**Atonal homolog 1 Is a Tumor Suppressor Gene**

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Colon cancer accounts for more than 10% of all cancer deaths annually. Our genetic evidence from Drosophila and previous in vitro studies of mammalian Atonal homolog 1 (Atoh1), also called Math1 or Hath1, suggest an anti-oncogenic function for the Atonal group of proneural basic helix-loop-helix transcription factors. We asked whether mouse Atoh1 and human ATOH1 act as tumor suppressor genes in vivo. Genetic knockouts in mouse and molecular analyses in the mouse and in human cancer cell lines support a tumor suppressor function for ATOH1. ATOH1 antagonizes tumor formation and growth by regulating proliferation and apoptosis, likely via activation of the Jun N-terminal kinase signaling pathway. Furthermore, colorectal cancer and Merkel cell carcinoma patients show genetic and epigenetic ATOH1 loss-of-function mutations. Our data indicate that ATOH1 may be an early target for oncogenic mutations in tissues where it cellsulellar differentiation.

Introduction

The Atonal (Ato) proneural transcription factors form a highly conserved group of key developmental regulators in multiple neural and neuroendocrine tissues. The mammalian Ato (CG7508) ortholog, ATOH1 (Ensembl accession number: ENSG00000172238), is essential for cell fate commitment of mecanorheptive Merkel cells in the skin [1] and the secretory goblet, Paneth, and enteroendocrine cells in the intestine [2,3] in addition to multiple neuronal lineages [4,5]. The functional conservation between the fly and mammalian proteins is underscored by the fact that Drosophila aito can fully rescue the Atoh1 (Ensembl: ENSMUSG00000073043) null mutant mouse [6].

Genetic analyses in Drosophila [7] suggest that aito regulates the formation and progression of tumors in fly retina, where it acts as a master regulator of cell fate specification. In mammal, two aggressive human cancers derive from tissues where ATOH1 instructs cell fate commitment, namely Merkel cell carcinoma (MCC) and colorectal cancer (CRC). MCC is a rare, but very aggressive, neuroendocrine cancer of the skin with approximately 40% mortality [8]. CRC is a highly prevalent cancer with high mortality (36%) representing 11% of all cancer deaths annually [9]. Recent studies in colon cancer cell lines suggest that ATOH1 can inhibit tumor cell growth in vitro [10]. If the anti-oncogenic function of Drosophila aito is conserved in its mammalian counterparts, one would predict that the loss and gain of function of ATOH1 would enhance and suppress tumor formation, respectively, in MCC and CRC models. In addition, the ATOH1 should be subject to loss-of-function mutations in a significant number of human cancer patients.

We tested this prediction in two different mouse models for colon cancer, as well as human MCC and CRC cell lines. In addition, we examined the status of the ATOH1 locus in primary human tumors derived from human MCC and CRC patients. Our data show that loss of ATOH1 strongly enhances the formation and progression of tumors in mice and human cell lines. Conversely, gain of ATOH1 function strongly inhibits the oncogenic phenotypes in human cell lines. Furthermore, we find genetic and epigenetic loss-of-function mutations with very high frequency in primary human tumors derived from ATOH1-dependent tissues and provide biochemical insight into how these epigenetic mutations may arise. Finally, we describe a highly conserved anti-oncogenic molecular signaling pathway that links ATOH1 activity to the stress sensor Jun N-terminal kinase (JNK) pathway mediated via cell type-specific differential co-option of receptor tyrosine kinases (RTKs). Together with the genetic analysis in Drosophila [7], these data support a novel, highly conserved tumor suppressor function for the Atonal group of transcription factors.

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Abbreviations: AOM, azoxymethane; CGH, comparative genomic hybridization; CRC, colorectal cancer; GALT, gut-associated lymphoid tissue; JNK, Jun N-terminal kinase; MCC, Merkel cell carcinoma; NTRK, Neurotrophic tyrosine kinase receptor type 1; RTK, receptor tyrosine kinase; RT-qPCR, quantitative reverse-transcriptase PCR

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Results

Loss of Atoh1 Promotes Tumor Formation in Two Colorectal Cancer Mouse Models

In the mouse, Atoh1 is a master regulator of secretory cell fate commitment in the intestinal epithelium [2,11]. To investigate whether Atoh1 plays a role in intestinal tumors, we analyzed the function of the mammalian ato homolog, Atoh1, in colon tumorigenesis. We assessed the tumor susceptibility of mice with an intestine-specific deletion of Atoh1 (Atoh1\textsuperscript{Dintestine}) [3] in two different established mouse models of CRC.

