Published in final edited form as:

Cancer Res. 2010 October 15; 70(20): 8149–8158. doi:10.1158/0008-5472.CAN-10-0315.

Cyclin D2-CDK4/6 is required for efficient proliferation and tumorigenesis following Apc loss

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Abstract

Inactivation of the *Apc* gene is recognized as the key early event in the development of sporadic colorectal cancer where its loss leads to constitutive activation of β -catenin/TCF4 signaling and hence transcription of Wnt target genes such as c-Myc. Our and others previous studies have shown that although Cyclin D1 is required for adenoma formation, it is not immediately upregulated following Apc loss within the intestine, suggesting proliferation following acute Apc loss may be dependent on another D-type Cyclin.

In this study we investigated the expression and functional relevance of Cyclin D2 following Apc loss in the intestinal epithelium. Cyclin D2 is upregulated immediately following Apc loss, which corresponded with a significant increase in CDK4 and hyper-phosphorylated Rb levels. Deficiency of Cyclin D2 resulted in a reduction in enterocyte proliferation and crypt size within Apc deficient intestinal epithelium. Moreover Cyclin D2 dramatically reduced tumor growth and development in *Apc*^{Min/+} mice. Importantly Cyclin D2 knockout did not affect proliferation of normal enterocytes and furthermore CDK4/6 inhibition also suppressed the proliferation of adenomatous cells and not normal cells from *Apc*^{Min/+} mice.

Taken together, these results indicate that Cyclin D-CDK4/6 complexes are required for the efficient proliferation of cells with deregulated Wnt signaling and inhibiting this complex may be an effective chemopreventative strategy in colorectal cancer.

Introduction

The *Apc* gene encodes the Adenomatous Polyposis Coli tumor suppressor protein, the germ line mutation of which characterizes Familial Adenomatous Polyposis (FAP), an autosomal syndrome characterized by multiple colorectal adenoma (1). Inactivation of the *Apc* gene is recognized as a key early event in the development of sporadic colorectal cancers (CRC) with up to 80 percent of CRC having mutations in the Apc gene. The major tumor suppressor function of Apc is thought to be as a negative regulator of Wnt signaling, where it forms part of the β -catenin destruction complex, comprising Axin, GSK3 β and CK1. Mutations in Apc lead to the stabilization of β -catenin and consequently to the deregulation of the Wnt pathway through the activation of TCF/LEF target genes (2, 3).

Previous studies have shown that acute loss of the Apc gene within the murine intestinal epithelium *in vivo* leads to a 'crypt progenitor cell-like' phenotype (4, 5), which is characterized by an increase in proliferation of intestinal enterocytes with reduced intestinal differentiation and migration. Coincident with the onset of this 'crypt progenitor cell-like phenotype' is the accumulation of nuclear β -catenin and the transcription of Wnt target genes such as c-Myc and CD44. The upregulation of c-Myc following Apc loss is critical to all the phenotypes observed, with double Apc c-Myc knockout intestines showing proliferation at equivalent levels to wild type intestines (6).

Previous studies have shown that CyclinD/CDK4/6 complexes may be essential downstream mediators of c-Myc dependent proliferation (7, 8). Consistent with this, depending on cellular context CDK4, Cyclin D1 and Cyclin D2 have all been proposed to be transcriptional targets of c-Myc (9). Therefore these studies predict that Apc deficient cells may be dependent on high levels of Cyclin D/CDK4-6 complexes. Pertinently, Cyclin D1 has been proposed to be a canonical Wnt target gene (10, 11) and thus would provide a ready mechanism to elevate proliferation following Apc loss. However we and others have shown that Cyclin D1 is not upregulated immediately following Apc loss and genetic deletion of Cyclin D1 makes no impact on the immediate phenotype (including levels of proliferation). Cyclin D1 is however upregulated at later stages following Apc loss, required for efficient adenoma formation (12) and acts as a modifier of disease severity in the *Apc^{Min/+}* mouse (13, 14).

Cyclin D2 is normally expressed at the base of the intestinal crypt, where there are the highest levels of Wnt signaling (15). Studies have shown that Cyclin D2 is overexpressed in 53% of colon tumors and that over expression of Cyclin D2 may be related to a higher TMN stage of tumor (16). Mechanistically it has been suggested that Cyclin D2 is a direct c-Myc target gene (7) implying that Cyclin D2 levels should be deregulated following *Apc* mutation. Here, we show that Cyclin D2 is rapidly deregulated following Apc loss where it plays a functional role to promote proliferation and tumorigenesis.

