Cancer Biology & Therapy 14:7, 672–678; July 2013; © 2013 Landes Bioscience

Cables1 is a tumor suppressor gene that regulates intestinal tumor progression in *Apc*^{Min} mice

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Keywords: *Cables1*, chromosome 18q, colorectal cancer, *Apc*^{Min} mice, tumor suppressor gene, β-catenin, adenocarcinoma

Abbreviations: APC, adenomatous polyposis coli; CDK, cyclin-dependent kinase; CRC, colorectal cancer; H&E, hematoxylin and eosin; IHC, immunohistochemistry; LOH, loss of heterozygosity

The transformation of colonic mucosal epithelium to adenocarcinoma requires progressive oncogene activation and tumor suppressor gene inactivation. Loss of chromosome 18q is common in colon cancer but not in precancerous adenomas. A few candidate tumor suppressor genes have been identified in this region, including *CABLES1* at 18q11.2–12.1. This study investigates the role of *CABLES1* in an in vivo mouse model of intestinal adenocarcinoma and in human colon cancer cell culture. *Apc*$^{Min/+}$ mice were crossed with mice harboring targeted inactivation of the *Cables1* gene (*Cables1*$^{-/-}$). The intestinal tumor burden and tumor expression of β-catenin and PCNA was compared in *Cables1*$^{+/+}$*Apc*$^{Min/+}$ and *Cables1*$^{-/-}$*Apc*$^{Min/+}$ mice. β-catenin activity in human colon cancer cells with *CABLES1* inactivation and intestinal progenitor cell function in *Cables1*$^{-/-}$ mice were assayed in vitro. The mean number of small intestinal tumors per mouse was 3.1 ± 0.6 in *Cables1*$^{+/+}$ *Apc*$^{Min/+}$ mice, compared with 32.4 ± 3.5 in the *Cables1*$^{-/-}$*Apc*$^{Min/+}$ mice ($P < 0.0001$). Fewer colonic tumors were observed in *Cables1*$^{+/+}$*Apc*$^{Min/+}$ mice (mean 0.6 ± 0.1) compared with the *Cables1*$^{-/-}$*Apc*$^{Min/+}$ mice (mean 1.3 ± 0.3, $P = 0.01$). Tumors from *Cables1*$^{-/-}$*Apc*$^{Min/+}$ mice demonstrated increased nuclear expression of β-catenin and an increased number of PCNA-positive cells. In vitro studies revealed that *CABLES1* deficiency increased β-catenin dependent transcription and increased intestinal progenitor cell activity. Loss of *Cables1* enhances tumor progression in the *Apc*$^{Min/+}$ mouse model and activates the Wnt/β-catenin signaling pathway. *Cables1* is a tumor suppressor gene on chromosome 18q in this in vivo mouse model and likely has a similar role in human colon cancer.

Introduction

In the pathogenesis of colorectal cancer (CRC) via the classic chromosomal instability pathway, the colonic epithelium is thought to require an ordered activation of specific oncogenes and inactivation of tumor suppressor genes as the tumor progresses through an adenomatous intermediate prior to adenocarcinoma. Inactivation of the adenomatous polyposis coli (*APC*) tumor suppressor gene is one of the earliest events, followed by oncogenic *KRAS* mutations.[1] Later events include activation of *PIK3CA*, inactivation of the tumor suppressor gene *TP53* and loss of heterozygosity (LOH) on chromosome 18q.[1]

There is allelic deletion of chromosome 18q in approximately 70% of human CRCs, and several candidate tumor suppressor genes, including Deleted in Colorectal Carcinoma (*DCC*), *SMAD4*, and *SMAD2*, have been identified in this region.[2,3] The expression of *DCC* is lost or markedly reduced in the majority of colorectal cancers.[4] However, based on the observations that *DCC* encodes a cell surface receptor for the neuronal protein netrin-1, *DCC* mutations are rare in human colorectal cancers (6%), and mice with inactivating mutations of *Dcc* do not develop intestinal tumors, some doubts have been raised about the function of *DCC* as a tumor suppressor in colon cancer.[4,5] The SMAD proteins are intracellular mediators of the transforming growth factor-β pathway that regulate cell growth, differentiation, and apoptosis. Although biallelic inactivation of *SMAD4* occurs in greater than 60% of pancreatic tumors, mutations of *SMAD4* and *SMAD2* have been found in less than 20% and 10% of colon cancers, respectively.[6,7] Thus, alterations in the *DCC* and *SMAD* genes are unlikely to fully account for all the 18q deletions in colorectal cancer, suggesting the existence of other tumor suppressor genes on chromosome 18q.