We first treated Atoh1\textsuperscript{Dintestine} mice with azoxymethane (AOM), a chemical carcinogen that preferentially induces colon tumors. We found a significant enhancement in polyp formation in the large intestines of Atoh1\textsuperscript{Dintestine} compared to wild-type littermate mice, characterized by increased incidence (33% [4/12] vs. 100% [8/8], \( p < 0.005 \); Figure 1A), multiplicity (0.75 vs. 10.3 polyps/colon, \( p < 0.0008 \); Figure 1C), and size (2.1 vs. 4.1 mm/polyp, \( p < 0.0004 \); Figure 1E) when examined under the dissecting microscope. Histological analysis of colons from AOM-treated Atoh1\textsuperscript{Dintestine} mice

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**Figure 1. Loss of Atoh1 Enhances Tumor Formation in the Mouse Colon**

(A) shows the incidence of polyps in AOM-treated mice. (B) shows the incidence of polyps in the Atoh1\textsuperscript{WT} and Atoh1\textsuperscript{Dintestine} mice in an APC\textsuperscript{min} background. In (A) and (B), the two-tailed Fisher exact test was used. (C) Bar graph shows the average number of macroscopically visible polyps (>1 mm) in the colons of AOM-treated Atoh1\textsuperscript{WT} (WT; \( n = 9 \) polyps in 12 mice, white bars) and Atoh1\textsuperscript{Dintestine} (\( n = 82 \) polyps in 8 mice, black bars). (D) The average number of polyps in the colons of APC\textsuperscript{min} (\( n = 27 \) polyps in 27 mice) and APC\textsuperscript{min}; Atoh1\textsuperscript{Dintestine} (\( n = 123 \) polyps in 10 mice). (E and F) The average maximum diameter of each polyp is shown as a bar graph for Atoh1\textsuperscript{WT} or Atoh1\textsuperscript{Dintestine} mice; and (F) from APC\textsuperscript{min} and APC\textsuperscript{min}; Atoh1\textsuperscript{Dintestine} mice. For (C–F), the two-tailed Student \( t \)-test was used to measure significance. Error bars indicate the standard error of the mean. Double asterisks (**) indicate \( p < 0.01 \); triple asterisks (***') indicate \( p < 0.001 \).

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confirmed these findings and showed a range of histological phenotypes, from severely hyperplastic mucosa with embedded multifocal adenomas, to large, highly dysplastic adenomas, with one animal having invasive adenocarcinoma (Figure 2A, 2A′, and 2A″). In contrast, histological examination of AOM-treated Atoh1wt littermates showed none with adenomatous changes; with all polyp-like structures shown to be gut-associated lymphoid tissue (GALT; Figures 2B and S1). These data support the notion that loss of Atoh1 can be an initiating event in mammalian cancer formation.

In previous work, we found that ablation of Atoh1 leads to an increase of proliferation but has no effect on apoptosis [3]. We therefore examined proliferation and apoptosis in the “preneoplastic” normal-appearing epithelium within the colons of AOM-treated Atoh1wt and Atoh1Δintestine mice. As in the Drosophila model, we find more proliferation of epithelial cells in Atoh1Δintestine crypts compared to nonrecombined wild-type crypts within the same animals, or compared to crypts from Atoh1wt littermates (Figures 2E and S2D–S2F) but no obvious difference in apoptosis (Figures 2F and S2G–S2I).

We extended our tumor analysis with an independent genetic mouse model for CRC by crossing Atoh1Δintestine mice to APCmin mice (APC: ENSMUSG00000005871), in which Wnt signaling is constitutively activated [12,13]. APCmin mice develop spontaneous adenomas primarily in the small intestine, with only occasional polyps in the colon [14]. Interestingly, Wnt signaling is thought to interact negatively with a to during sense organ formation in the developing Drosophila epithelium [15]. Comparing the large intestines of APCmin, Atoh1wt mice to APCmin, Atoh1Δintestine littermates at 16 wk, we find significantly more polyps in the colons of double-mutant mice, characterized by increased incidence (52% [14] vs. 100% [10/10], p < 0.007; Figure 1B), multiplicity (1.1 vs. 12.3 polyps/colon, p < 0.0001; Figure 1D), and size (3.3 vs. 4.3 mm/colon, p < 0.0002; Figure 1F). Histological examination confirmed these polyps as adenomas in both APCmin; Atoh1wt and APCmin; Atoh1Δintestine mice, with two polyps in APCmin; Atoh1Δintestine mice having progressed to invasive adenocarcinoma (Figure 2C and 2D). The polyps in the APCmin; Atoh1Δintestine mice originated from the Atoh1 mutant crypts as indicated by the absence of goblet cells in the polyps (Figure S3A and S3B).

