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

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ABSTRACT The adenomatous polyposis coli (APC) tumor suppressor protein is associated with the regulation of Wnt signaling; however, APC also controls other cellular processes including the regulation of cell adhesion and migration. The expression of full-length APC in SW480 colorectal cancer cells (SW480+APC) not only reduces Wnt signaling, but increases membrane E-cadherin and restores cell–cell adhesion. This report describes the effects of full-length, wild-type APC (fl-APC) on cell–cell adhesion genes and p120-catenin isoform switching in SW480 colon cancer cells: fl-APC increased the expression of genes implicated in cell–cell adhesion, whereas the expression of negative regulators of E-cadherin was decreased. Analysis of cell–cell adhesion-related proteins in SW480+APC cells revealed an increase in p120-catenin isoform 3A; similarly, depletion of APC altered the p120-catenin protein isoform profile. Expression of ESRP1 (epithelial splice regulatory protein 1) is increased in SW480+APC cells, and its depletion results in reversion to the p120-catenin isoform 1A phenotype and reduced cell–cell adhesion. The ESRP1 transcript is reduced in primary colorectal cancer, and its expression correlates with the level of APC. Pyrvinium pamoate, which inhibits Wnt signaling, promotes ESRP1 expression. We conclude that re-expression of APC restores the cell–cell adhesion gene and posttranscriptional regulatory programs leading to p120-catenin isoform switching and associated changes in cell–cell adhesion.

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INTRODUCTION

Colorectal cancer (CRC) is a leading cause of cancer-related deaths worldwide (World Health Organization, 2018). More than 80% of CRCs arise from biallelic, truncating mutations in the tumor suppressor gene adenomatous polyposis coli (APC) (Kinzler and Vogelstein,

1996; Clevers and Nusse, 2012). APC is a multifunctional protein implicated in a range of cellular processes including cell migration, differentiation, and cell–cell adhesion and contributes to normal intestinal crypt homeostasis (Nathke *et al.*, 1996; Faux *et al.*, 2004;

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Data and materials availability: The data sets generated and/or analyzed during the current study are available in the Genomics Data repository, <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76307> (King *et al.*, 2016).

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Abbreviations used: APC, adenomatous polyposis coli; CDH1, E-cadherin; CDH3, P-cadherin; CRC, colorectal cancer; CTNND1, catenin delta (p120-catenin); EMT, epithelial-to-mesenchymal transition; ESRP1, epithelial splice regulatory protein 1; ID2, DNA-binding protein inhibitor 2; ZEB1, Zinc Finger E-Box Binding Homeobox 1.

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Sansom *et al.*, 2004; Dow *et al.*, 2015); truncation of APC stimulates increased crypt production (Paulsen *et al.*, 2001). APC is best characterized for its role in the regulation of Wnt/ β -catenin signaling (Clevers and Nusse, 2012). Wnt signaling is essential for normal embryonic development (Nusse and Varmus, 1992; Logan and Nusse, 2004) and stem cell maintenance (Reya and Clevers, 2005). APC mutations result in aberrant Wnt/ β -catenin signaling and increased transcription of Wnt target genes (e.g., *AXIN2* and *myc*) (Peifer and Polakis, 2000; Giles *et al.*, 2003; Huang and He, 2008). SW480 is a CRC cell line where only truncated APC is present. We have shown previously that re-expression of full-length, wild-type APC (fl-APC) in SW480 CRC cells (SW480+APC) reduces Wnt/ β -catenin signaling, increases membrane-associated E-cadherin, and restores cell–cell adhesion (Faux *et al.*, 2004). However, neither the detailed mechanisms controlling APC induction of E-cadherin membrane association nor the mechanisms associated with the role of APC in increasing cell–cell adhesion have been identified.

E-cadherin is a transmembrane protein that mediates calcium-dependent cell–cell adhesion through homophilic interactions with its extracellular domains (Gul *et al.*, 2017). The cytoplasmic tail of E-cadherin interacts with the β - and p120-catenins through binding to their armadillo repeats and indirectly with α -catenin, which connects the complex to the actin cytoskeleton (Ozawa *et al.*, 1989; Takeichi, 2014). β - and p120-catenins are multifunctional proteins with roles in determining the strength of cell–cell adhesion junctions (Nelson, 2008; Pieters *et al.*, 2012) as well as transcriptional signaling (Park *et al.*, 2005; Clevers and Nusse, 2012; Lee *et al.*, 2014). β -catenin is a central component in the canonical Wnt signaling pathway: Wnt stimulation increases β -catenin binding to the Tcf family of transcriptional regulators, consequentially driving Wnt target gene expression (Giles *et al.*, 2003; Huang and He, 2008). p120-catenin is required for tuning the role of E-cadherin activity at cell–cell contacts (Yap *et al.*, 1998; Ireton *et al.*, 2002; Davis *et al.*, 2003). At the cell junctions p120-catenin stabilizes E-cadherin (Davis *et al.*, 2003) and reduces E-cadherin endocytosis by masking the endocytic dileucine motif (Nanes *et al.*, 2012). p120-catenin also modulates RhoGTPase activity through its RhoA-binding sites (Anastasiadis *et al.*, 2000) and activates Rac1 and consequentially cell motility (Noren *et al.*, 2000), as well as participating in gene transcription by interacting with Kaiso (Park *et al.*, 2005), thereby modulating the range of gene targets stimulated by Wnt/ β -catenin.

p120-catenin is crucial for E-cadherin function in cell–cell adhesion, and as such it is essential for normal development (Pieters *et al.*, 2016) and homeostasis in both the intestinal mucosa (Smalley-Freed *et al.*, 2010) and the skin (Perez-Moreno *et al.*, 2006). The p120-catenin protein is present as multiple isoforms (Pieters *et al.*, 2012), which occur as a result of alternative splicing of the p120-catenin (*CTNND1*) gene (Keirsebilck *et al.*, 1998). The p120-catenin isoform 1A is the longest isoform and contains nine armadillo domains in the central part of the molecule and an extended N-terminus (Pieters *et al.*, 2012). p120-catenin isoforms 3A and 4 are missing part and all of the N-terminal region, respectively. The relative levels of p120-catenin 1A (long) and 3A (short) are different depending on the tissue and cell type (Mo and Reynolds, 1996; Ireton *et al.*, 2002). In particular, there is a switch from the short 3A isoform to the longer 1A isoform when epithelial cells transition to mesenchymal cells (Ireton *et al.*, 2002). Accordingly, short and long isoforms are reported to behave differently in human cancers, where the 1A isoform has been linked to increased cell migration and invasion (Seidel *et al.*, 2004; Yanagisawa *et al.*, 2008; Slorach *et al.*, 2011; Zhang *et al.*, 2014). The p120-catenin isoform 1A inhibits RhoA activity, thereby promoting cell invasiveness, whereas the shorter 3A

isoform does not (Yanagisawa *et al.*, 2008). The mechanisms by which the splicing of the p120 precursor mRNA have been investigated (Warzecha *et al.*, 2009), but the relationship of the splicing to Wnt/ β -catenin signaling and cell–cell adhesion are not well understood.