Material and Methods

Mouse experiments

All experiments were performed under the UK Home Office guidelines. The alleles used for this study were as follows: *c-Myc^{fl}* (17), *AlbCre*, *Apc^{580S}* (18), (4), *Cyclin D2^{-/-}* and Cyclin D1 (19). For precise details of mouse genetic backgrounds for each experiments and cre inductions regime see Supplemental Methods.

CDK4/6 inhibitor treatment

PD0332991 was obtained from Pfizer (La Jolla, CA) and mice were treated with 150mg/kg daily by gavage or vehicle (lactic acid buffer, pH 4.0). This regime was comparable to the previous *in vivo* studies (20).

Assaying apoptosis, mitosis, and crypt size *in vivo*

Apoptosis, crypt size and mitotic index were scored from H&E stained sections as previously described. (4). For each analysis, 25 full crypts were scored from at least 3 mice of each genotype. Apoptosis was independently confirmed by immunohistochemical staining with an antibody against active caspase 3 (1:750, R & D systems).

Tumour scoring

For cohort and timepoint analyses, the entire intestine and colon was removed and flushed with water. Both intestine and colon were mounted 'en face' and fixed overnight in

methacarn. Lesions were then scored macroscopically. Intestines were then wound into a “swiss” roll which were subsequently embedded in paraffin, sectioned at 10 μ M and stained with hematoxylin and eosin prior to microscopic analysis.

Assaying proliferation *in vivo*

In order to examine levels of proliferation, mice were injected with 250 μ l of BrdU (Amersham) 2 hours prior to being sacrificed. Immunohistochemical staining for BrdU was then performed using an anti-BrdU antibody (BD biosciences 1:500). At least 3 mice were used for each genotype and timepoint. For scoring of proliferation within Apc^{Min/+} tumors, mice were injected with BrdU 2 hours prior to euthanasia. 500 cells per adenoma were scored to give an average level of proliferation per adenoma and multiple tumors per mouse were scored until a consistent running mean for each mouse was obtained. At least 3 mice were scored per genotype.

Immunohistochemistry

Primary antibodies used for immunohistochemistry: β -catenin (1:50 Transduction labs), BrdU (1:500 BD Biosciences), Cyclin D1 (1:100 Nova Castra DCS-6), Cyclin D2 (1:200 Santa Cruz SC-593). Immunohistochemistry was performed on formalin fixed intestinal sections.

Epithelial extractions, Quantitive PCR and immunoblotting

In order to obtain a population of cells enriched for epithelial cells, an epithelial extraction protocol based on that of Bjerknes & Cheng (21) was performed (see 22). Quantitive PCR was performed as previously described upon epithelial extractions (6). At least 3 different epithelial extractions were used per genotype.

The primers used for this study were as follows:

Cyclin D2:

F- CTACCGACTTCAAGTTTGCC

R- GCTTTGAGACAATCCACATCAG

CDK4:

F- AATGTTGTACGGCTGATGGA

R- AGAAACTGACGCATTAGATCCT

Opticon Monitor analysis software (Version 2.03, MJ Research) to calibrate and run the reaction.

For immunoblotting, primary antibodies and conditions used to probe blots were anti-Cyclin D2 (1:1000; MS214, M-20 and SC-593 1:1000), pRB (Cell Signaling 9803, 1:1000), Cdk4 (sc-260, 1:200) and mouse anti- β -actin (1:5000; Sigma). Appropriate HRP-conjugated secondary anti-rabbit or anti-mouse antibodies were used (Amersham Biosciences). Western analysis was performed using standard protocols (see 22).

Results

Deletion of Apc leads to an upregulation of Cyclin D2 and CDK4

First, we wished to assess whether Cyclin D2 was upregulated following Apc loss *in vivo*. We have previously deleted *Apc* in the intestinal epithelium using Cre-lox technology. Here mice carrying an inducible knockout *Apc* allele *Apc*^{580S} (from here on referred to as *Apc*^{d1})

are intercrossed to mice carrying the *AhCre* transgene which yields near constitutive inducible Cre recombinase expression within the intestinal epithelium following exposure to β -naphthoflavone (18). Four days following Cre induction these mice develop a robust phenotype, crypts become hyperplastic and β -catenin accumulates within the nucleus of cells. Our previous microarray analysis suggested that Cyclin D2 is immediately upregulated following Apc loss *in vivo* (4). To confirm this we performed *in situ* hybridization (ISH) on wild type and Apc deficient intestine 4 days following Apc gene deletion (4 mice of each genotype). A clear upregulation of Cyclin D2 mRNA was observed suggesting that Cyclin D2 is transcriptionally activated (Figure 1A). To investigate protein levels we performed immunohistochemistry (IHC) and immunoblotting (IB) for Cyclin D2 and found it to be clearly deregulated (Figure 1B and 1C). β -catenin was localized to the nucleus of all Apc deficient cells (Supplementary Figure 1A-B) suggesting that every cell with high β -catenin had Cyclin D2 expression (Supplementary Figure 1C-D).