In summary, thus far, loss-of-function analysis of Atoh1 in two mouse models of colon tumorigenesis supports a role for Atoh1 as a key switch in tumor formation and progression. This role appears to be mediated by increased cell proliferation in the absence of Atoh1. Importantly, the mouse tumors lack the secretory cell types that depend on Atoh1 for their formation. Thus, the role of Atoh1 in tumor formation is likely linked to its function as a master regulator of differentiation.

**Loss of ATOH1 Expression in Primary Human CRC and MCC Tumors**

Given the remarkable evolutionary conservation of ato/Atoh1 function in cancer development, we decided to analyze whether loss of the human ortholog, ATOH1, might be selected for during malignant transformation in human cancer. To this end, we investigated the role of ATOH1 in CRC and MCC. In vitro overexpression experiments in CRC cell lines suggest that ATOH1 gain of function decreases the population growth potential of CRC cells [10]. Similarly, expression analysis in MCC cell lines suggests an inverse correlation between ATOH1 levels and the population growth of MCC cells (Figure S4A–S4C) [16]. These observations hint at a potential role for ATOH1 in these cancers.

To test this possibility in vivo in primary human tumors, we began by asking whether the expression of ATOH1 is down-regulated in primary tumor samples from 42 CRC and four MCC patients. As MCC is a very rare cancer, only four samples were available for analysis. Seventy percent of the CRC samples show a significant decrease of ATOH1 mRNA expression compared to tissue-matched colon samples from normal controls as analyzed by quantitative reverse-transcriptase PCR (RT-qPCR), suggesting that loss of ATOH1 expression is a highly common feature of CRC oncogenesis (Figures 3F and S4). Furthermore, ATOH1 mRNA levels are significantly lower in adenocarcinoma samples compared to adenomas (t-test: p = 0.017), indicating progressive loss of ATOH1 expression levels with increasing tumor severity (Figure 3A). Similarly, in MCC, the two samples with lower ATOH1 expression levels were derived from the two patients showing metastases (Figure S5).

**CRC and MCC Patients Show Deletions in the ATOH1 Locus**

One mechanism to explain loss of gene expression during oncogenesis is the deletion of the locus, which we tested for using quantitative PCR on genomic DNA in all 46 patient samples, one MCC cell line (MCC14.2), and one CRC cell line (Ht29). At least one deletion in the locus was detected in 57% of the samples (Figure 3B and S3F). A second primer set yielded similar results (49% deletion rate, Figure 3B). One allele was also deleted in both Ht29 and MCC14.2. Comparative genomic hybridization (CGH) array experiments [17] on three samples did not show a deviation of the clones flanking the ATOH1 locus (Figures S5 and S6), indicating that the deletions observed in patients are likely to be ATOH1-specific microdeletions. When only one copy is deleted, complete loss of gene function could be achieved by point mutations in the remaining allele. Sequencing of the ATOH1 open reading frame of 24 samples, however, did not reveal any mutations in any of the samples (unpublished data). Together with the loss of ATOH1 mRNA expression in patients, these results suggest that an epigenetic silencing mechanism may be involved—a possibility we tested in further detail.