Here we demonstrate that restoration of full-length, wild-type APC (fl-APC) results in altered cell–cell adhesion, changes in gene expression profiles for cell adhesion components, and the redistribution of key cell–cell junctional adhesion proteins. p120-catenin mRNA levels are not altered at the transcriptional level, but the levels of the p120-catenin isoforms change because of changes to mRNA splicing. SW480+APC cells predominantly express the 3A short isoform, whereas the parental cells express both 3A (short) and 1A (long) isoforms. p120-catenin isoform switching is stimulated by an epithelial splice regulatory protein (ESRP1), which has increased expression in SW480+APC cells. We show that ESRP1 levels are reduced in primary CRC tissue and that inhibition of Wnt signaling further increases ESRP1 expression and consequentially the E-cadherin function in cell–cell adhesion junctions, suggesting a mechanism by which APC and Wnt signaling regulate the epithelial phenotype.

RESULTS

Altered cell–cell adhesion transcript profiles in SW480 cells with restored APC expression

We have previously shown that expression of full-length APC protein in SW480 CRC cells (SW480+APC) reduces Wnt signaling, increases membrane β -catenin and E-cadherin, and restores cell–cell adhesion (Faux *et al.*, 2004). The gene expression profiles that are altered by expression of wild-type APC have been determined by RNAseq analysis of SW480, SW480 control (empty vector-transfected control), and SW480+APC cells (King *et al.*, 2016). The transcriptome sequencing identified a total of ~25,130 genes, of which 1735 were significantly differentially expressed (King *et al.*, 2016). Among the top differentially expressed genes were Wnt targets such as *AXIN2*, *CCND2*, and *ID2* (DNA-binding protein inhibitor 2) and cell–cell adhesion genes, such as *CDH1*, *CDH3*, and *MMP9* (Supplemental Table S1). Gene set enrichment analysis identified Wnt signaling, cell–cell adhesion, and epithelial-to-mesenchymal transition (EMT) as significant pathways induced by APC (King *et al.*, 2016). Because re-expression of normal APC restored the cell–cell adhesion phenotype (Faux *et al.*, 2004), we focused on changes in gene expression related to cell–cell adhesion. Hierarchical clustering of cell–cell adhesion and EMT gene expression profiles show differential expression in SW480+APC cells in comparison to SW480 and SW480 control cells (Figure 1, A and B). Our transcriptome analysis shows 22 cell–cell adhesion and 24 EMT genes as significantly differentially expressed (genes with an adjusted two-sided *P* value <0.05 and >2-fold change in expression) (Figure 1, A and B) (Supplemental Table S1). The analysis revealed that restoration of normal APC is associated with increased transcript expression of cell–cell adhesion genes, such as *CDH1* (E-cadherin) and *CDH3* (P-cadherin), intercellular adhesion molecule 1 (*ICAM1*), and epithelial cell adhesion molecule (*EPCAM*) as well as cell adhesion tight junction genes *OCLN*, *CLDN 1, 4, 7, and 9* (Figure 1C). In contrast, there was no change in transcript level for *CTNNA1*, *CTNND1*, or tight junction gene *ZO-1*, whereas *CTNNB1* and *ZEB1* (Zinc Finger E-Box Binding Homeobox 1), a negative regulator of E-cadherin expression (Spaderna *et al.*, 2006) were reduced at the transcript level in SW480+APC cells. The fold changes (log₂) for significantly differentially expressed cell–cell adhesion genes are shown in Figure 1D. The reduction in the β -catenin transcripts in SW480+APC cells is consistent with changes in

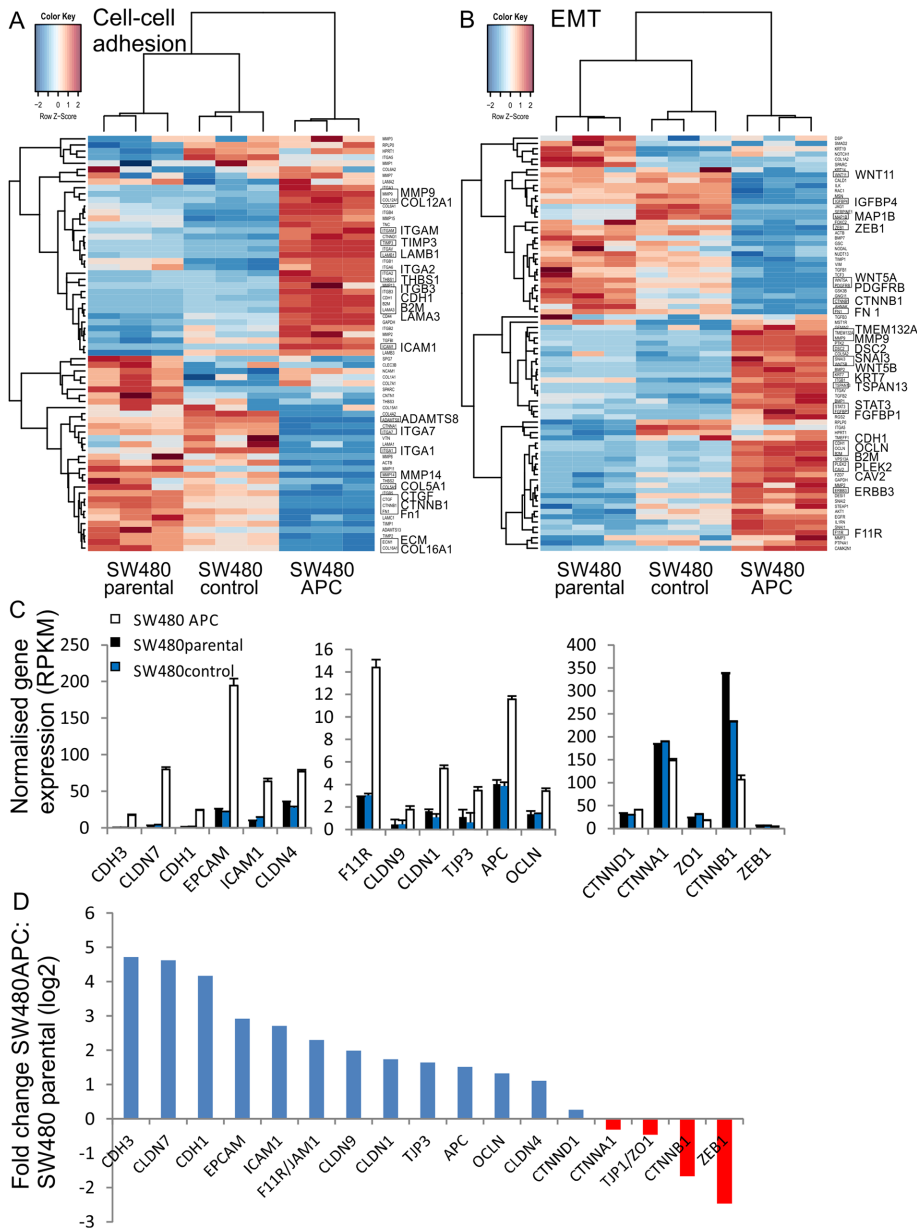


FIGURE 1: Restoration of full-length APC promotes the expression of cell adhesion genes. (A, B) Heatmaps displaying hierarchical clustering for differentially expressed cell adhesion (A) and EMT (B) genes in SW480+APC compared with SW480 and SW480 control. The heatmap was drawn using $\log_2(+1 \text{ offset})$ expression values, mean centered and scaled by gene. Gene and sample dendrograms were generated using divisive hierarchical clustering (DIANA). (C) Selected cell–cell adhesion genes showing differential expression in SW480+APC, SW480 parental, and SW480 control cells expressed as normalized gene expression (RPKM). Mean \pm SD ($n = 3$). (D) Log₂ fold change in cell adhesion genes (from C) in SW480+APC compared with SW480 parental cells. Genes with an adjusted two-sided P value of less than 0.05 and showing a greater than twofold change in expression were considered differentially expressed. *CTNND1*, *CTNNA1*, and *ZO1* are not differentially expressed.