Our previous microarray analysis also suggested that CDK4 was upregulated. This was confirmed by QRT-PCR and IB performed on intestinal epithelial extracts, which showed a significant upregulation of CDK4 levels following Apc loss (Figure 1D-1E). To assess if this upregulation of CDK4 led to an increased activity, we assessed whether there was increased levels of phosphorylated (and therefore inhibited) pRB^{S807/811} following Apc loss by IB. Figure 1E showed a marked induction of phosphorylated pRB following Apc loss.

As Cyclin D2 and CDK4 have previously been shown to be transcriptional targets of the key Wnt target genes c-Myc, we investigated whether their overexpression following Apc loss was c-Myc dependent. Analysis by QRT-PCR (from epithelial extractions) showed significant reduction in Cyclin D2 and CDK4 mRNA levels in *AhCre⁺ Apc^{fl/fl} C-Myc^{fl/fl}* compared to *AhCre⁺ Apc^{fl/fl} C-Myc^{+/+}* intestinal enterocytes (Figure 1D) (6). To test whether this led to a functional reduction in CDK4 activity we analyzed phosphorylated pRB levels and found this to be significantly reduced in *AhCre⁺ Apc^{fl/fl} C-Myc^{fl/fl}* compared to *AhCre⁺ Apc^{fl/fl} C-Myc^{+/+}* intestinal enterocytes (Figure 1E). Importantly, neither Cyclin D2 nor CDK4 levels were downregulated by QRT-PCR, in mice where only c-Myc was deleted (*AhCre⁺ Myc^{fl/fl}*) suggesting that the induction of hyperproliferation following Apc loss, but not normal proliferation, may be dependent on the Cyclin D2/CDK4 (Myc v Wt, Cdk4 0.9, Cyclin D2 2.2, n=3).

Cyclin D2 deficiency reduces crypt size and proliferation in Apc deficient intestinal crypts

We next wanted to test the functional importance of Cyclin D2 upregulation. Therefore, we intercrossed *Cyclin D2^{-/-}* mice to *AhCre⁺ Apc^{fl/fl}* mice, and analyzed the intestinal phenotype 4 days following Apc deletion. Although Cyclin D2 deficiency alone had no impact on crypt size in mice (*AhCre⁺ Apc^{+/+} Cyclin D2^{-/-}*) (Figure 2A, Mann-Whitney U test, p=1.00, n=5), Cyclin D2 deficiency significantly reduced the crypt size of *AhCre⁺ Apc^{fl/fl} CyclinD2^{-/-}* mice when compared to *AhCre⁺ Apc^{fl/fl} CyclinD2^{+/+}* mice (Figure 2A, 2C, Mann-Whitney U test, p=0.01, n=5). To investigate whether this was due to reduced proliferation, we examined BrdU incorporation and found that *AhCre⁺ Apc^{fl/fl} CyclinD2^{-/-}* mice had significantly reduced proliferation compared to *AhCre⁺ Apc^{fl/fl} CyclinD2^{+/+}* mice (Figure 2B, Mann-Whitney U test, p=0.04, n=5). Once again, no impact of Cyclin D2 deficiency alone was observed (Figure 2B, *AhCre⁺ Apc^{+/+} CyclinD2^{-/-}* v *AhCre⁺ Apc^{+/+} CyclinD2^{+/+}*, Mann-Whitney U test, p=0.1914, n=5). No differences were observed in the other phenotypes associated with Apc loss (apoptosis, migration and differentiation, data not shown). Cyclin D2 deficiency did not affect β -catenin accumulation or localization following Apc loss, displaying that Cyclin D2 is downstream of Wnt signaling (Figure 2D).