**The ATOH1 Locus Is Methylated in CRC and MCC Patients**

To address a putative epigenetic transcriptional silencing mechanism, we began by asking whether transcription could, in principle, be initiated from the ATOH1 locus in cancer cells. Drosophila ato and mouse Atoh1 are both known to be autoregulatory [18,19]. This provides the opportunity to test whether transcription could be activated from the ATOH1 locus by ATOH1 itself in normal versus cancer cells. We took advantage of expressing the mouse ortholog in human cells to distinguish the expression of endogenous ATOH1 from that of Atoh1. For the tests in human cancer cell lines, we used the MCC14.2 cell line, which is derived from MCC and has strongly reduced expression of ATOH1. Surprisingly, Atoh1 overexpression failed to activate endogenous human ATOH1 expression in the MCC14.2 cell line (Figure 3C), despite the virtually complete conservation of the two proteins. This may be due to a key difference between the two proteins, or to a
Figure 2. Increased Proliferation Contributes to Tumorigenesis in Atoh1Δintestine Colon

(A) Hematoxylin and eosin staining of a representative adenoma in AOM-treated Atoh1Δintestine colon (5× magnification). The section is in the rectal region and is characterized by cystic structures with severe crypt hyperplasia with multifocal embedded adenomas and severe lymphocyte infiltration of the submucosa. (L, lumen; M, muscle). (A′) and (A″) show higher magnification (20×) of two areas of the adenoma, indicating the aberrant branched crypt structures that are imbedded in the submucosa.

(B) The colons of AOM-treated Atoh1wt mice showed large GALT (highlighted by arrows) that were macroscopically counted as polyps. Magnification is 5×. Note the relative size of the GALT compared to adjacent normal-appearing crypts.

(C) Histological analysis of colon in APCmin.
Histological analysis in APC\textsuperscript{min}, Atoh1\textsuperscript{null} mice show highly dysplastic adenomas with cystic structures and lymphocyte infiltration.

Proliferation was measured by determining the percentage of BrdU-positive epithelial cells in AOM-treated Atoh1\textsuperscript{wt} and Atoh1\textsuperscript{null} normal-appearing colonic crypts. The white bar represents crypts from Atoh1\textsuperscript{wt} mice; the gray bar represents nondeleted Atoh1\textsuperscript{wt} crypts, and the black bar Atoh1-null crypts in Atoh1\textsuperscript{null} mice.

Apoptosis was measured by determining the percentage of cleaved caspase-3 (c-Caspase 3)-positive epithelial cells in AOM-treated Atoh1\textsuperscript{wt} and Atoh1\textsuperscript{null} normal-appearing colonic crypts. Bar shading indicates crypt genotype as in (E).

For each graph in (E and F), the two-tailed Student t-test was used to measure significance: a single asterisk (*) indicates \( p < 0.05 \); double asterisks (**) indicate \( p < 0.01 \). Error bars indicate the standard error of the mean.

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**Figure 3.** Loss of ATOH1 Expression and Genomic Deletions in CRC Patient Samples

(A) ATOH1 expression normalized to GADPH and control colon samples: ATOH1 expression is lower in tumor compared to control samples. Additionally, adenocarcinomas have significantly lower ATOH1 expression than adenomas. Box plot indicating 25–75 percentiles, central line indicates the median, red cross indicates the mean. Error bars indicate next data point. Outliers are represented as dots. Double asterisks (**) indicate \( p < 0.01 \) (t-test).

(B) Deletions in the ATOH1 locus. Upper dashed line indicates the upper limit of single-deletion detection set at 0.70 of the ratio of ATOH1 locus to control locus. White and gray bars indicate different primer sets.

(C) Endogenous ATOH1 expression in different MCC14.2-derived cell lines and human keratinocytes (HK) in response to Atoh1 expression.

(D) Detection of methylation at the ATOH1 locus using pull-down assay of methylated DNA. Bands in “M-CpG pulldown” indicate positive for ATOH1 methylation. “Input” shows DNA input before the pull-down. Samples were processed blindly. Internal methylated and unmethylated controls are shown in the last four lanes.

(E) Inhibition of methyltransferase activity with 5-aza-deoxycytosine for 7 d leads to an increase of ATOH1 expression in the Ht29 cell line. Error bars indicate the standard deviation.

(F) Graph representing observations on the genomic and mRNA levels. First column: percentage of patients showing deletions or duplications (black: deletions, gray: two copies, and white: duplications). Second column: percentage of patients showing methylation using the pull-down of methylated DNA assay (black: methylated, and white: not methylated). Third column: mRNA expression of CRC samples versus control colon (black: low expression, grey: normal expression, and white: high expression).