protein levels as reported previously (Faux *et al.*, 2004). EMT is characterized by reduction in *CDH1* and increased expression of cadherins such as *CDH2* or *CDH11* (Pal *et al.*, 2018). While neither *CDH2* or *CDH11* is expressed, *CDH23* expression is up-regulated in SW480 cells and *CDH1* as well as *CDH3* are rescued upon APC expression (Supplemental Table S1). Thus our transcriptome analysis suggests that expression of APC in SW480 cells results in specific gene expression changes and supports a role for APC in promoting cell–cell

adhesion, possibly mediated through regulation of E-cadherin and/or Wnt signaling.

p120-catenin subcellular distribution and isoform levels are altered upon APC expression

Given the changes in transcript for cell–cell adhesion genes, we investigated the subcellular distribution of cell–cell adhesion proteins (Figure 2A). As reported previously, β -catenin and E-cadherin redistribute to sites of cell–cell contact in SW480+APC cells, consistent with the altered morphology of the cells (Faux *et al.*, 2004) (Figure 2A). The tight junction proteins ZO-1 and claudin-7 also demonstrate strong cell–cell contact staining in SW480+APC cells, in striking contrast to the control cells, which demonstrate diffuse cytoplasmic and some nuclear staining. We note that claudin-7 expression (*CLDN7*) is significantly increased at the transcript level in SW480+APC cells, whereas ZO-1 is not altered. p120-catenin (*CTNND1*) also demonstrates a strong redistribution to sites of cell–cell contact (Figure 2A) but as with ZO-1 is not differentially expressed at the transcript level (see Figure 1D).

There are two species of p120-catenin protein in SW480 parental and SW480 control cells, but in SW480+APC cells the faster migrating species predominates (Figure 2B). p120-catenin can be expressed as multiple isoforms due to alternative splicing (Keirsebilck *et al.*, 1998) and four alternative transcriptional start sites. The migration pattern observed in SW480 cells is consistent with p120-catenin isoforms 1A and 3A (Pieters *et al.*, 2012), the most common isoforms. In SW480 and SW480 control cells, isoforms 1A and 3A are equally distributed; however, in SW480+APC cells isoform 3A is increased by >2 -fold ($P < 0.005$) and isoform 1A is barely detectable (Figure 2B). The increased expression of isoform 3 correlates with the increased membrane-associated E-cadherin and the pronounced epithelial morphology of the SW480+APC cells (Faux *et al.*, 2004) (see Figure 2A). Thus, p120-catenin isoform expression is significantly altered upon expression of full-length APC.

To exclude that the aberrant migration of the p120-catenin species on SDS–PAGE was due to differences in phosphorylation, cell extracts were treated with phosphatase (Figure 2C). Phosphatase treatment did not affect the migration of p120-catenin species (Figure 2C, top panel). As a control, these analyses show that the truncated APC protein is phosphorylated in both the parental and SW480+APC cells (Figure 2C). Thus, altered migration of p120-catenin proteins is not due to phosphorylation. p120-catenin 1A and 3A isoform expression was also analyzed with quantitative-PCR using isoform-specific primers