Cyclin D2 deficiency leads to a dramatic reduction of tumor burden within the *Apc^{Min/+}* mouse

Given the reduced crypt size and proliferation observed in *AhCre⁺ Apc^{fl/fl} CyclinD2^{-/-}* mice, we next wished to assess if this was functionally relevant for tumor formation. First, we examined whether Cyclin D2 was overexpressed in the *Apc^{Min/+}* adenomas and found this to be the case (Figure 3A). To investigate the importance of Cyclin D2 for tumor formation and survival, we crossed *Cyclin D2^{-/-}* mice to the *Apc^{Min/+}* model of intestinal tumorigenesis. Two studies were performed. First, in order to determine the effect of Cyclin D2 on survival, a cohort containing *Apc^{Min/+} Cyclin D2^{+/+}* mice (n=29), *Apc^{Min/+} Cyclin D2^{+/-}* mice (n=26) and *Apc^{Min/+} Cyclin D2^{-/-}* mice (n=21) were aged until showing signs of intestinal illness. This included paling feet, starry coats and hunching. Figure 3B shows that deletion of Cyclin D2 significantly increased the survival of *Apc^{Min/+} Cyclin D2^{-/-}* mice when compared to *Apc^{Min/+} Cyclin D2^{+/+}* mice (Log rank test, $p < 0.001$). Heterozygous deletion of Cyclin D2 did not significantly affect survival (Log rank test, $p = 0.2516$). At death, tumor burden of *Apc^{Min/+} Cyclin D2^{-/-}* mice (Supplementary Figure 2, Mann-Whitney U test, $p = 0.08$) and average tumor size (Supplementary Figure 2, Mann-Whitney U test, $p = 0.1788$), were similar to those in *Apc^{Min/+} Cyclin D2^{+/+}* mice, confirming that mice were being euthanized at the same stage of disease.

To ensure that the increased lifespan was due to delayed tumorigenesis we aged a second cohort to a timepoint of 110 days (*Apc^{Min/+}* n=30, *Apc^{Min/+} Cyclin D2^{+/-}* n=30, *Apc^{Min/+} Cyclin D2^{-/-}* n=23). *Apc^{Min/+} Cyclin D2^{-/-}* mice displayed a significant reduction in total tumor burden (Figure 3C, Mann-Whitney U test, $p < 0.001$, Bonferroni correction, $p < 0.01$), number and size (Figure 3D-E Mann-Whitney U test, $p < 0.001$ Bonferroni correction, $p < 0.01$). As tumor size and number were reduced, this would suggest that Cyclin D2 deficiency was suppressing both tumor initiation and progression. Heterozygous deletion of Cyclin D2 did not affect total tumor number (Figure 3D, Mann-Whitney U test, $p = 0.2451$) or average tumor size (Figure 3E, Mann-Whitney, $p = 0.1452$).

We next investigated levels of β -catenin and proliferation within adenomas from *Apc^{Min/+}* and *Apc^{Min/+} Cyclin D2^{-/-}* mice at a timepoint of 110 days. Figure 4A (and Supplementary Figure 3) shows strong nuclear staining of β -catenin within the polyps of *Apc^{Min/+}* and *Apc^{Min/+} Cyclin D2^{-/-}* mice. Immunohistochemical staining for Cyclin D2 shows a complete ablation of protein from both the colonic epithelium and polyps from *Apc^{Min/+} Cyclin D2^{-/-}* mice (Figure 4B). Importantly, polyps from *Apc^{Min/+} Cyclin D2^{-/-}* mice were significantly less proliferative than *Apc^{Min/+}* mice as scored through total number of BrdU positive cells per 500 tumor cells (Figure 4C-D, Mann-Whitney U test, $p = 0.04$, n=3 mouse per genotype, at least 5 tumors per mouse).

CDK4/6 inhibition suppresses proliferation within established *Apc^{Min/+}* mouse adenomas

Given recent studies showing that Cyclin D proteins can affect processes outwith cell cycle control (22), it remains theoretically possible that Cyclin D2 was affecting the proliferation of cells via a CDK independent mechanism. Therefore we wanted to assess whether inhibition of CDK4/6 could specifically reduce the proliferation of *Apc* deficient cells *in vivo*. To achieve this, we used a novel small molecule pharmacological inhibitor of CDK4/6, PD0332991, which has been shown to selectively inhibit CDK4/6 *in vivo* (20). The use of an inhibitor also allowed us to treat established tumors. Thus we identified *Apc^{Min/+}* mice that had developed a tumor burden (at approximately 80 days) and treated these mice with a daily gavage of 150mg/kg of inhibitor for 5 days. At the end of the treatment regime, mice were injected with BrdU and intestines harvested two hours later. Analysis of BrdU incorporation showed a strong reduction in the levels of proliferation in the PD0332991 treated tumors compared to vehicle treated tumors (Figure 5A-C). Indeed

adenoma proliferation was inhibited to comparable levels that are observed in Cyclin D2 knockout tumors. Importantly, no impact on normal intestinal enterocyte proliferation was observed (Figure 5A-C). Hence this suggests that Apc deficient cells are dependent on Cyclin D-CDK4/6 complexes for efficient proliferation.

