1 is more likely to regulate p120-catenin isoform switching in SW480 cells. Interestingly, depletion of *ZEB1* resulted in increased ESRP2 but not ESRP1 in SW480 cells but the knockdown of *ZEB1* did not affect p120-isoform distribution (Figure S4).

We investigated junctional p120-catenin and E-cadherin staining in SW480+APC cells treated with ESRP1 siRNA. The SW480+APC cells show evident cell-cell contact staining with junctional markers such as E-cadherin, ZO1 and p120-catenin, consistent with an epithelial phenotype (see Figure 2A, Figure 4G). p120-catenin junctional staining was disrupted upon ESRP1 depletion with less tightly packed cells and an increase in nuclear signal (Figure 4G). The increased nuclear signal in ESRP1-depleted cells resembles the p120-catenin distribution in the SW480 parental cells (Figure 2A). Similarly, E-cadherin staining at cell junctions was reduced. This is in contrast to mock- treated (control) cells which display p120-catenin and E-cadherin at sites of cell adhesion (Figure 4G). Thus treatment with ESRP1 siRNAs partially reverted the epithelial phenotype and cell-cell junction morphology towards a more mesenchymal phenotype. Collectively these data show that restoring full-length APC expression in SW480 cells promotes ESRP1/2 expression leading to altered epithelial p120-catenin isoform expression and that ESRP1 depletion shifts the splicing program to partially mesenchymal."

5. Depletion of ESPR1, or ESPR2 has some effects on p120 isoform switch only in APC-overexpressed, but not parental cancer cells. Does this mean that these splicing factors fail to regulate p120 catenin in APC truncated cells? What about their role in regulating p120 catenin expression in normal epithelial and mesenchymal type cells?

Does this mean that these splicing factors fail to regulate p120 catenin in APC truncated cells?
Response:
The expression of ESRP1 and 2 is significantly increased in SW480+APC cells compared to SW480. This is evident in the transcriptional data (RNAseq for ESRP1 and ESRP2 (Figure 4A) and qRT-PCR for ESRP1 (Figure 4D). The low level of ESRP1 and 2 in SW480 parental cells was confirmed by immunoblot analysis which show little detectable ESRP1/2 (Figure 4C; Figure S3, S4). It is therefore not surprising that there is less of an effect of depletion of the already low levels of ESRPs in parental cancer cells. There is also reduced ESRP1 and ESRP2 in primary CRC (revised Figure 5B, C) which correlates with APC expression. These data suggest that regulation of p120-catenin isoforms by ESRPs is limited in APC truncated cells due to reduced expression of ESRPs. By expression of wild-type APC in SW480 cells, ESRP1 and ESPR2 expression is increased suggesting that APC can regulate ESRP1/2 expression and thereby regulate isoform splicing, including p120-catein.

We have investigated p120-catenin isoform expression in a panel of cell lines by immunoblot analysis and have included this data in revised Figure 5 (5A). In cells with APC mutations (eg Colo320, Dld1, Caco2) p120-catenin isoform1A is expressed: in Colo320 cells isoform 1A is the dominant isoform, whereas LIM2537 cells (which contain wtAPC) predominantly express isoform 3A, similar to SW480+APC cells and correlates with *ESRP1* and *ESRP2* transcript analysis in these cells (Wang et al. 2017 Gastroenterology). This supports the data in SW480/SW480+APC cells.

Both DLD1 and Caco2 cells express a higher level of the epithelial 3A isoform compared to 1A which likely reflects the epithelial morphology of these cells (Hidalgo et al., 1989 Gastroenterology; Tanaka et al., 2016 Biochem Biophys Rep). We show that the distribution of

p120-catenin isoforms shows a strong correlation with E-cadherin expression and this is supported in the literature (Warzecha et al., 2009 Mol Cell, 2010 EMBO J; Horiguchi et al., 2012 Oncogene). Moreover, cells with a mesenchymal phenotype, such as RKO and HEK293 cells (Ayinde et al 2017 Oncotarget) show a similar distribution of p120-catenin isoforms to SW480 cells and similarly express low levels of E-cadherin (Fig 5A) and *ESRP1* (Wang et al 2018; Warzecha et al., 2009). This data suggests that ESRP expression is important in maintaining the epithelial phenotype, including regulation of p120-catenin isoform distribution, *rather than APC status*, but there are also likely to be other contributing factors, such as additional mutations, eg Ras, TGFb signalling.

What about their role in regulating p120 catenin expression in normal epithelial and mesenchymal type cells?

Response:

The role for ESRPs in regulating p120-catenin expression in normal epithelial and mesenchymal type cells is well characterised (Warzecha et al., 2009; Shapiro et al 2012 PLOS Genetics, reviewed in Pradella et al 2017 Mol Cancer, Lamouille et al 2014 Nat Rev Mol Cell Biol). ESRPs are reported to be specifically expressed by epithelial cells (Warzecha et al 2010, Horiguchi et al., 2012 Oncogene, Mager et al 2017 Elife) and reduced expression in CRC results in a switch to *FGFR2* and *CTNND1* mesenchymal variants (Deloria et al 2016 Oncotarget).