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possible disruption of the autoregulatory loop during oncogenesis. To test whether Atoh1 can activate ATOH1 expression in normal noncancerous skin cells, we transduced normal human primary keratinocytes with an Atoh1 expression vector and tested the expression of endogenous ATOH1 40 h after lentiviral transduction. In contrast to the cancer cell lines, endogenous ATOH1 is up-regulated 38-fold by Atoh1 (Figure 3C). This indicates that the expressibility of the ATOH1 locus is inhibited during oncogenesis.

Genomic database searches show that all known ATOH1 orthologs, from human to Drosophila, reside in a CpG island covering at least the promoter and the transcription start site, suggesting that CpG methylation may be a mechanism of ATOH1 loss of function. We therefore assayed all patient samples and both cell lines for ATOH1 methylation using three independent assays: pull-down of methylated DNA, methylation-sensitive restriction digest, and methylation-specific PCR for bisulfite DNA modification. We found that up to 81% of the patients, as well as both cell lines, show methylation of the ATOH1 locus in both the coding sequence and the promoter sequences directly upstream of the ATG. In contrast, none of the control samples show ATOH1 methylation (Figures 3D, 3F, and S7A–S7C). This suggests that methylation is likely causal to the transcriptional silencing of the locus during oncogenesis as this is accompanied by lower ATOH1 expression. In a few cases, however, the correlation was not observed. In two cases (samples 27 and 30), this may be due to the presence of a putative duplication, which is yet to be methylated. Alternatively, but not exclusively, it may be due to heterogeneity of the degree of methylation in different cells comprising the tumor samples analyzed. To provide evidence that methylation silences the ATOH1 locus, we inhibited DNA methyltransferases (Dnmts) with 5-aza-deoxycytidine in the Ht29 cell line. This results in an approximately 8-fold increase in ATOH1 expression (Figure 3E). Thus, the ATOH1 locus is methylated in cancer patients and derived cell lines, and this methylation can be reversed, resulting in the transcriptional reactivation of the locus. The autoregulatory function of ATOH1 combined with the methylation of its regulatory sequences might hint to the involvement of ATOH1 in the silencing of its own promoter. To gain some insight into whether this may indeed be the case, we asked whether Atoh1 can physically interact with Dnmt proteins. We tested the binding of Atoh1 to Dnmt1 (ENSG00000130816), Dnmt3a (ENSG00000119772), and Dnmt3b (ENSG00000088505) using GST pull-down assays. We find that Atoh1 binds to all three Dnmts. This is further supported by the ability of GST-Atoh1 to pull down DNA methyltransferase activity from nuclear extracts, similar to the positive control (EED: embryonic ectoderm development, ENSG00000074266) [20]. Focusing on Dnmt1, the maintenance DNA methyltransferase, we confirmed this observation using coimmunoprecipitation experiments in 293T cells transfected with HA-tagged Atoh1. Lastly, mapping experiments identified several regions of Dnmt1 as mediating the association with Atoh1 (Figure S8A–S8D).

In summary, deletions and epigenetic silencing via methylation combine to cause loss-of-function mutations of the human ATOH1 locus in primary human cancers. Together with loss-of-function evidence in five independent cancer models in human cells, Drosophila, and mouse, these data strongly support a role for the Atoh1/ATOH1 transcription factors as key modulators of oncogenic transformation.

ATOH1 Functions via JNK-Dependent Inhibition of the Cell Cycle and Induction of Apoptosis

To better understand the role that ATOH1 plays in cancer, we sought to determine the molecular mechanism by which it acts to suppress the formation and progression of tumors. Gain- and loss-of-function analyses point to Atoh1-dependent regulation of proliferation in mouse colon tumors (Figures 2E and S3D–S3F). Analysis of the role of Drosophila ato in fly retinal tumors [7] shows that this function is mediated by the JNK signaling pathway. We hypothesized that the Ato-JNK-p21 pathway may mediate the tumorigenic phenotype in our mouse models of CRC as well. If loss of ATOH1 were indeed an initiating event in tumor formation, we reasoned that the effects of Atoh1 function would have to be detectable in preneoplastic tissue as this is where oncogenesis begins. We therefore first examined the expression levels of Atoh1, Cdkn1a (p21, ENSG00000124762), Cdkn1b (p27, ENSG00000111276), and Cdkn1c (p57, ENSG00000129757) and the JNK target cJun (ENSG00000177606) in colon crypts isolated from Atoh1wt and Atoh1intact (mouse) (Figure 4A). We observe an 6.8-fold reduction in Atoh1 expression and an approximately 2.8-fold reduction in expression of all three Cdkn1 isoforms in colon crypts from Atoh1intact mice. We also observe a similar reduction in cjun mRNA levels.