The *CABLES1* gene maps to chromosome 18q11.2–12.1 and may be a proximal 18q gene involved in human colon cancer.[8]

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Submitted: 02/11/13; Revised: 04/17/13; Accepted: 05/19/13
http://dx.doi.org/10.4161/cbt.25089

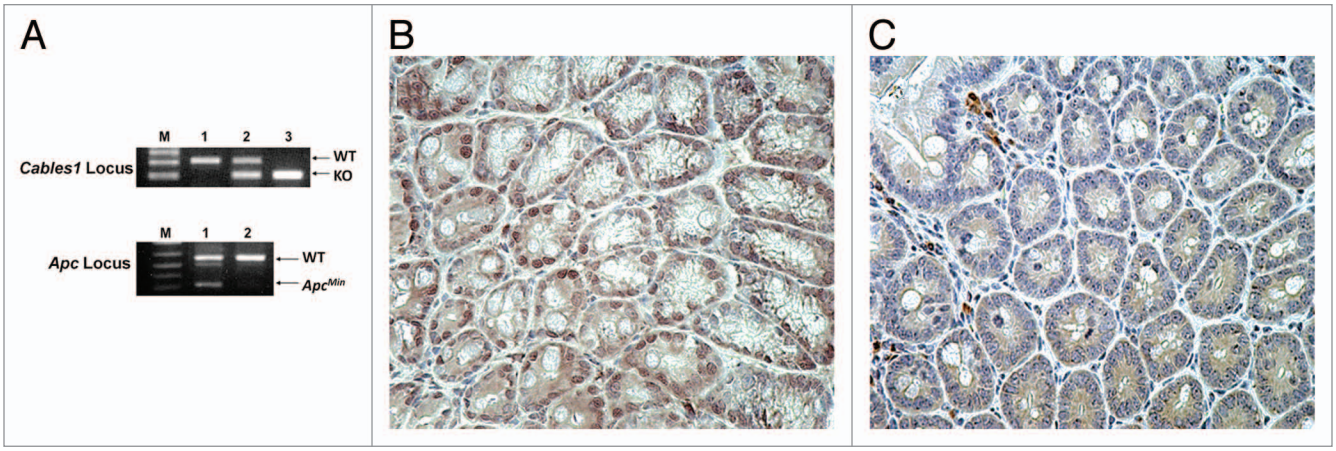

Figure 1. (**A**) PCR genotyping of *Cables1* and *Apc*. In the upper panel, lanes 1, 2, and 3 represent the *Cables1* wild-type, heterozygous, and homozygous genotypes, respectively. WT, wild-type fragment (~300 bp); KO, knockout fragment (~200 bp). In the lower panel, lanes 1 and 2 represent the $Apc^{Min/+}$ and *Apc* wild-type genotypes, respectively. WT fragment (~600 bp); Apc^{Min} fragment (~340 bp). (**B**) Intact nuclear Cables1 expression by immunohistochemistry in $Cables1^{+/+}$ $Apc^{Min/+}$ mouse intestinal epithelial cells. (**C**) Loss of nuclear Cables1 expression by immunohistochemistry in $Cables1^{-/-}Apc^{Min/+}$ mouse intestinal epithelial cells. A few inflammatory cells retain Cables1 expression.

Functionally, the Cables1 protein acts as a linker or "cable" enhancing cyclin-dependent kinase (CDKs 2, 3, and 5) tyrosine phosphorylation by non-receptor tyrosine kinases (Src, Abl, Wee1) to modulate CDK activity.[8,9] Cables1 also interacts with p53 and has been reported to potentiate p53-induced cell death. In neurons, Cables1 links Robo-bound Abl kinase to N-cadherin bound β-catenin, resulting in a rapid loss of cadherin-mediated adhesion and activation of gene transcription.[10] Loss of nuclear Cables1 expression has been observed in head and neck, lung, ovarian, endometrial and colon cancers.[8,11,12] Cables1 expression, evaluated by immunohistochemistry, is decreased or absent in 65% of primary CRCs.[13] Loss of Cables1 expression is likely due to hypermethylation of CpG islands in the promoter of *CABLES1,* coupled with 18q LOH.[13] In *Cables1*-knockout mice ($Cables1^{-/-}$), there is an increased incidence of both endometrial hyperplasia and endometrial cancer following prolonged exposure to unopposed estrogen.[14] Furthermore, in response to the carcinogen 1,2-dimethylhydrazine, $Cables1^{-/-}$ mice have an increased incidence of intestinal tumors and reduced survival.[15] Primary mouse embryonic fibroblasts from $Cables1^{-/-}$ mice exhibit an increased rate of cell proliferation and delayed onset of senescence.[16]

To determine whether the *Cables1* gene modulates the progression of colonic tumors in a mouse model, we crossed $Cables1^{-/-}$ mice with mice carrying a truncating mutation in the *Apc* tumor suppressor gene ($Apc^{Min/+}$ mice). Our analysis of tumor burden in $Cables1^{+/+}$ $Apc^{Min/+}$ mice compared with $Cables1^{-/-}$ $Apc^{Min/+}$ mice indicates a role for *Cables1* in intestinal tumor progression. In vitro assays suggest that loss of Cables1 expression results in activation of the Wnt/β-catenin signaling pathway.