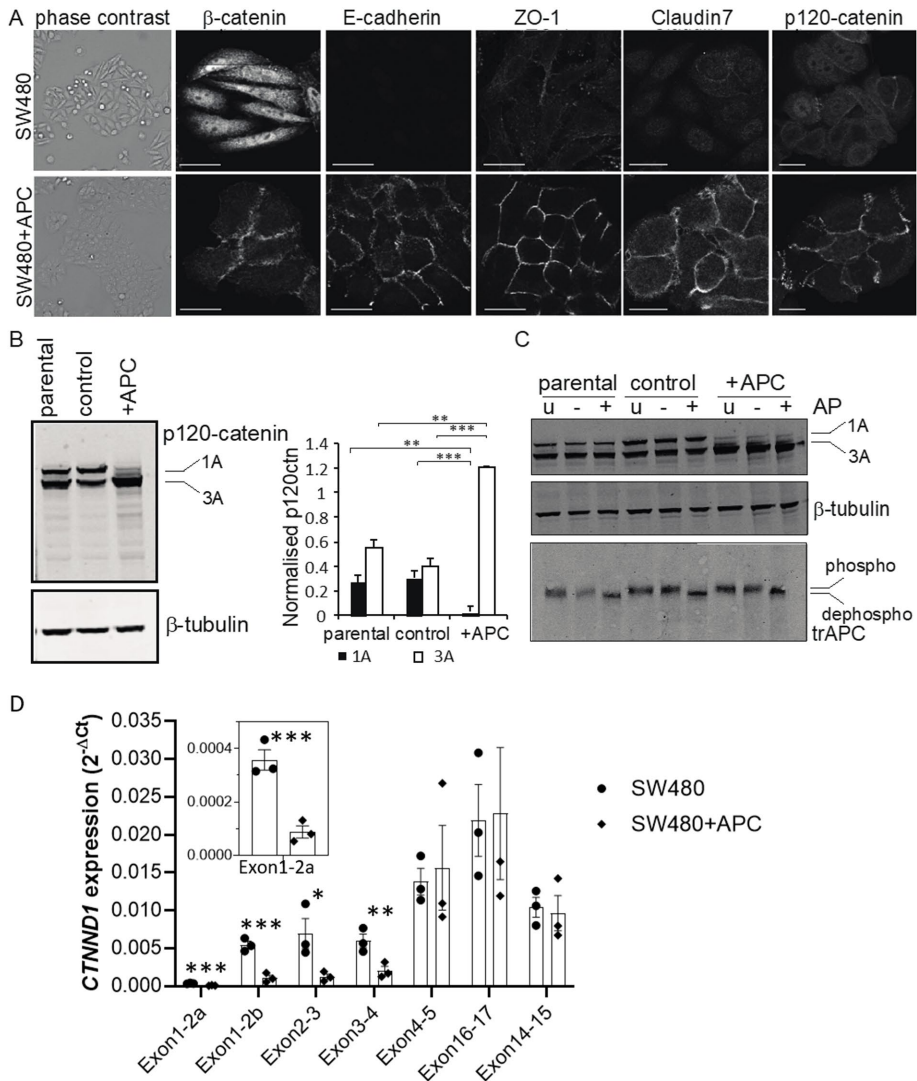


FIGURE 2: Altered p120-catenin localization and isoform expression upon restoration of APC. (A) Cell adhesion proteins are localized to cell–cell contacts in SW480+APC but not SW480 parental cells. Phase contrast images (left-hand side) of SW480 parental and SW480+APC cells. Cells were immunostained with antibodies to β -catenin, E-cadherin, ZO-1, claudin-7, and p120-catenin. Shown are single confocal sections; scale bar 20 μ m. (B) Immunoblot analysis of p120-catenin in SW480, SW480 control, and SW480+APC. p120-catenin migrates as two species in SW480 and SW480 control cells, but the faster migrating form predominates in SW480+APC cells. β -tubulin was used as a loading control. Quantified levels of normalized p120-catenin isoforms are shown on the right. Graph shows mean \pm SEM, $n = 5$; $^{**}P < 0.005$; $^{***}P < 0.001$, unpaired Student’s t test. (C) Altered migration of p120-catenin is not due to phosphorylation. SW480, SW480 control, and SW480+APC cells were untreated (u) or treated without (–) or with (+) Antarctic phosphatase (AP; 50 U) for 30 min at 37°C and proteins resolved by SDS–PAGE. Immunoblot analysis shows that phosphatase treatment did not alter the migration of p120-catenin and β -tubulin but resulted in the collapse of truncated APC to a faster migrating species (dephospho). (D) qRT-PCR analysis of *CTNND1* mRNA expression with isoformspecific primers with extracts from SW480 and SW480+APC cells. SW480 cells express isoforms 1A and 3A, and SW480+APC cells express isoform 3A (mean \pm SEM, $n = 3$; unpaired Student’s t test; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$). Inset shows *CTNND1* expression (exon2a).

(Figure 2D; Supplemental Figure 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 with SW480+APC, that is, 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 *CTNND1* expression with the primers

that detect both 1A and 3A isoforms, that is, those spanning exons 4–5 and 14–15 (Figure 2D). Expression of APC in SW480 CRC cells therefore results in a redistribution of cell–cell adhesion proteins to cell contacts, mirroring altered distributions in β -catenin and E-cadherin and altered p120-catenin isoform distribution. This suggests that relative levels p120-catenin isoforms may affect the adhesive and/or promigratory phenotype of SW480 cells.

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 whether APC regulates p120-catenin isoform levels, we depleted APC using small interfering RNA (siRNA). The truncated APC protein, present in SW480, SW480 control, and SW480+APC cells and full-length APC, present only in SW480+APC cells, is efficiently depleted at 72 h posttransfection (Figure 3A; Supplemental Figure S2A). 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 3, B and 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 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 with mock control-treated cells (Supplemental Figure S2B). 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 and Supplemental Figure S2C). The increase in p120-catenin isoform 1 following depletion of APC demonstrates that full-length APC controls p120-catenin isoform levels in SW480 cells.

ESRP1 controls p120-catenin isoform switching in SW480+APC cells

To gain mechanistic insight into how APC mediates alterations in p120-catenin isoform switching and the generation of the epithelial phenotype, we investigated genes that can induce a p120-catenin isoform switch. EMT genes such as *c-FOS*, *SNAI1*, *SNAI2* (slug),

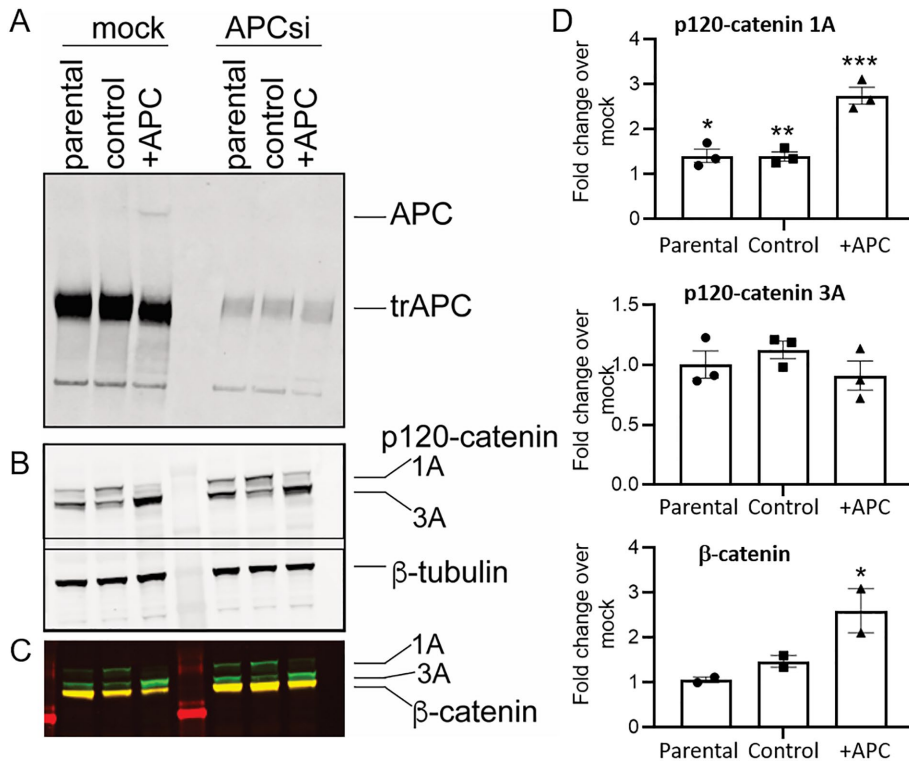


FIGURE 3: p120-catenin isoform expression is dependent on APC. (A) Depletion of APC by siRNA at 72 h. APC levels were assessed by immunoblot analysis. Full-length APC is present in SW480+APC cells only; the truncated APC protein (trAPC) is indicated. (B) siRNA-mediated depletion perturbs p120-catenin isoform expression. SW480 parental, SW480 control, and SW480+APC cells were transfected with mock control and APC siRNA and p120-catenin levels assessed by immunoblot analysis. β -tubulin was used as a loading control. (C) β -catenin is reduced in mock-transfected SW480+APC cells compared with parental and control cells and increased in APC siRNA transfected cells. Immunoblots in B were probed with antibodies to β -catenin (yellow); p120-catenin is visible in green. (D) Quantitation of p120-catenin 1A (top) and 3A (middle) isoforms and β -catenin (bottom) normalized to β -tubulin in APC-depleted cells (72 h); graph shows fold change over mock control transfected cells, mean \pm SEM ($n = 3$) for p120-catenin isoforms and mean \pm SD ($n = 2$) for β -catenin, * $P < 0.05$; ** $P < 0.01$, *** $P < 0.005$, unpaired Student's t test.