We further examined the Ato-JNK-Cdkn1 pathway by immunoblotting proteins from colon polyps and normal-appearing colon tissue from APCmin/–; Atoh1wt and APCmin/–; Atoh1intact mice. Colon polyps show higher levels of cjun and p21wt protein compared to normal colonic tissue. Importantly, however, we find a reduction in cjun levels in APCmin/–; Atoh1intact polyps compared to APCmin/–; Atoh1wt polyps, consistent with the mRNA analysis showing less cjun (Figure 4B). Importantly, we find a reduction in p27 protein and a trend toward less p21 in Atoh1-mutant colon tissues and polyps, consistent with our analysis of mRNA levels (Figure 4B). We also find a specific reduction in pJNK1 levels (Figure 4C) in APCmin/–; Atoh1wt versus APCmin/–; Atoh1intact tissues, in agreement with earlier reports that JNK1 (MAPK8; ENSG00000107643) may have anti-oncogenic activity in the mouse intestine [21], whereas JNK2 (MAPK9; ENSG00000050748) deficiency enhances tumorigenesis in other epithelia [22]. Finally, we examined the pattern of JNK activation in the colon of Atoh1wt and Atoh1intact mice, and observe identical numbers of pJNK1/2-positive cells in Atoh1-null compared to control crypts (Figure S9). Together with western blot analysis demonstrating selective reduction in pJNK1, but not pJNK2, our data suggest that the level of pJNK1 in individual crypt cells is decreased upon loss of Atoh1. These data are consistent with a preneoplastic function of Atoh1 in regulating proliferation, by a JNK-dependent induction of cell cycle inhibitors.

Next we asked whether the molecular mechanism of ATOH1 function in human cancer is similar to the mouse. We took advantage of several established MCC cell lines [23,24] with either high ATOH1 expression (MCC1 and MCC6, derived from less aggressive tumors) or low ATOH1 expression (MCC13, MCC14.2, and MCC26, derived from highly aggressive metastatic tumors; Figure S4A and S4C). We find that the growth rate of cell lines, measured as their doubling
time, correlates inversely with levels of ATOH1 expression (Figure S4B). To determine whether the reduction in ATOH1 levels might be causal to decreased doubling time, we restored ATOH1 function by creating stable cell lines expressing Atoh1 using lentiviral vectors (Figure 5A and 5B).

Stable Atoh1-expressing cell lines (MCC14.2-Atoh1.1a, MCC14.2-Atoh1.1b, MCC14.2-Atoh1.2a, and MCC14.2-Atoh1.2b) have a significantly slower population doubling time compared to control cell lines (MCC14.2 and MCC14.2-GFP; p < 0.0001; Figure 5G). To assess whether this increase in doubling time reflects decreased malignancy, we tested these cell lines for growth in soft agar. Cell lines with high levels of Atoh1 expression display a marked decrease in growth in soft agar compared to control cell lines (Figure 5D, p < 0.001). The change in population doubling time could be due to a slower cell cycle, an increased apoptotic rate, or both.

Although the distribution of cells in the cell cycle appears unaltered (Figure S10A), the speed of the cell cycle is 25% lower in Atoh1-expressing cells, as assayed by BrdU pulse-chase experiments (Figure 5E, p < 0.01).

In addition to slower proliferation, we find a specific and strong increase in apoptotic cell death, as measured by annexin-V and cleaved caspase-3 (ENST00000308394), in MCC (MCC14.2 series) and CRC (Ht29) cell lines transduced or transfected with Atoh1 (Figures 6A, 6B, and S10B). This increase in cell death is mediated by the intrinsic apoptosis pathway, as suggested by enhanced caspase-9 (ENST00000333868) cleavage (Figure 6A and 6B).