Results

Identification of $Cables1^{-/-}$ $Apc^{Min/+}$ and $Cables1^{+/+}$ $Apc^{Min/+}$ mice. To determine whether *Cables1* modulates late events in colorectal cancer progression, $Cables1^{-/-}$ mice were crossed with $Apc^{Min/+}$ mice, which are predisposed to the development of multiple adenomas in the small intestine and in the colon. *Cables1* and *Apc* genotypes were determined by PCR analysis of tail-snip genomic DNA using specific primers (**Fig. 1A**). Nine $Cables1^{-/-}$ $Apc^{Min/+}$ mice were identified following genotype analysis, forming the *Cables1* gene knockout group. These were compared with 15 $Cables1^{+/+}$ $Apc^{Min/+}$ mice, which also had their genotypes confirmed by PCR. Immunohistochemistry confirmed loss of Cables1 nuclear staining in the $Cables1^{-/-}$ $Apc^{Min/+}$ and intact Cables1 nuclear staining in $Cables1^{+/+}$ $Apc^{Min/+}$ mice (**Fig. 1B and C**). Seventeen mice heterozygous for *Cables1* ($Cables1^{+/-}$ $Apc^{Min/+}$ mice) were also identified as a control group for comparison with the two study groups.

Quantification of tumor burden in $Cables1^{-/-}$ $Apc^{Min/+}$ and $Cables1^{+/+}$ $Apc^{Min/+}$ mice. Nine $Cables1^{-/-}$ $Apc^{Min/+}$ mice and 15 $Cables1^{+/+}$ $Apc^{Min/+}$ mice were euthanized at a mean age of 15.4 and 15.8 weeks respectively. Several mice in the $Cables1^{-/-}$ $Apc^{Min/+}$ group died or became moribund before termination of the experiment as a consequence of intestinal obstruction and perforation. The number of intestinal and colonic tumors was counted histologically by assessment of a single H&E section of the intestine "Swiss" roll (**Fig. 2A and B**). A total of 304 tumors were found in the 9 $Cables1^{-/-}$ $Apc^{Min/+}$ mice (292 in the small intestine and 12 in the colon), as compared with a total of 58 tumors in the 15 $Cables1^{+/+}$ $Apc^{Min/+}$ control mice (49 in the small intestine and 9 in the colon, respectively; $P < 0.001$). As shown in **Table 1**, the mean number of small intestinal tumors per mouse was significantly higher ($P < 0.0001$) in the $Cables1^{-/-}$ $Apc^{Min/+}$ mice (mean 32.4 ± 3.5 tumors) compared with the $Cables1^{+/+}$ $Apc^{Min/+}$ mice (mean 3.1 ± 0.6 tumors). Similarly, the mean number of colonic tumors per mouse was increased ($P = 0.01$) in the $Cables1^{-/-}$ $Apc^{Min/+}$ mice (mean 1.3 ± 0.3 tumors) compared with the $Cables1^{+/+}$ $Apc^{Min/+}$ mice (mean 0.6 ± 0.1 tumors). Seventeen mice heterozygous for *Cables1* ($Cables1^{+/-}$ $Apc^{Min/+}$) were sacrificed and there was no significant difference ($P = 0.08$) in mean tumor number between

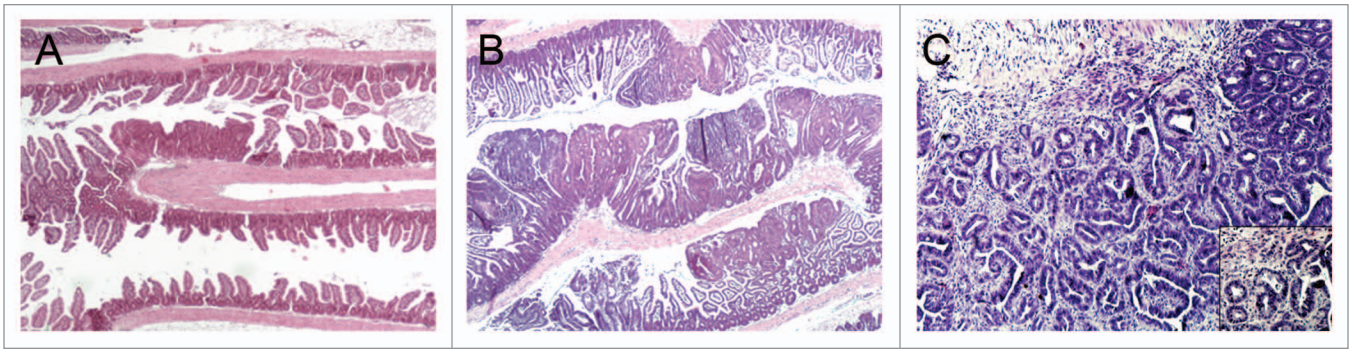

Figure 2. (**A**) Hematoxylin and eosin (H&E) stained small intestine from a *Cables1*$^{+/+}$*Apc*$^{Min/+}$ mouse with rare adenomas. (**B**) H&E stained small intestine from a *Cables1*$^{-/-}$ *Apc*$^{Min/+}$ mouse with frequent adenomas. (**C**) An adenocarcinoma in the small intestine of a *Cables1*$^{-/-}$ *Apc*$^{Min/+}$ mouse. Inset: higher magnification.