SIP1/ZEB2, *TWIST*, and *ZEPO* (Shapiro *et al.*, 2011) have been associated with the p120-catenin isoform switch. Epithelial splice regulatory proteins (ESRP1 and ESRP2) coordinate an epithelial cell type-specific splicing program and have been shown to regulate splicing of several genes including p120-catenin (Warzecha *et al.*, 2009). Indeed, we found that both *ESRP1* and *ESRP2* were significantly increased at the transcript level in SW480+APC cells (Figure 4A) (log₂-fold change in SW480+APC compared with SW480 parental and SW480 control was 6.00 and 4.734 for *ESRP1* and 1.3 and 1.178 for *ESRP2*, respectively). Moreover, siRNA-mediated depletion of APC resulted in a marked reduction in *ESRP1* mRNA in SW480+APC cells, whereas Wnt target genes *AXIN2* and *ID2* mRNA levels increased, as expected (Figure 4B). In concordance with mRNA levels, *ESRP1* and *ESRP2* protein levels were also markedly increased in SW480+APC compared with SW480 parental cells (Figure 4C). Depletion of *ESRP1* resulted in reduced *ESRP1* protein and mRNA in SW480+APC cells (Figure 4, C and D). *ESRP2* siRNA treatment resulted in reduced *ESRP2* protein (Figure 4C). We found that *ESRP1* was also reduced upon *ESRP2*-mediated siRNA treatment but to a lesser extent than upon *ESRP1*-siRNA, and likewise, *ESRP2* was reduced when *ESRP1* was depleted (Figure 4C). Depletion of *ESRP1* did not reverse the shift from 1A to 3A isoforms but did result in a greater than fourfold

increase in the p120-catenin isoform 1 in SW480+APC cells (Figure 4, E and F). p120-catenin isoform 1 was also increased when *ESRP2* was depleted (Figure 4, E and 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 an increase similar to that of the knockdown of *ESRP1* alone (Supplemental 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 (Supplemental 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 Figures 2A and 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 toward 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.

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 *ESRP2*, 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 DLD1 and Caco2 cells express a higher level of the epithelial 3A isoform compared with 1A (Figure 5A). These cells contain mutated APC but

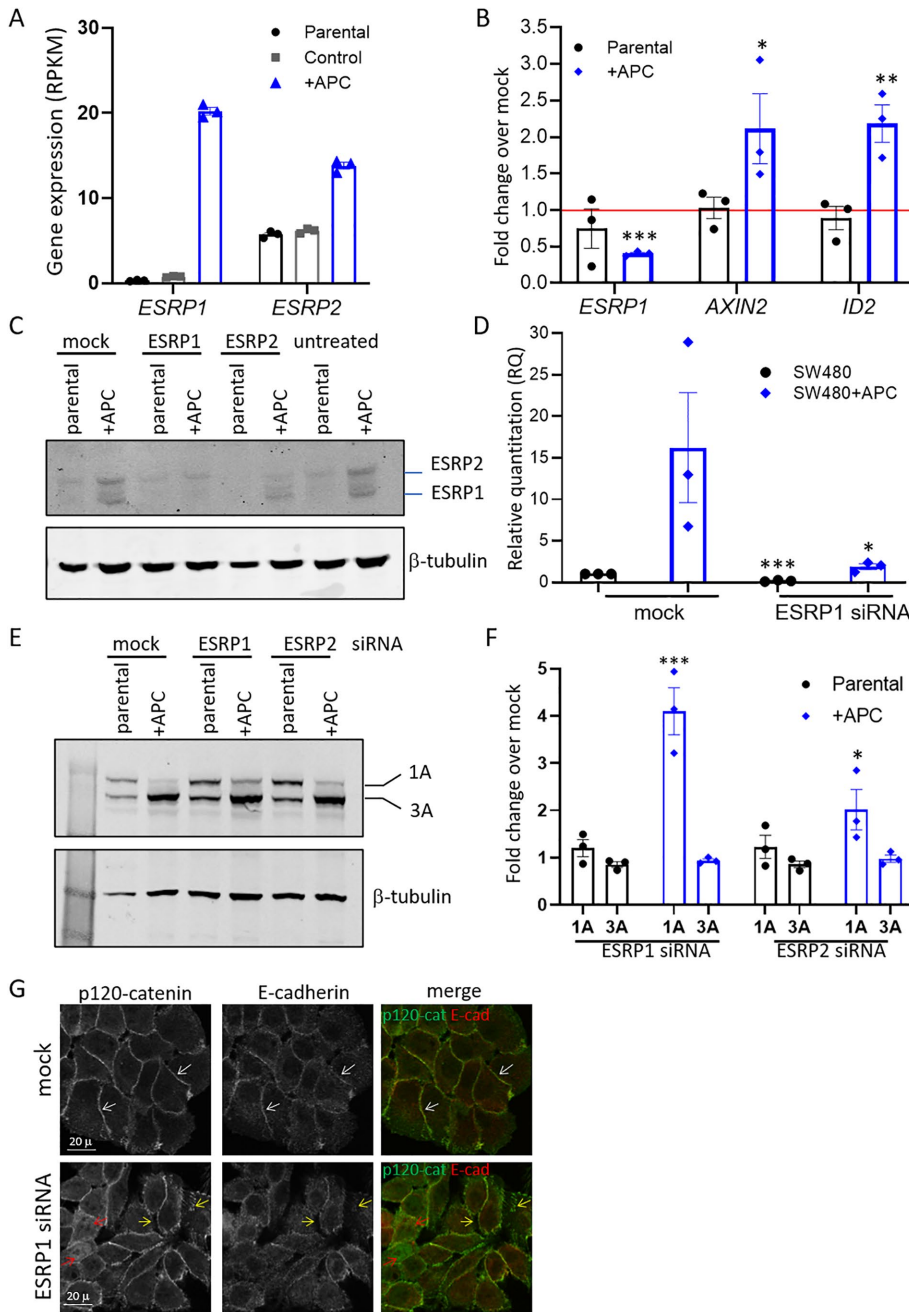


FIGURE 4: ESRP1 is regulated by APC and promotes p120-catenin isoform switching in SW480+APC cells. (A) Increased expression of *ESRP1* and *ESRP2* transcript in SW480+APC cells from RNAseq analysis of SW480 parental, SW480 control, and SW480+APC (APC+) cells. Graph shows mean \pm SD gene expression (RPKM) ($n = 3$). (B) siRNA-mediated depletion of APC results in reduced *ESRP1* mRNA levels and increased Wnt target gene expression. qRT-PCR analysis of *ESRP1*, *AXIN2*, and *ID2* mRNA in APC siRNA transfected SW480 parental and SW480+APC cells. Data are expressed as fold change over mock transfected cells, mean \pm SEM from three independent experiments, * $P < 0.05$, ** $P < 0.001$, *** $P < 0.005$, unpaired Student's t test. (C) Increased *ESRP1* and *ESRP2* in SW480+APC cells is reduced following siRNA-mediated depletion of *ESRP1* and *ESRP2*, respectively. Immunoblot analysis of *ESRP1/2*. β -tubulin was used as a loading control. Shown is representative of three independent experiments. (D) siRNA-mediated depletion of *ESRP1*. SW480 parental and SW480+APC cells were transfected with siRNAs targeting *ESRP1*. Graph shows quantitation of *ESRP1* mRNA assessed by qRT-PCR for mean \pm SEM, $n = 3$. * $P < 0.05$, *** $P < 0.005$, unpaired Student's t test. (E) Depletion of *ESRP1* by siRNA results in increased p120-catenin isoform 1A in SW480+APC cells. Immunoblot analysis of p120-catenin. β -tubulin was used as a loading control. (F) Quantitation of p120-catenin isoforms 1A and 3A normalized to β -tubulin in *ESRP1* and *ESRP2*-depleted cells expressed as fold change over mock-transfected cells. Shown is mean \pm

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 (Warzecha *et al.*, 2009, 2010; Baum and Georgiou, 2011). To assess whether the morphology of the cells was indicative of the isoform distribution, we analyzed 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 (Buck *et al.*, 2007; Warzecha *et al.*, 2009; Ayinde *et al.*, 2017), show a similar distribution of p120-catenin isoforms to SW480 cells and also express low levels of E-cadherin (Figure 5A) and *ESRP* (Warzecha *et al.*, 2009, 2010; Wang *et al.*, 2017). The distribution of p120-catenin isoforms therefore correlates well with E-cadherin expression and underscores the importance of *ESRP* and epithelial isoform expression in governing the epithelial phenotype.