Combination of Cyclin D2 deficiency with Cyclin D1 haploinsufficiency further reduces tumor burden

Previous studies have suggested there may be compensation between the different Cyclin D family members. To investigate this, we examined the expression of Cyclin D1 in the Cyclin D2 knockout intestines following Apc loss. IHC staining for Cyclin D1 failed to reveal an upregulation of Cyclin D1 levels in *AhCre⁺ Apc^{fl/fl} CyclinD2^{-/-}* deficient intestinal epithelium, with only a small subset of cells showing high levels of nuclear Cyclin D1 (Supplementary Figure 4). Cyclin D1 expression was also comparable in adenomas from *Apc^{Min/+}* and *Apc^{Min/+} Cyclin D2^{-/-}* mice (Supplementary Figure 4). Mice lacking both Cyclin D1 and D2 die shortly after birth which precludes analysis of double knockout intestines. However, as studies have shown that Cyclin D1 heterozygosity can slow intestinal tumorigenesis in the *Apc^{Min/+}* mouse, this allowed us to assess if suppression of tumorigenesis by Cyclin D1 and D2 was epistatic, additive or cooperative. Therefore we backcrossed Cyclin D1 mice to C57Bl6J mice for 5 generations and then intercrossed these animals with *Apc^{Min/+} Cyclin D2^{-/-}* animals. Cohorts of *Apc^{Min/+} CyclinD2^{-/-} CyclinD1^{+/+}*, *Apc^{Min/+} Cyclin D2^{+/+} Cyclin D1^{+/+}*, *Apc^{Min/+} CyclinD2^{-/-} CyclinD1^{+/-}*, *Apc^{Min/+} CyclinD2^{-/-} CyclinD1^{+/-}* were generated (at least 10 per group) and aged 110 days. In agreement with the findings of Hult et al we saw a significant suppression of tumorigenesis in the *Apc^{Min/+} Cyclin D2^{+/+} Cyclin D1^{+/+}* animals ($p=0.006$, Mann Whitney $n=10$ Bonferroni correction, $p<0.01$) (Figure 6A) (13). Again we saw a significant reduction in tumorigenesis in the *Apc^{Min/+} CyclinD2^{-/-} CyclinD1^{+/+}* animals ($p=0.005$, Mann Whitney $n=10$, Bonferroni correction, $p<0.01$). Importantly *Apc^{Min/+} CyclinD2^{-/-} Cyclin D1^{+/-}* knockout animals showed a further reduction in tumorigenesis when compared to *Apc^{Min/+} CyclinD2^{-/-} CyclinD1^{+/+}* and *Apc^{Min/+} Cyclin D2^{+/+} Cyclin D1^{+/+}* intestines definitively showing that Cyclin D1 and Cyclin D2 are not epistatic (Figure 6A). Instead the data was most consistent with an additive impact of Cyclin D1 haploinsufficiency and Cyclin D2 knockout. Finally to assess whether the reduction of tumorigenesis correlated with reduced proliferation, we examined BrdU labelling within the rare adenomas that formed within *Apc^{Min/+} CyclinD2^{-/-} Cyclin D1^{+/+}* mice and observed a further reduction of proliferation within the tumors of these mice compared to *Apc^{Min/+} CyclinD2^{-/-} CyclinD1^{+/+}* mice (Figure 6B-C). No impact in normal small intestinal proliferation rates was observed (Figure 6D).

Discussion

In this study we have shown that Cyclin D2 is required for efficient proliferation and tumorigenesis following Apc loss. This finding is particularly pertinent to human CRC as previous studies have shown that overexpression of Cyclin D2 has been reported to be the most considerable aberration among G1-phase regulators in human colonic polyps (23). Moreover, a positive relation has also been reported between overexpression of Cyclin D2 and higher TNM stage of tumor, suggesting that overexpression of Cyclin D2 correlates to a high metastatic degree of tumor (16).