Table 1. Tumor burden in *Cables1*$^{+/+}$ *Apc*$^{Min/+}$ mice and *Cables1*$^{-/-}$ *Apc*$^{Min/+}$mice

Group	*Cables1*$^{+/+}$ *Apc*$^{Min/+}$	*Cables1*$^{-/-}$ *Apc*$^{Min/+}$	Significance (Student's t-test)
Number of mice examined	15	9	-
Mean number of small intestine tumors (± SE)	3.1 ± 0.6	32.4 ± 3.5	$P < 0.0001$
Mean number of colon tumors (± SE)	0.6 ± 0.1	1.3 ± 0.3	$P = 0.01$

this group and mice with homozygous wild-type *Cables1* genotype (*Cables1*$^{+/+}$ *Apc*$^{Min/+}$ mice). Furthermore, although histopathological analysis revealed that the majority of tumors in the *Cables1*$^{-/-}$ *Apc*$^{Min/+}$ mice were adenomas, three adenocarcinomas, one in the small intestine and two in the colon, were observed in two *Cables1* knockout mice (**Fig. 2C**). No adenocarcinomas were observed in the *Cables1*$^{+/+}$ *Apc*$^{Min/+}$ mice.

β-catenin and PCNA IHC in tumor tissue from *Cables1*$^{-/-}$ *Apc*$^{Min/+}$ and *Cables1*$^{+/+}$ *Apc*$^{Min/+}$ mice. Adenomas in the *Cables1*$^{+/+}$ *Apc*$^{Min/+}$ mice demonstrated membranous and weak cytoplasmic staining for β-catenin by IHC, with minimal nuclear immunopositivity (**Fig. 3A**). Tumor tissue from *Cables1*$^{-/-}$ *Apc*$^{Min/+}$ mice revealed an increased percentage of cells with nuclear β-catenin staining and an increased intensity of nuclear β-catenin staining (**Fig. 3B**). When evaluated semi-quantitatively, there was a significantly higher mean nuclear β-catenin IHC score ($P = 0.002$) in the *Cables1*$^{-/-}$ *Apc*$^{Min/+}$ mice (162 ± 25.2) compared with *Cables1*$^{+/+}$ *Apc*$^{Min/+}$ mice (20.6 ± 13.1), as shown in **Figure 4A**. The mean number of PCNA-positive cells per high power field of tumor tissue from the *Cables1*$^{-/-}$ *Apc*$^{Min/+}$ mice was 310.2 ± 13.4 as compared with 119.2 ± 6.7 in the *Cables1*$^{+/+}$ *Apc*$^{Min/+}$ mice ($P < 0.0001$). Whereas proliferating cells were confined to the proliferative zone in the lower half of the crypt adjacent to the base (transient amplifying cell region) in the wild type mice, a more diffuse distribution of positive cells was observed in the *Cables1*-knockout mice. A significant correlation (r = 0.70, $P = 0.0006$) was observed between nuclear expression of β-catenin and PCNA.

In vitro assessment of β-catenin expression with siRNA induced *CABLES1* knockdown. We investigated whether Cables1 regulates β-catenin-dependent transcription in vitro in human colon cancer cell culture (SW480 cells) using a TOP/FOP reporter assay. Knockdown of *CABLES1* gene expression by siRNA resulted in an increase in TOP/FOP reporter activity of almost 2-fold when compared with cells transfected with a control siRNA (**Fig. 4B**). Conversely, overexpression of *CABLES1* led to a 46% decrease in TOP/FOP reporter activity compared with controls.

Intestinal progenitor cell function in *Cables1*$^{-/-}$ mice. To test how loss of Cables1 influenced intestinal progenitor function we isolated small intestinal crypts from 1-year-old wild type and *Cables1* knockout (*Cables1*$^{-/-}$) mice and assayed the ability of these crypts to give rise to "mini-intestines" or organoid bodies in culture.[17-19] These small intestinal crypts posses all of the intestinal stem and progenitor cells that give rise to all of the differentiated intestinal epithelial cell types. We found that Cables1-deficient crypts were nearly 2-fold more clonogenic in culture than crypts from wild-type mice, indicating that they contain more stem cell or progenitor cell activity (**Fig. 4C**).

Discussion

Loss of heterozygosity (LOH) for chromosome 18q has been implicated in the stepwise progression of human colonic mucosal epithelium to dysplasia and adenocarcinoma. Approximately 1/3 of colonic adenomas with low-grade dysplasia have LOH of 18q.[20] The proportion of adenomas with high-grade dysplasia and adenocarcinomas with LOH of 18q has been reported to exceed 70%.[20] However, the identity of the relevant tumor suppressor gene on 18q has remained elusive. *DCC* was one of the first candidate genes proposed, but multiple lines of evidence have not supported such a role. In particular, a *Dcc* null mutation does not affect intestinal polyp initiation, number, size, or morphology in *Apc*$^{Min/+}$ mice.[5] *SMAD2* and *SMAD4* also localize to chromosome 18q. One study demonstrated that tumor growth and invasion were accelerated by loss of *Smad2* in *Apc* mutant mice, but another study failed to support a significant role in the malignant