Our data for p120-catenin isoform distribution are consistent with analysis of the expression profile of ESRPs in cell lines which show higher expression of ESRP1 and 2 in epithelial cells compared to mesenchymal cells (eg. NC160 panel 10 of 11 epithelial cell lines show 10-fold higher ESRP1 expression compared to 36 mesenchymal cell lines, Warzecha et al., 2009 Mol Cell). Our data show that cells with an epithelial morphology show increased expression of p120-catenin isoform 3A compared to 1A. ESRPs have been reported to regulate splicing of a number of epithelial genes, including *FGFR2*, *CD44* and *CTNND1* and this is linked to epithelial phenotype (Warzecha et al., 2009; Shapiro et al 2012 PLOS Genetics, reviewed in Pradella et al 2017 Mol Cancer, Lamouille et al 2014 Nat Rev Mol Cell Biol). In particular, prolonged ESRP1 knockdown or overexpression was shown to result in altered *CTNND1* transcript although p120-catenin protein was not shown (Warzecha et al., 2009 Mol Cell). The 'prolonged' ESRP1 knockdown (using shRNA) may result further reversion in *CTNND1* transcription. Expression of ESRP1 has been shown to lead to increased recruitment of p120-catenin to sites of cell-cell adhesion (Shapiro et al 2012 PLOS Genetics). As evident from our immunofluorescent analysis of ESPR1-depleted cells, we show reduced p120-catenin at cell contacts, confirming a role for ESRP in promoting an epithelial phenotype (see new Figure 4G). These studies support a role for ESRPs in EMT and tumour progression by changing alternative isoform expression of genes important for epithelial and mesenchymal cell morphology, such as p120-catenin.

We have revised the manuscript text to incorporate these changes:

"p120-catenin isoform expression in colon cancer cells

Expression of wild-type full-length APC in SW480 cells leads to increased expression of ESRP1 and ESPR2 suggesting that APC can regulate ESRP1/2 expression and thereby regulate isoform splicing, including p120-catenin. The corollary of this observation is that truncated APC is unable to regulate ESRP and p120-catenin isoform splicing. To investigate the contribution of APC to ESRP and isoform distribution, we investigated p120-catenin isoform expression in a panel of cell lines (Figure 5A). In cells with APC mutations (Colo320, DLD1, Caco2), p120-catenin isoform 1A is expressed: in Colo320 cells isoform 1A is the dominant isoform. In contrast, LIM2537 cells which contain full-length APC predominantly express isoform 3A, similar to SW480+APC cells (Figure 5A). This correlates with ESRP1 and ESRP2 transcript analysis in these cells (Wang et al., 2017) and supports our findings in SW480/SW480+APC cells. Both DLD1and Caco2 cells express a higher level of

the epithelial 3A isoform compared to 1A (Figure 5A). These cells contain mutated APC but also demonstrate an epithelial morphology with intact cell adhesion junctions(Hidalgo et al., 1989; Tanaka et al., 2016). A defining feature of epithelial cells is E-cadherin expression at cell-cell junctions which is absent in mesenchymal cells and this correlates with ESRP and epithelial isoform expression(Baum and Georgiou, 2011; Warzecha et al., 2010; Warzecha et al., 2009). To assess whether the morphology of the cells was indicative of the isoform distribution, we analysed E-cadherin expression in the cell lines (Figure 5A). E-cadherin is expressed in cells with an epithelial phenotype that also express higher levels of isoform 3A. Moreover, cells with a mesenchymal phenotype, including RKO and HEK293(Ayinde et al., 2017; Buck et al., 2007; Warzecha et al., 2009), show a similar distribution of p120-catenin isoforms to SW480 cells and also express low levels of E-cadherin (Figure 5A) and ESRP(Wang et al., 2017; Warzecha et al., 2010; Warzecha et al., 2009). The distribution of p120-catenin isoforms therefore correlates well with the E-cadherin expression and underscores the importance of ESRP and epithelial isoform expression in governing the epithelial phenotype."

6. I wonder if loss of ESPR1 and ESPR2 could reverse the mesenchymal-to-epithelial transition caused by APC overexpression.

Response:
We have performed *ESRP1*, *ESRP2* and *ESRP1* and 2 depletion experiments and see only negligible effects on EMT markers E-cadherin and Vimentin. We note that Horiguchi et al (2012 Oncogene) reported an attenuated effect of ESRPs in breast cancer cells as overexpression of ESRP failed to affect the organisation of actin or expression of mesenchymal markers, including fibronectin and N-cadherin and Warzecha et al (2010 EMBO J) report no effect on E-cadherin but increased vimentin, fibronectin and N-cadherin upon prolonged knockdown of ESRP. While ESRPs are major regulators of alternative splicing in epithelial cells, Warzecha et al (2010) propose that there is likely to be a combination of factors that collaborate, for example other splicing factors that contribute to EMT-associated switches, such as RBM47 and Quaking (Yang et al., 2016 Mol Cell Biol).

7. What is the effect of Wnt inhibitor on expression of two p120 catenin isoforms?

Response:
Wnt inhibition results in only a slight reduction in p120-catenin isoform 1A expression, this is consistent with the increase in ESRP.
We have added this data to Supplementary Material Figure S5 of the revised manuscript.

We have revised the manuscript text to describe this data:

"Wnt inhibition stimulated *ESRP1* expression in both SW480 and SW480+APC cells (Figure 6A) with concomitant reductions in the expression of Wnt target genes, e.g. *AXIN2* and *ID2* (Figure 6B and C) but there was only a modest effect on the p120-catenin isoform distribution (Figure S5)."

RE: Manuscript #E20-05-0321R-A
TITLE: "APC regulation of ESRP1 and p120-catenin isoforms in colorectal cancer cells."

Dear Maree and Tony,

Thank you for your revised manuscript. We appreciate the extensive efforts that you've made to respond to the reviewers' comments. Everything now seems to be in order and so I am delighted to accept your paper for publication in MBoC.

Best wishes,

Alpha

Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Faux:

Congratulations on the acceptance of your manuscript.

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Eric Baker
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Reviewer #2 (Remarks to the Author):

I was the least positive reviewer but the revised manuscript addresses all my major concerns. I have nothing to add.