***ESRP1* and *ESRP2* are reduced in primary CRC**

We next explored whether the alterations in *ESRP1* and *ESRP2* observed in SW480 cells are representative of APC loss of heterozygosity (LOH) in CRCs. We interrogated RNA transcript data from 87 primary CRCs and 48 normal colon tissues (Wang *et al.*, 2017) for expression of *ESRP1*, *ESRP2*, and *APC* (Figure 5, B–D). *ESRP1* transcript levels were significantly higher in normal colon tissue in comparison to CRC tumor samples (Figure 5B), consistent with our findings in SW480+APC rescue cells compared with SW480 CRC cells. Similarly, *ESRP2* showed a robust increase in normal tissue compared with CRC tumor tissue, albeit with lower overall expression (Figure 5C). As most CRCs (>70%) have truncating mutations in

SEM, $n = 3$, * $P < 0.05$, *** $P < 0.005$, Student's t test. (G) Depletion of *ESRP1* by siRNA results in reduced cell-cell contact staining in SW480+APC cells. Mock or *ESRP1* siRNA-treated cells were stained with antibodies to p120-catenin and E-cadherin. Shown are confocal sections of the fluorescence images for the p120-catenin (p120-cat) green and E-cadherin (Ecad) red, in merged image. Cell-cell junctions (white arrows), disrupted junctional staining (yellow arrows), and p120-catenin nuclear staining (red arrows) are indicated. Scale bars 20 μ m.

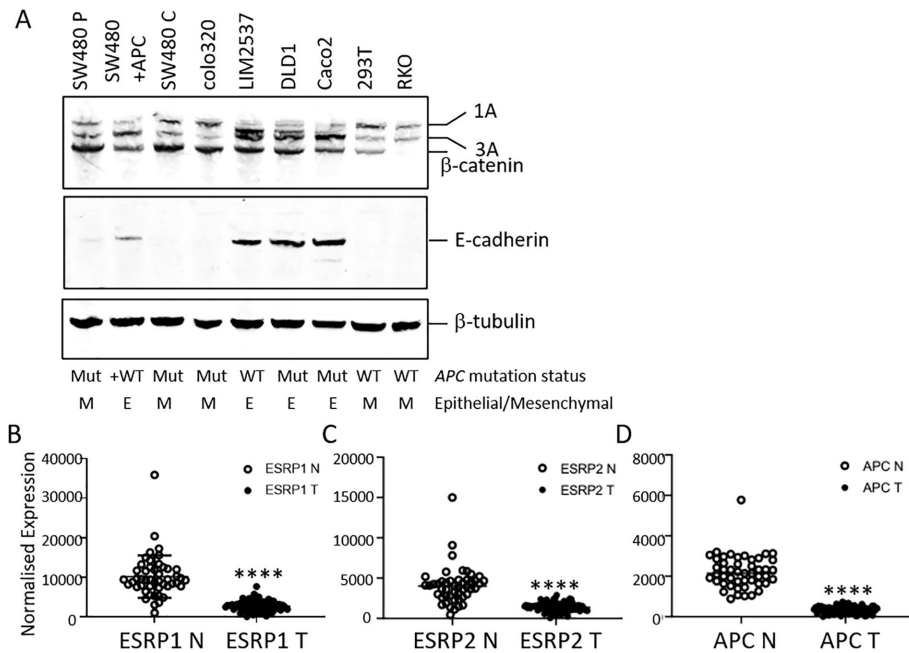


FIGURE 5: p120-catenin isoform distribution and ESRP1 expression in CRC. (A) p120-catenin isoform distribution in a panel of cell lines. Blots were probed with antibodies to p120-catenin, β -catenin, E-cadherin, and β -tubulin. The APC mutation status and epithelial/mesenchymal phenotype of each cell line are indicated. (B–D) *ESRP1* (B), *ESRP2* (C), and *APC* (D) transcript levels in normal colon (N) and CRC primary tumors (T). N $n = 48$, T $n = 87$; **** $P < 0.0001$, unpaired t test with Welch's correlation.

the *APC* gene and frequently demonstrate LOH or nonsense-mediated decay, *APC* transcript levels were significantly reduced in CRC tumor tissue in comparison to normal colon (Figure 5D). Moreover, we found significant correlation between *ESRP1* and *APC* transcript levels in normal colon (Pearson $r = 0.6943$, $P < 0.0001$) and in primary CRC tissue (Pearson $r = 0.4752$, $P < 0.0001$). While the correlation between *ESRP2* and *APC* was significant in normal colon (Pearson $r = 0.5649$, $P < 0.0001$), there was no correlation with *ESRP2* and *APC* in primary CRC samples (Pearson $r = 0.111$, $P = 0.3060$). This may be reflected in the larger changes in *ESRP1* compared with *ESRP2* in SW480/SW480+APC cells. The reduced *ESRP1* transcript in primary CRC and correlation with *APC* indicate a role for *APC* in regulation of *ESRP1* and subsequent epithelial gene splicing events.

Wnt signaling inhibition promotes ESRP1 expression

To investigate the Wnt regulatory role, SW480 and SW480+APC cells were treated with the Wnt signaling inhibitor pyrvinium

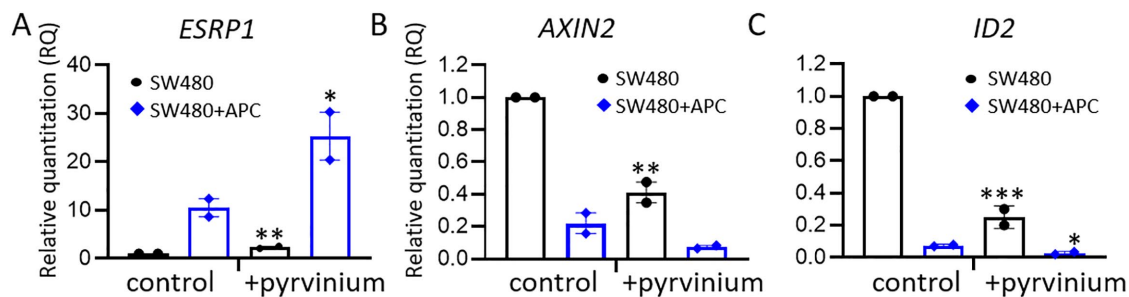


FIGURE 6: Inhibition of Wnt signaling promotes *ESRP1* expression. SW480 parental and SW480+APC cells were treated with either vehicle control or Wnt inhibitor pyrvinium pamoate (1 μ M, 18 h) and mRNA levels (RQ) assessed by qRT-PCR for *ESRP1* (A), *AXIN2* (B), and *ID2* (C). Graphs show mean \pm SEM, $n = 2$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, unpaired Student's t test.