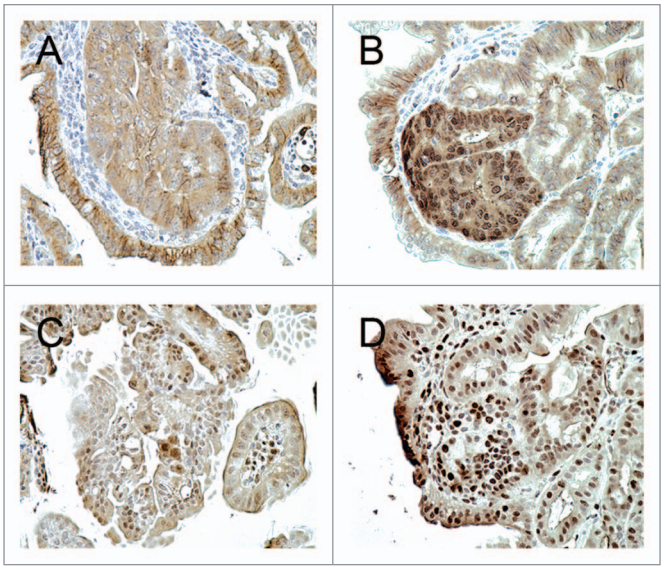

Figure 3. (**A**) Membranous and cytoplasmic β-catenin immunohistochemical staining was observed in the adenomas of *Cables1*$^{+/+}$ *Apc*$^{Min/+}$ mice, but there was minimal nuclear staining. (**B**) In the adenomas of *Cables1*$^{-/-}$ *Apc*$^{Min/+}$ mice, there was nuclear immunopositivity for β-catenin. (**C**) A low proportion of epithelial cells were PCNA positive in adenomas from *Cables1*$^{+/+}$ *Apc*$^{Min/+}$ mice. (**D**) In the adenomas of *Cables1*$^{-/-}$ *Apc*$^{Min/+}$ mice, a high proportion of epithelial cells were PCNA-positive by immunohistochemistry.

progression of polyps.[21,22] Deletions of *Smad4* appear to more consistently accelerate tumor progression in *Apc* mutant mice.[23,24] However, *SMAD4* mutations are present in less than 20% of human colorectal cancers.[6] Cables1 expression, evaluated by immunohistochemistry, is decreased or absent in 65% of primary CRCs and there is evidence for frequent methylation induced epigenetic silencing of the *CABLES1* gene in human colorectal cancer.[13] LOH has been shown to be more frequent in the proximal portion of chromosome 18q containing the *CABLES1* gene (18q11.1-q12.2) than in the more distal region, which is the location of *DCC*, *SMAD2* and *SMAD4* (18q21.1–q21.3).[13]

In this study, inactivation of *Cables1* was associated with a marked (approximately 10-fold) increase in small intestinal tumor burden in vivo in the *Apc*$^{Min/+}$ model compared with *Apc*$^{Min/+}$ mice with wild type *Cables1.* The mean tumor burden of 32.4 ± 3.5 small intestinal tumors counted in the *Cables1*$^{-/-}$ *Apc*$^{Min/+}$ mice may actually fall short of the true tumor burden in mice with this genotype, as several of these mice died due to intestinal obstruction and perforation prior to termination of the experiment and were not included in the final analysis. It was observed that mice heterozygous for *Cables1* had no significant difference in mean number of intestinal tumors from the *Cables1*$^{+/+}$ *Apc*$^{Min/+}$ mice, suggesting that inactivation of both alleles of *Cables1* is required to promote tumorigenesis. The finding of adenocarcinomas in the *Cables1*$^{-/-}$ *Apc*$^{Min/+}$ mice, compared with none in the other groups, suggests that loss of *Cables1* may accelerate intestinal tumorigenesis in the *Apc*$^{Min/+}$ mouse model. Overall, these results suggest that *Cables1* is a relevant 18q tumor suppressor gene in intestinal adenocarcinoma.