There has been some debate over the role of Cyclin D1 as a Wnt target gene in colorectal cancer. Our previous studies have shown that Cyclin D1 is not immediately upregulated following Apc loss in the murine intestinal epithelium and our data shown here suggests that unlike Cyclin D1, Cyclin D2 can reduce the level of proliferation of intestinal enterocytes immediately following Apc loss. However, it has been clearly shown that Cyclin D1 is

upregulated in adenomas of *Apc^{Min/+}* mouse and Cyclin D1 deficiency can suppress tumorigenesis (12-14). Thus our finding that Cyclin D2 loss can also suppress tumorigenesis is important, as there is still expression of Cyclin D1 within these adenomas which cannot completely compensate for the lack of Cyclin D2. This is in contrast to normal intestinal epithelium which shows no defect in proliferation in the absence of Cyclin D2, highlighting that Apc deficient intestinal enterocytes are somewhat dependent on high Cyclin D2 levels. This finding is reinforced by our experiments analyzing the impact of Cyclin D1 heterozygosity in the context of Cyclin D2 genetic knockout. The finding that a further reduction in tumorigenesis was observed suggests that the roles that Cyclin D1 and D2 play in intestinal tumorigenesis are distinct. This data therefore supports the notion, of complementary but distinguishable roles for Cyclin D1 and Cyclin D2 in normal development and cancer. Combined deletion of Cyclin D1 and D2 results in death in the first three weeks of life due to hypoplastic cerebellum (24), suggesting some compensation between D-type Cyclins. Given the dramatic suppression of tumorigenesis in the Cyclin D1 heterozygous, Cyclin D2 knockout mice, it is therefore tempting to speculate that Apc deficient epithelium would be unable to proliferate in the absence of both Cyclin D1 and Cyclin D2.

Apc deletion within the intestinal epithelium, leads to a 'crypt progenitor cell-like phenotype' of hyperproliferation, failed differentiation and failed migration. In this study, we show that reduction of just one of these phenotypes, hyperproliferation can strongly suppress tumorigenesis. This raised the possibility that other strategies such as inhibiting CDK4/6 complexes (which we have shown are upregulated following Apc loss) may also suppress tumorigenesis. Importantly, unlike Cyclin D2 there are small molecule kinase inhibitors to these complexes currently under preclinical development and in Phase 1 trials. In this study we have used Pyridopyrimidine (PD-0332991 from Pfizer), which selectively inhibits CDK4 and CDK6, and is currently in phase 1 clinical trials for mantle cell lymphoma and phase 2-3 clinical trials for multiple myeloma and hormone receptor-positive advanced breast cancer (25, 26). This inhibitor suppressed proliferation within preformed adenomas from *Apc^{Min/+}* mice but had no impact on the normal intestinal epithelium. This therefore suggests a clear therapeutic window for the use of this inhibitor in Apc deficient tumors. Of interest to our study, PD -0332991 was shown to cause regression of tumors in mice with a human CRC xenograft (27). However despite this finding PD-0332991 has not yet begun clinical trial testing for CRC [reviewed in (26)]. One question that remains is whether this selectivity for Apc deficient cells will remain in more advanced tumors where there are numerous other mutations. It will be thus of interest to assess whether PD-0332991 can cause a reduction in proliferation of the more aggressive mouse models of CRC that have recently been developed (28, 29). It will also be interesting to evaluate its effect in combination with conventional chemotherapeutics such as 5 Fluorouracil.

Our previous studies have shown that c-Myc deletion rescues all the phenotypes following Apc loss and thus blocks tumor formation and indeed heterozygosity for c-Myc also strongly suppresses tumorigenesis. Although this makes c-Myc an excellent target, given the difficulty in targeting a transcription factor such as c-Myc, it may be a more efficacious strategy to inhibit in combination a number of pathways downstream of Apc loss where drugs are already available. It is interesting to note in this study that expression of Cyclin D2 and CDK4 following Apc loss were c-Myc dependent, illustrating that the upregulation of these Cyclin D2/CDK4 complexes is key for c-Myc dependent proliferation following Apc loss. This raises the possibility that cancer cell cycles may be more dependent on CyclinD-CDK4/6 complexes than normal cells. Recent studies have suggested that this may be the case in intestinal epithelial cells as although deletion of E2F1-3 suppressed the proliferation induced following pRB loss, there was no impact on normal intestinal proliferation (30).

Taken together, these results indicate the important proliferative role that CyclinD/CDK4-6 complexes play following Wnt activation in driving tumor formation. Moreover, this raises the possibility that inhibition of Cyclin D/CDK4-6 may be useful in those individuals with a high risk of CRC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by Cancer Research UK and R01 CA083688 and CA108420 (to PS). PS is a Scholar of the Leukemia & Lymphoma Society. Thanks to BSU, histology and Roger Brisewitz.