pamoate (Thorne *et al.*, 2010) and the effect on the *ESRP1* transcript level assessed. Wnt inhibition stimulated *ESRP1* expression in both SW480 and SW480+APC cells (Figure 6A) with concomitant reductions in the expression of Wnt target genes, for example, *AXIN2* and *ID2* (Figure 6, B and C), but there was only a modest effect on the p120-catenin isoform distribution (Supplemental Figure S5). These results suggest that APC-mediated negative regulation of Wnt signaling promotes the expression of *ESRP1* and consequentially p120-catenin switching to isotype 3A, which is associated with increased cell–cell adhesion and the epithelial phenotype (Figure 7).

DISCUSSION

We have identified that APC is a modulator of cell–cell adhesion through altered expression of cell–cell adhesion genes and the master epithelial splice regulator *ESRP1*. We had previously shown that expression of full-length APC in SW480 cells eradicates tumor growth and re-establishes cadherin-mediated cell–cell adhesion (Faux *et al.*, 2004). Here we show a novel role for APC in promoting *ESRP1* expression, which in turn modifies the splicing of p120-catenin.

Expression of the epithelial p120-catenin isoform promotes cell–cell adhesiveness by stabilization of E-cadherin, allowing reactivation of E-cadherin function (Ireton *et al.*, 2002; Davis *et al.*, 2003). We propose that *ESRP1* gene expression is regulated by APC and that the lack of *ESRP1*-mediated splicing of p120-catenin reduces cell–cell adhesion in CRC.

In this report we show that in addition to regulating Wnt signaling genes (King *et al.* 2016), APC regulates the expression of EMT-associated genes. These alterations could either be a consequence of Wnt signaling (Brembeck *et al.*, 2006) or a direct effect of APC on cytoskeletal-dependent processes (McCartney and Nathke, 2008; Nelson and Nathke, 2013). The APC-induced changes in cell–cell adhesion may not be a direct outcome of effects of Wnt signaling. A Wnt-independent function is suggested by the increase in p120-catenin 1A following APC depletion even in cells containing only truncated APC. The master splicing regulator, *ESRP1*, has not previously been reported as a Wnt target gene (Nusse and Clevers, 2017) and yet is among the most abundantly increased genes when

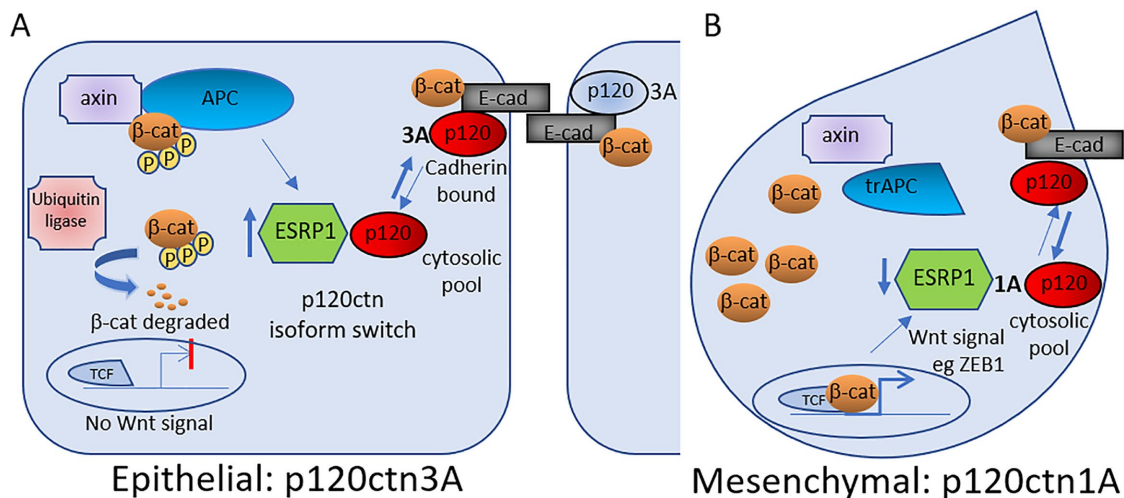


FIGURE 7: Model for APC regulation of p120-catenin isoform splicing and cell–cell adhesion. Intact APC inhibits Wnt/β-catenin signaling and can promote ESRP1, which controls p120-catenin isoform splicing and contributes to maintaining cell–cell adhesion and an epithelial phenotype (A). Truncated APC leads to increased Wnt/β-catenin signaling and reduced ESRP1 and isoform switching and a mesenchymal cell type (B).

full-length APC is expressed in SW480 cells. It is intriguing that ESRP1 expression is increased further by inhibition of Wnt signaling, but this did not translate into detectable changes in p120-catenin isoform switching. ESRP1 regulates splicing of p120-catenin, which functions to stabilize E-cadherin at cell junctions (Ireton *et al.*, 2002; Warzecha *et al.*, 2009). Thus, APC expression effectively reduces Wnt signaling but also has effects on cell–cell adhesion and EMT, underscoring the impact of APC mutation on a range of cellular processes important in tumorigenesis (Hankey *et al.*, 2018).

Despite efficient depletion of ESRP1 (>80%) and reversion to p120-catenin isoform 1A, we detected only modest decreases in isoform 3A and E-cadherin. Similarly, Warzecha *et al.* (2010) did not detect a change in E-cadherin upon ESRP1 knockdown, and ESRP1 overexpression results in an attenuated response (Shapiro *et al.*, 2011; Horiguchi *et al.*, 2012). We cannot rule out the possibility that other genes, such as transcriptional repressor ZEPPO, are involved in repression of E-cadherin and p120-catenin isoform regulation (Slorach *et al.*, 2011). However, the striking increase in ESRP1 expression reported here, along with its function in isoform splicing (Warzecha *et al.*, 2009, 2010; Lamouille *et al.*, 2014; Pradella *et al.*, 2017), point to ESRP1 as a key mechanism for promoting p120-catenin isoform splicing in the SW480+APC cells.