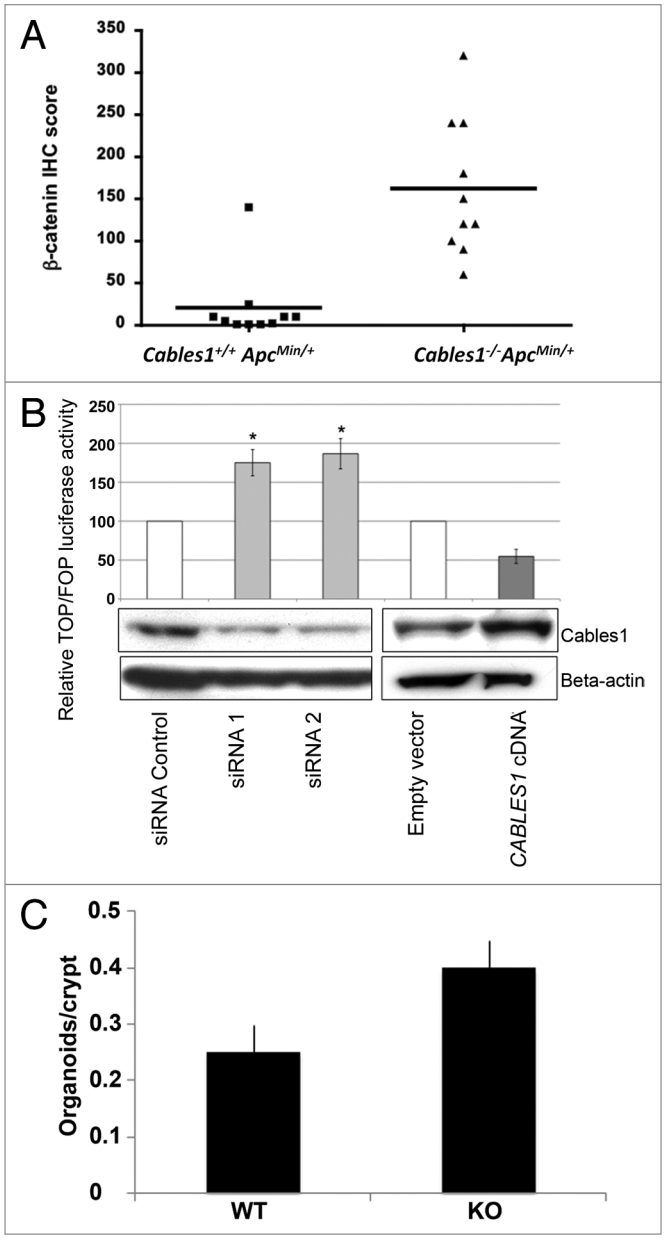

Figure 4. (**A**) β-catenin immunohistochemistry (IHC) scores of *Cables1*$^{+/+}$ *Apc*$^{Min/+}$ and *Cables1*$^{-/-}$ *Apc*$^{Min/+}$ mice. A horizontal line indicates the median in each group. There was a significantly higher mean nuclear β-catenin IHC score ($P = 0.002$) in the *Cables1*$^{-/-}$ *Apc*$^{Min/+}$ mice. (**B**) SW480 cells were transfected with either small interfering RNA (siRNA) targeting endogenous *CABLES1* or with *CABLES1* cDNA. Twelve hours later, cells were transfected with a β-catenin responsive (pGL3-OT) or a mutant (pGL3-OF) reporter construct and luciferase activity was measured. Results are expressed as the TOP/FOP ratio. The *CABLES1* siRNA was associated with significantly increased TOP/FOP reporter activity compared with cells transfected with a control siRNA. Overexpression of Cables1 following *CABLES1* cDNA transfection was associated with decreased TOP/FOP reporter activity compared with controls. Columns: average of at least two independent experiments; bars: standard error of mean (SEM). *$P < 0.05$ as compared with control cells. Protein levels of Cables1 were confirmed by western blot in lower panel. (**C**) Small intestinal crypts isolated from *Cables1* knockout mice (KO) were capable of nearly 2-fold greater ($P < 0.05$) organoid formation than crypts from wild type mice (WT), indicating increased intestinal progenitor cell activity in the *Cables1* knockout mice.

Apc$^{Min/+}$ mice develop multiple intestinal adenomas by 16 weeks of age and indeed, all of the *Cables1*$^{-/-}$ *Apc*$^{Min/+}$mice and *Cables1*$^{+/+}$ *Apc*$^{Min/+}$ mice in this study did develop intestinal adenomas. The mean number of colonic adenomas (0.6 tumors) was consistent with the literature.[25] The mean number of small intestinal adenomas in the *Cables1*$^{+/+}$ *Apc*$^{Min/+}$ group (3.1) was lower than is typically reported in literature for the 16 week age group, which often number approximately 30.[25] There are factors that may have contributed to the relatively low tumor burden observed in the *Cables1*$^{+/+}$ *Apc*$^{Min/+}$ group in this study, including a sterile housing facility and the method of counting polyps. Most studies report tumor counts using a dissecting microscope to analyze the entire mucosal surface. In contrast, our study did not analyze the entire mucosal surface, as polyps were only counted in the single plane sectioned from the "Swiss" roll for H&E staining. Since the tumor counting method and housing conditions were the same for both the *Cables1*$^{-/-}$ *Apc*$^{Min/+}$mice and *Cables1*$^{+/+}$ *Apc*$^{Min/+}$ mice, these factors would have caused a similar degree of underestimation of the total tumor burden in both groups. The other possible explanation for lower than expected tumor burden is the presence of a modifier gene. Several genes, known as *modifiers of Min* (*Mom1*, *Mom2*, *Mom3*, and *Mom7*), have been identified that can modify the Min phenotype.[26,27] Mutation of one of the *Mom* genes can result in a reduction of up to 90% in the polyp burden in *Apc*$^{Min/+}$ mice.[27] While it is possible that a modifier gene is responsible for the relatively low tumor burden observed in this study, this would have likely affected both the *Cables1*$^{+/+}$ *Apc*$^{Min/+}$ mice and the *Cables1*$^{-/-}$ *Apc*$^{Min/+}$mice. Consequently, the difference in tumor burden between these two groups is of greater interest than the absolute tumor burden in either group.