Data from mouse models indicate an involvement of JNK-mediated regulation of cell proliferation for the tumor suppressor effect of Atoh1. To assess whether the same mechanisms are operating in human cancer, we tested the
expression levels of the caspase-3 and caspase-9, p21waf1, and p-JNK in MCC and CRC cells lines. We note a clear and specific up-regulation of p21waf1 and p-JNK levels upon Atoh1 expression (Figures 6A, 6B, and S10C–S10H). Similarly, transfection of wild-type Drosophila Ato results in the up-regulation of cleaved caspase-3, p21waf1, and p-JNK (Figure 6C), in contrast to a null-mutant form of the protein that completely fails to bind DNA [25], indicating the conservation and specificity of the Ato/Atoh1 effect.

Next, we tested the expression of these proteins in a loss-of-function setting. We generated and expressed the transcriptional repressor form of ATOH1 by fusing it to the engrailed repressor domain (ATOH1ERD) [26], which specifically inhibits the Atoh1-induced effects on MCC cell lines (Figure 6D). In the MCC1 cell line, which shows higher endogenous ATOH1 expression compared to MCC14.2 (Figure S4C), expression of ATOH1 ERD leads to a down-regulation of p21waf1 and p-JNK as well as inhibition of caspase-3 cleavage (Figure 6E).

In summary, gain- and loss-of-function studies in mouse

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**Figure 5. ATOH1 Suppresses Growth by Interfering with the Cell Cycle**

(A) Constructs used for creating lentiviral and transfection vectors.

(B) Western blot analysis for Atoh1 and chromogranin on untransduced MCC14.2 cells (lane 1), GFP-transduced MCC14.2 cells (lane 2), and two independently derived MCC14.2 cell lines transduced with Atoh1-IRES-eGFP (lane 3: MCC14.2-Atoh1.1a, and lane 4: MCC14.2-Atoh1.2a). Quantifications in Figure S12E and S12F.

(C) Doubling time in hours for MCC14.2 (lane 1), MCC14.2-GFP (lane 2), and four lines independently transduced with Atoh1-IRES-eGFP (lanes 3–6: MCC14.2-Atoh1.1a, MCC14.2-Atoh1.1b, MCC14.2-Atoh1.2a, and MCC14.2-Atoh1.2b, respectively).


The triple asterisks (*** in (C and D) indicate a significant difference from MCC14.2 (*t*-test: *p* < 0.001).

(E) Percentage of cells positively labeled for BrdU and past S-phase in a BrdU pulse-chase experiment; double asterisks (**) indicate *p* < 0.01 (*t*-test). doi:10.1371/journal.pbio.1000039.g005
colon cancer models and human cancer cells support a conserved antitumor function for ATOH1 mediated by JNK and p21.

**ATOH1 Activates RTK Expression in Human Cancer Cells**

How does ATOH1 expression lead to JNK activation in cancer cells? ATOH1 and its orthologs are known to exert their developmental functions by modulating Notch; Atonal orthologs inhibit Notch signaling to induce differentiation during development, whereas Notch inhibits ato expression by its target gene Hes1 (ENSG00000114315) [27–29]. Notch signaling has also been described to be upstream of JNK [30]. However, three lines of evidence suggest that the Atoh1-mediated effects are not Notch-dependent. First, Atoh1 expression levels do not influence expression of HES1, a Notch signaling target gene (Figure S11A). Second, we do not detect cleaved intracellular Notch in the MCC14.2 cell line, which expresses low levels of ATOH1 (Figure S11B). Finally,
blocking of Notch activation by selective inhibition of 
\(\gamma\)-secretase [31] has no effect on the growth of MCC cells
(Figure S11C). Other known upstream activators of JNK are
RTKs [32]. As Atoh1 is a transcription factor, we checked
mRNA expression of all 90 human RTKs upon
\(\text{Atoh1}\) expression in both the MCC14.2 and the Ht29 cell line
compared to control cells. We observed a significant and
specific \(\text{Atoh1}\)-dependent up-regulation of Neurotrophic
tyrosine kinase receptor type 1 (NTRK1: ENSG00000198400),
a hallmark for differentiation in Merkel cells [33,34] in
MCC14.2, and of FGF receptors in Ht29 (Figure S11D). The
increase in NTRK1 expression in MCC14.2 seen on the
mRNA level was confirmed using RT-qPCR (Figure S10E). We
also observed elevated NTRK1 levels in the endogenously
\(\text{ATOH1}\)-expressing MCC1 cells (Figure S10E). This was
accompanied by higher protein levels of both the NTRK1
receptor and one of its ligands, Neurotrophin-3 (NT3:
ENSG00000185652; Figure S10E–S10H), also a marker for
Merkel cell differentiation. Therefore, Ato gain of function
results in tumor type-specific elevation of RTK levels.