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Figure 1. Cyclin D2 is upregulated immediately following loss of Apc and is c-Myc dependent
A) ISH for Cyclin D2 on wild type crypts (labelled Wt) and *AhCre⁺ Apc^{fl/fl}* crypts (labelled *Apc^{fl/fl}*) at day 4 post induction. Note strong upregulation of Cyclin D2 in *AhCre⁺ Apc^{fl/fl}* crypts. Scale bar represents 20μm **B)** IHC for Cyclin D2 showing high upregulation of Cyclin D2 in *AhCre⁺ Apc^{fl/fl}* crypts. Scale bar represents 20μm. **C)** Immunoblotting for Cyclin D2 shows strong upregulation of protein from intestinal epithelial cells extracts of *AhCre⁺ Apc^{fl/fl}* mice compared to wild type. **D)** QRT-PCR showing a significant upregulation of both Cyclin D2 (4.292, p=0.004) and CDK4 (2.194, p= 0.005) following loss of Apc. Note a decrease in expression of both Cyclin D2 (0.193, p= 0.01) and CDK4 (0.32, p= 0.004) in *AhCre⁺ Apc^{fl/fl} Myc^{fl/fl}*, illustrating that the upregulation of both Cyclin D2 and CDK4 are c-Myc dependent. **E)** Immunoblotting for CDK4, Cyclin D2 and p-Rb in wild type, *AhCre⁺ Apc^{fl/fl}* (labelled *Apc^{fl/fl}*) and *AhCre⁺ Apc^{fl/fl} Myc^{fl/fl}* (labelled *Apc^{fl/fl} Myc^{fl/fl}*), 4 days follow cre induction.

Figure 2. Cyclin D2 deficiency reduces proliferation following Apc loss

A) Boxplot showing Cyclin D2 deficiency does not affect crypt size in wild type crypts (Mann-Whitney U test, $p = 1.00$, $n = 5$). However following Apc loss, Cyclin D2 deficiency does significantly decrease crypt size (Mann-Whitney U test, $p = 0.01$, $n = 5$). **B)** Boxplot showing Cyclin D2 deficiency does not affect proliferation in wild type crypts (Mann-Whitney U test, $p = 0.1914$). However following Apc loss, deletion of Cyclin D2 does significantly decrease proliferation levels (Mann-Whitney U test, $p = 0.04$, $n = 5$). **C)** H&E staining showing crypt size. Black bars highlight crypt size. Crypts have a single cell crypt-villus axis in wild type (*Wt*) mice, whilst *AhCre⁺ Apc^{fl/fl}* (labelled *Apc^{fl/fl}*) deficient crypts are much larger. Double mutant *AhCre⁺ Apc^{fl/fl} Cyclin D2^{-/-}* (labelled *Apc^{fl/fl} Cyclin D2^{-/-}*) have smaller crypts compared to *AhCre⁺ Apc^{fl/fl}* mice. **D)** IHC for β -catenin. Wild type intestinal crypts only have a small subset of enterocytes with nuclear β -catenin at the base of the crypt (arrowed inset). Both *AhCre⁺ Apc^{fl/fl}* and *AhCre⁺ Apc^{fl/fl} Cyclin D2^{-/-}* intestinal crypts have enterocytes throughout with nuclear β -catenin (red arrows). Scale bars represents 20 μ m.

Figure 3. Cyclin D2 deficiency reduces tumorigenesis in the *Apc^{Min/+}* mouse

A) Cyclin D2 IHC performed on intestinal polyp (black arrow) from *Apc^{Min/+}* mouse showing increased expressed of Cyclin D2 compared to surrounding normal epithelium (red arrow). Inset shows nuclear staining of Cyclin D2. **B)** Kaplan Meier survival plot showing that *Apc^{Min/+} Cyclin D2^{-/-}* mice (green line) survive significantly longer than *Apc^{Min/+}* mice (black line) (Log rank test, $p < 0.001$, $n = 20$). Heterozygosity for *Cyclin D2* (red line) did not affect survival compared to *Apc^{Min/+}* mice (Log rank test, $p = 0.2516$, $n = 20$). **C)** Boxplot showing that *Apc^{Min/+} Cyclin D2^{-/-}* mice have a significantly smaller tumor burden in the small intestine than *Apc^{Min/+}* mice (Mann-Whitney U test, $p < 0.001$, $n = 20$), when taken at a timepoint of 110 days. Heterozygosity for *Cyclin D2* did not affect total tumor burden compared to *Apc^{Min/+}* mice (Mann-Whitney U test, $p = 0.58$, $n = 20$). **D)** Boxplot showing that *Apc^{Min/+} Cyclin D2^{-/-}* mice have significantly less tumors in the small intestine than *Apc^{Min/+}* mice (Mann-Whitney U test, $p < 0.001$, $n = 20$), when taken at a timepoint of 110 days. Heterozygosity for *Cyclin D2* did not affect total tumor number compared to *Apc^{Min/+}* mice (Mann-Whitney U test, $p = 0.2451$). **E)** Boxplot showing *Apc^{Min/+} Cyclin D2^{-/-}* mice have a significantly smaller average tumor size than *Apc^{Min/+}* mice (Mann-Whitney U test, $p < 0.001$, $n = 20$). Similarly to tumor burden, heterozygous deletion for Cyclin D2 did not affect average tumor size compared to those of *Apc^{Min/+}* mice (Mann-Whitney U test, $p = 0.1452$, $n = 20$).