In the Wnt signaling pathway, the *APC* gene product targets β-catenin for degradation by the proteosome, in association with GSK-3β, CK1 (casein kinase 1), and axin.[28] Mutant *APC* disrupts the complex formation and creates a permissive condition whereby free, unphosphorylated β-catenin may accumulate in the cytoplasm, and following its translocation into the nucleus, drive the transcription of multiple genes implicated in tumor growth and invasion. In this study, increased β-catenin expression was detected in tumor tissue from *Cables1*$^{-/-}$ *Apc*$^{Min/+}$ mice when compared with adenomas from *Cables1*$^{+/+}$ *Apc*$^{Min/+}$ mice. We also found that an siRNA induced *CABLES1* knockdown in human colon cancer cell culture resulted in increased β-catenin-dependent transcription. Overall, this suggests that in colon cancer, inactivation of Cables1 may further deregulate Wnt pathway signaling, leading to increased accumulation of nuclear β-catenin and increased β-catenin-dependent transcription. Of note, this is in contrast with our current understanding of Cables1 function in neurons, in which Cables1 is thought to be an essential linker protein between Robo-associated Abl and N-cadherin-associated β-catenin, preventing nuclear targeting of β-catenin and activation of transcription.[10]

Because *Cables1*$^{-/-}$ *Apc*$^{Min/+}$ mice exhibit increased tumor formation and Cables1 has been reported to play a role in regulating the activity of cyclin-dependent kinases, we measured proliferative activity by immunohistochemical staining for proliferating cell nuclear antigen.[8,9] PCNA was significantly increased in tumor tissue from *Cables1*$^{-/-}$ *Apc*$^{Min/+}$ mice. The strong correlation between nuclear expression of β-catenin and PCNA, suggests that nuclear accumulation of β-catenin increases during tumor progression and may have a direct effect on proliferation in vivo. Increased cell proliferation (shortened cell culture doubling time) has been previously identified in *Cables1*-deficient mouse embryonic fibroblasts.[15] The *Cables1*-deficient cells in these experiments were shown to have decreased expression of cyclinD1 and the cyclin dependant kinase inhibitors p16 and p27. We have performed immunohistochemistry for cyclinD1, p16, and p27 on tissue from *Cables1*$^{-/-}$ *Apc*$^{Min/+}$ mice and *Cables1*$^{+/+}$ *Apc*$^{Min/+}$ mice, but we have not identified differences in expression of these proteins in tumor tissue and background non-neoplastic tissue between the two groups (results not shown). The study of cyclins and cyclin dependent kinase inhibitors in a *Cables1*-deficient in vivo model, such as this, is an area of interest for future research, but likely requires more sensitive techniques than immunohistochemistry, such as western blotting, for assessment of cell cycle proteins.

Finally, to test how loss of Cables1 influences intestinal progenitor cell function, we isolated small intestinal crypts from 1-year-old wild-type and *Cables1* knockout mice and found that Cables1 deficient crypts were nearly twice as capable of forming organoids in culture than wild type crypts. This data suggests that there may be more stem cell or progenitor cell activity in Cables1 deficient crypts, since only long-lived stem cells can self-renew and differentiate into the various mature intestinal cell types required for organoid body maintenance in vitro.

In summary, loss of *Cables1* enhances tumor progression in the *Apc*$^{Min/+}$ mouse model. Inactivation of *Cables1* results in activation of the Wnt/β-catenin signaling pathway, indicating that *Cables1* is a tumor suppressor gene on chromosome 18q in this in vivo mouse model and likely has a similar role in human colon cancer.

Materials and Methods

Generation of *Cables1*$^{+/+}$ *Apc*$^{Min/+}$ and *Cables1*$^{-/-}$ *Apc*$^{Min/+}$ mice and genotyping. C57BL/6J *Apc*$^{Min/+}$ mice were obtained from the Jackson Laboratory. Mice harboring a targeted inactivation of the *Cables1* gene (*Cables1*$^{-/-}$) have been previously described.[12,13] *Cables1*-knockout mice had been previously backcrossed with C57BL/6J mice before mating with C57BL/6J *Apc*$^{Min/+}$ mice. *Cables1*$^{-/-}$ females were mated to *Apc*$^{Min/+}$ males to obtain *Cables1*$^{+/-}$ *Apc*$^{Min/+}$ breeding pairs, which were then intercrossed to generate both *Cables1*$^{+/+}$ *Apc*$^{Min/+}$ mice and *Cables1*$^{-/-}$ *Apc*$^{Min/+}$ mice. All offspring were genotyped by PCR analysis of tail-snip genomic DNA using specific primers. The three primer pairs used for genotyping *Apc*$^{Min/+}$ mice were: IMR033 5'-GCCATCCCTT CACGTTAG-3', IMR034 5'-TTCCACTTTG GCATAAGGC-3' and IMR758 5'-TTCTGAGAAA GACAGAAGTT A-3'. The PCR conditions used are those recommended by the Induced Mutant Resource of The Jackson Laboratory. The *Cables1* PCR assay involved the use of three primers: 5'-TTGGTTATTG GGTCGGGTGC-3', 5'-GGAGGAAAAC AAAGCCGCAG G-3' and 5'-GGATGTGGAA TGTGTGCGAG GC-3'.

Reaction mixtures containing all three primers were subjected to 35 cycles of standard PCR condition with a 63 °C annealing temperature. Products were resolved using 1.5% agarose gel electrophoresis and stained with ethidium bromide. The wild type product is ~300 bp long and the disrupted product is ~200 bp long. By evaluating both of these PCR reaction products, the knockout status of the mice was determined. The proportion of mice with the desired *Cables1*$^{-/-}$ *Apc*$^{Min/+}$ genotype was small, which was expected because *Cables1* and *Apc* both map to mouse chromosome 18, whereas in humans they map to different chromosomes (5 and 18).