In addition to modulation of ESRP1 by re-expression of APC or APC depletion, we show striking decreases in ESRP1 and 2 in primary CRC and detected that ESRP1 can be increased directly by inhibition of Wnt signaling. ESRP2 expression was also increased in SW480+APC cells but to a lesser extent than ESRP1; the depletion of ESRP2 resulted in only a modest increase in the level of the p120-catenin isoform 1A, and the combination of ESRP1 and ESRP2 siRNA did not result in any further increase. Significantly, increased expression of ESRP1 and ESRP2 has been shown to correlate with epithelial splice signatures and favorable CRC outcomes (Deloria *et al.*, 2016; Mager *et al.*, 2017). ESRP1 expression is reduced in a number of other cancers, including head and neck (Ishii *et al.*, 2014), breast (Fici *et al.*, 2017), ovarian (Jeong *et al.*, 2017), pancreas (Ueda *et al.*, 2014), and lung (Walser *et al.*, 2018), in which it is recognized as a favorable prognostic factor for patients with metastatic disease. The transcriptional repressors SNAI and ZEB1 as well as the miR200 family have been implicated in regulation of ESRP expression

(Haraguchi *et al.*, 2016; Larsen *et al.*, 2016; Walser *et al.*, 2018). Indeed the high levels of ESRP1 with correspondingly low ZEB1 in SW480+APC cells are consistent with direct regulation of ESRP1 by ZEB1; however, knockdown of ZEB1 resulted in an increased level of ESRP2 but not ESRP1. Wnt inhibition did modulate ESRP1 expression further, indicating that ESRP1 can be controlled by a Wnt-ZEB1-axis. Our findings implicate APC and Wnt signaling in EMT processes through alterations in transcription as well as isoform splicing of cell–cell adhesion genes.

In conclusion, the restoration of full-length APC in SW480 colon cancer cells that express only truncated APC returns the cells to an epithelial phenotype with functional cell–cell adhesion junctions. In addition to the well-characterized changes in β-catenin and Wnt signaling targets, we report significant changes in cell–cell adhesion and other regulatory signatures. In particular, we identified ESRP1 as a key gene involved in the regulation of p120-catenin isoform splicing. These data imply that the alteration in p120-catenin splicing, upon re-expression of full-length APC, contributes to E-cadherin-mediated cell–cell adhesion. While APC's role as a negative regulator of Wnt signaling is important, the impact of APC on Wnt-independent pathways such as cell–cell adhesion signaling needs to be considered. The switch from an epithelial to a mesenchymal-type tumor cell can involve significant changes in many signaling and regulatory pathways, including the levels of ESRP1 and ESRP2 (Warzecha *et al.*, 2009). ESRP1 is not usually considered a Wnt target gene (Cong *et al.*, 2004) and does not appear to be influenced directly by changes in cell–cell signaling. Presumably, complex responses to changes in kinase activity (e.g., CK1 and GSK3) and location will have Wnt-independent effects on cell morphology, metabolism, and motility. The levels of cytoplasmic β-catenin and the interactions of β-catenin with proteins in the nucleus, cytoplasm, and cell membrane change with tyrosine kinase activation and cell–cell adhesion. The consequential effects of the APC-induced p120-catenin isoform switch on E-cadherin turnover and cell–cell adhesion may be separate from Wnt-driven events but significant contributors to the suppression of the tumor phenotype.

MATERIALS AND METHODS

Request a protocol through Bio-protocol.

Antibodies

The following antibodies were used: anti-E-cadherin (HECD1 #ab1416; Abcam; 24E10 #3195; Cell Signaling; #610182; BD Transduction Laboratories), anti-claudin-7 (#349100; Invitrogen), anti-p120-catenin (#610134; BD Transduction Laboratories), anti-ZO1 (#61096; BD Transduction Laboratories), anti- β -tubulin (AA2 #T8328; Sigma Aldrich), anti-APC (H290; Santa Cruz), anti- β -catenin (#610153; BD Transduction Laboratories), and anti-ESRP1/2 (Rockland). Antibodies were used at 1:1000 for immunoblot analysis and 1:200 for immunostaining, unless otherwise stated. Secondary antibodies for immunoblots were Alexa488 goat anti-mouse/rabbit (#A-11001 and #A-11035; Thermo Scientific) and Alexa546 goat anti-mouse/rabbit (#A-11030 and #A-11035; Thermo Scientific), used at 1:10,000.

Cell culture conditions

SW480 cells (from the American Type Culture Collection, SW480 control, and SW480+APC were authenticated (King *et al.*, 2016) and were cultured in RPMI supplemented with 1.08% thioglycerol, 50 mg/ml hydrocortisone, 100 U/ml insulin, and 10% fetal calf serum (FCS) plus 1.5 mg/ml G418 for the control and +APC cells. Colo320, HEK293, RKO, DLD1, Caco2, and LIM2537 cells were cultured in DME supplemented with 10% FCS. Cells were tested for mycoplasma every 4–6 mo. All cell lines were authenticated by STR (short tandem repeat) profiling analysis at the Australian Genome Research Facility (AGRF) (Parkville, VIC, Australia) using the GenePrint 10 System (Promega) (Wang *et al.*, 2017).

RNAseq analysis

RNA from SW480, SW480+APC, and SW480 control cells were sequenced and analyzed as described (King *et al.*, 2016). Differential gene expression analysis for cell adhesion and EMT-specific genes was conducted using read counts with the Bioconductor edgeR package as described (King *et al.*, 2016).

Cell based assays

Cells were cultured and transfected with siRNA using Lipofectamine 2000 (Thermo Fisher). The following siRNA were used: APC (ON-TARGETplus L-03869), ESRP1 (ON-TARGETplus L-20672-01), ESRP2 (ON-TARGETplus L-014523), and control ON-TARGETplus nontargeting pool D-001810-10) (all from Dharmacon). Cells were harvested for immunoblot analysis or immunostained for confocal microscopy acquired with an Olympus confocal microscope. Cells were counterstained with fluorescently labeled 4',6-diamidino-2-phenylindole (DAPI) (#10236276001; Roche Diagnostics). For immunoblot analysis, the levels of protein were quantified using densitometry normalized to β -tubulin.

Quantitative real-time PCR analysis

mRNA was extracted and purified (Illustra RNAspin Mini Kit #25-0500-70; GE LifeSciences) as per the manufacturer's instructions. Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (#4368814 AB; Applied Biosystems). Real-time PCR amplification was achieved using PowerSYBR Green PCR Master Mix (#4367659; Applied Biosystems) and Taqman Fast Advanced Master Mix (ThermoFisherScientific Applied Biosystems; 4444557) reagents. The SYBR green primers were as follows: AXIN2: (#QT00037639; Qiagen); Primer sequences used: GAPDH FWD: CAATGACCCCTTCATTGACC, REV: TGATGACAAGCTTCCCGTTTC; ESRP1 FWD: TGCTGTTCTGGAAAGTCGTG, REV: CTTTG-CAGTCCTCCGTCAGT; ID2 FWD: TCAGCCTGCATCACCAGAGA, REV: CTGCAAGGACAGGATGCTGATA. The Taqman probes were

CTNND1 (HS00931672_m1; HS00931681_m1; HS00931673_m1; HS00609741_m1; HS00609742_m1; HS00931670_m1; HS00931671_m1), and GAPDH (HS99999905_m1). SYBR green samples were analyzed on a 7300 Real-Time PCR system (Applied Biosystems) using SDS software version 4.0 (Applied Biosystems). The Taqman samples were run on a Viia7 Real-Time PCR System (ThermoFisherScientific). Target gene expression is expressed relative to the housekeeping gene expression (GAPDH) and is presented in arbitrary units ($2^{-\Delta C_t}$).

Statistical analysis

Statistical analyses were performed using a Student's *t* test, unless otherwise described. Data presented graphically are the means \pm SEM for three independent experiments unless otherwise stated.

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