Figure 4. Cyclin D2 deficiency reduces proliferation rates in adenomas from the $Apc^{Min/+}$ mouse

A) IHC for β -catenin showing high levels of nuclear β -catenin in adenomas from both $Apc^{Min/+}$ and $Apc^{Min/+} Cyclin D2^{-/-}$ mice. Black arrows point to adenomatous cells with increased nuclear (and cytoplasmic) β -catenin, red arrows point to neighbouring wild type cells with only cytoplasmic staining. **B)** IHC for Cyclin D2 showing loss of all nuclear staining in adenomas from $Apc^{Min/+} Cyclin D2^{-/-}$ mice. Note cytoplasmic staining remains, suggesting this is non-specific staining. **C)** Boxplot showing a significant reduction in proliferation in adenomas arising from $Apc^{Min/+} Cyclin D2^{-/-}$ compared to $Apc^{Min/+}$ mice (Mann-Whitney U test, $p=0.04$, $n=3$). From each mouse at least 5 adenomas were scored, and at least 3 mice per genotype were used. **D)** IHC for BrdU showing higher BrdU incorporation within tumors from $Apc^{Min/+}$ mice compared to those of $Apc^{Min/+} Cyclin D2^{-/-}$ mice. Black arrows show areas of adenomas in the $Apc^{Min/+} Cyclin D2^{-/-}$ that lack BrdU incorporation. Red arrows show high proliferation in normal epithelium. Each scale bar represents 20 μ m.

Figure 5. Cdk4/6 inhibition suppresses proliferation in established adenomas from *Apc^{Min/+}* mice

A) Boxplot showing no change in intestinal enterocyte proliferation when *Apc^{Min/+}* mice are treated with 150mg/kg of PD0332991 daily for 5 days (Mann Whitney, $p=0.76$, $n=5$). **B)** Boxplot showing a significant reduction in proliferation in tumors arising from these *Apc^{Min/+}* treated with PD0332991 when compared to vehicle treated (Mann-Whitney U test, $p=0.01$, $n=5$ mice, 5 adenomas per mouse). **C)** IHC for BrdU showing lower levels of BrdU incorporation within adenomas from *Apc^{Min/+}* mice (black arrows) when compared to the surrounding normal tissues (red arrows). Scale bar represents 20 μ m.

Figure 6. Cyclin D1 haploinsufficiency further suppresses tumorigenesis and tumor proliferation when combined with Cyclin D2 deficiency

A) Boxplot showing that *Apc^{Min/+} Cyclin D2^{-/-} Cyclin D1^{+/-}* (*Min D2^{-/-} D1^{+/-}*) mice have a significantly smaller tumor burden in the small intestine than *Apc^{Min/+} Cyclin D2^{-/-} Cyclin D1^{+/+}* (*Min D2^{-/-} D1^{+/+}*) and *Apc^{Min/+} Cyclin D2^{+/+} Cyclin D1^{+/-}* (*Min D2^{+/+} D1^{+/-}*) mice (Mann-Whitney U test, $p < 0.001$, $n > 10$), when taken at a timepoint of 110 days. **B)** IHC for BrdU showing lower levels of BrdU incorporation within adenomas (black arrows), compared to surrounding normal tissue (red arrows). **C)** Boxplot showing a significant reduction in proliferation in tumors arising in *Apc^{Min/+} Cyclin D2^{-/-} Cyclin D1^{+/-}* compared to *Apc^{Min/+} Cyclin D2^{-/-} Cyclin D1^{+/+}* mice (Mann-Whitney $p = 0.01$, $n = 10$). **D)** Boxplot showing equivalent levels of proliferation in normal small intestinal *Apc^{Min/+} Cyclin D2^{-/-} Cyclin D1^{+/-}* compared to *Apc^{Min/+} Cyclin D2^{+/+} Cyclin D1^{+/+}* mice (Mann-Whitney, $p < 0.5$, $n = 5$).