All of the animal protocols were reviewed and approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee and were performed in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Harvesting and preparation of intestinal tissue samples. Mice were euthanized at a mean of 15–16 weeks of age with isoflurane and dissected immediately. The colon and small intestines were removed and carefully opened longitudinally. The intestines were rolled with the mucosa outwards, fixed in 10% buffered formalin, processed and embedded in paraffin. A single section of each intestine roll was cut by microtome and stained with hematoxylin and eosin. Small intestinal and colonic tumors were counted by light microscopy by a pathologist (LZ), who was blinded to the genotypes of the animals.

Immunohistochemical analysis. Immunohistochemical stains (IHC) were applied to formalin-fixed, paraffin-embedded tissue sections. A mouse anti-Cables1 monoclonal antibody was produced using a GST fusion protein containing the C-terminal sequence of human Cables1 as the immunogen. Nuclear staining identified intact Cables1 expression, whereas loss of Cables1 expression was based on absence of nuclear staining. Immunohistochemistry for the following primary antibodies was also performed: anti-β-catenin (monoclonal; clone 14; BD Transduction Laboratories) and anti-proliferating cell nuclear antigen (PCNA polyclonal; DAKO. Two investigators (MSP and LZ) blinded to the genotypes of the animals evaluated β-catenin and PCNA staining. For β-catenin, two randomly chosen cases for each mouse genotype group were evaluated by light microscopy in five high power fields (400× magnification) of tumor tissue. β-catenin expression was scored semi-quantitatively and expressed as an IHC score by multiplying the percentage of immunopositive cells and the staining intensity, as described previously.[29] Staining intensity was scored as follows: 0, negative; 1, weak; 2, moderate; 3, strong; and 4, very strong. The IHC score ranged from 0 to 400. To quantify PCNA expression, the number of positive cells in 10 randomly chosen high power fields (at 400× magnification) of sections of tumor tissue were counted in one case for each genotype group.

Cell culture, transfections and luciferase assay. Human SW480 colon cancer cells, which carry an *APC* mutation, were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-Invitrogen) supplemented with 10% fetal bovine serum (FBS; HyClone) and antibiotics (Penicillin-Streptomycin, Invitrogen). SW480 cells were transiently transfected with either *CABLES1* cDNA, using FuGENE6 (Roche) or small interfering RNA (siRNA) targeting endogenous *CABLES1*, using Lipofectamine RNAiMAX (Invitrogen). Nucleotide sequences corresponding to siRNAs were as follows: siRNA1, 5'-GGAGACCCTG GAAGATATT-3'; siRNA2, 5'-GUAUCGCGAC AGUACCCAA-3'. To measure β-catenin reporter activity, cells were transfected with either the pGL3-OT or pGL3-OF luciferase reporter constructs containing three copies of wild-type or mutated consensus TCF-4 response elements (gifts of Bert Vogelstein, John Hopkins University), using FuGENE6 transfection reagent (Roche). Each well was cotransfected with a Renilla luciferase expression vector (pRL-CMV; Promega) to normalize for transfection efficiency. Twenty-four hours after transfection, cells were lysed and firefly and Renilla luciferase activities were measured using the Dual Luciferase Reporter kit (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity.

Isolation and culture of intestinal stem cells from *Cables1*$^{-/-}$ mice. Intestinal stem cells were isolated from the intestinal tissue of one year old wild type and *Cables1*-deficient (*Cables1*$^{-/-}$) mice according to a previously published protocol.[17] Briefly, isolated crypts were counted and embeded in matrigel (BD Biosciences 356231 growth factor reduced) that contains 1 μM Jagged (ANASpec) at 5–10 crypts/μl and cultured in a modified form of medium as described by Sato et al.[18,19] Briefly, DMEM/F12 (Gibco) was supplemented by EGF 40 ng/ml (R&D Systems), Noggin 200 ng/ml (Peprotech), R-spondin 500 ng/ml (R&D or Sino Biological), N-acetyl-L-cysteine 1 μM (Sigma-Aldrich) and Y-27632 dihydrochloride monohydrate 20 ng/ml (Sigma-Aldrich). Thirty microliter drops of matrigel with crypts were plated onto a flat bottom 48-well plate (Corning 3548) and allowed to solidify for 20 to 30 min in a 37 °C incubator. Three hundred microliters of crypt culture medium was then overlaid onto the matrigel, changed every other day and maintained at 37 °C in fully humidified chambers containing 6% CO_2. Clonogenicity (colony-forming efficiency) was calculated by plating 50 to 400 crypts and assessing organoid formation 3 to 10 d after initiation of cultures.

Statistical analysis. All data are reported as mean ± standard error of the mean (SEM), unless otherwise specified. The Student *t* test was used to compare the means of each group and differences were considered to be significant if P values were < 0.05. For correlations, Pearson's correlation coefficients were calculated and considered statistically significant with an accompanying P value of < 0.05.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

Acknowledgments

The study was funded through National Institute of Health Grant R01 CA103883 entitled "Cables and the colonocyte". TA was funded through scholarships from the Royal College of Physicians and surgeons of Canada (Detweiler Traveling fellowship) and Dalhousie University (McLoughlin Scholarship).

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