\(\text{ATOH1 Functions by RTK-Mediated JNK Activation}\)

To test whether the RTKs may be functionally linked with
\(\text{ATOH1}\), we incubated the various MCC cell lines with K252a,
a narrow-specificity RTK inhibitor (NTRK, FGFR, PDGFR,
and IGFR) [35–37]. This results in a dose-dependent decrease
in doubling time of the MCC14.2-derived,
\(\text{Atoh1}\)-expressing cell lines, indicating that the
\(\text{Atoh1}\)-induced change in
doubling time is RTK-dependent (Figure 7A). In addition,
the induction of apoptosis by \(\text{Atoh1}\) in the MCC14.2 and the
Ht29 cell line is blocked by RTK inhibition (Figure 7B and
7C). These effects are accompanied by RTK inhibitor–
dependent decrease in the expression of \(\text{p21}^{\text{wt}}\) and \(\text{p-JNK}\)
(Figure 7D), suggesting that both \(\text{p21}^{\text{wt}}\) and \(\text{p-JNK}\) are
downstream effectors of \(\text{Atoh1}\)-induced RTK signaling. Next,
we asked whether \(\text{p-JNK}\) is required for \(\text{p21}^{\text{wt}}\) up-regulation
and apoptosis by treating MCC cells with SAPK Inhibitor II, a

![Figure 7. Molecular Mechanism for ATOH1 Function](plosbiology.org)

(A–D) show that ATOH1’s anti-oncogenic function acts through receptor tyrosine kinases. (A) Doubling times in hours of MCC14.2 and MCC14.2 transduced with \(\text{eGFP}\) (lane 2) or \(\text{Atoh1-}\text{IRES-eGFP}\) (lanes 3 and 4) with increasing concentrations of K252a. (B) AnnexinV-positive signals normalized to
transfected MCC14.2 cells (GFP). Cells transduced with \(\text{eGFP}\) (lanes 1 and 2) and \(\text{Atoh1-}\text{IRES-eGFP}\) (lanes 3 and 4) with 0.33 \(\mu\text{M} \text{K252a}\) (lanes 2 and 4) or
DMSO as a control (lanes 1 and 3). The double asterisks (***) indicate significant difference from GFP transacted without K252a (t-test: \(p < 0.05\)). (C) AnnexinV-positive signals normalized to transfected Hi29 cells (GFP). Cells transduced with \(\text{eGFP}\) (lanes 1 and 2) and \(\text{Atoh1-}\text{IRES-eGFP}\) (lanes 3 and 4) with 0.33 \(\mu\text{M} \text{K252a}\) (lanes 2 and 4) or DMSO as control (lanes 1 and 3). The double asterisks (***) indicate significant difference from GFP transacted without K252a (t-test: \(p < 0.05\)). (D) Inhibition of RTKs inhibits \(\text{p21}^{\text{wt}}\) and JNK phosphorylation. Western blot analysis for \(\text{p21}^{\text{wt}}\) and \(\text{p-JNK}\)
of MCC14.2 (lanes 1 and 5), MCC14.2 transduced with \(\text{eGFP}\) (lanes 2 and 6), and two MCC14.2-derived cell lines transduced with \(\text{Atoh1-}\text{IRES-eGFP}\) (lanes 3, 4, 7, and 8) with 0.3 \(\mu\text{M} \text{K252a}\) (lanes 1–4) and with DMSO as a control (lanes 5–8). Actin loading controls are shown below each blot. Quantifications
are shown in Figure S12U and S12V.

(E) JNK regulates apoptosis and \(\text{p21}^{\text{wt}}\) expression. Western blot analysis for \(\text{p21}^{\text{wt}}\) and cleaved caspase-3 of MCC14.2 (lanes 1 and 5), MCC14.2 transduced with \(\text{eGFP}\) (lanes 2 and 6), and two MCC14.2-derived cell lines transduced with \(\text{Atoh1-}\text{IRES-eGFP}\) (lanes 3, 4, 7, and 8) with 1 \(\mu\text{M} \text{JNK inhibitor}\)
(lanes 1–4) and with DMSO as a control (lanes 5–8). Actin loading control is shown below the blots. Phospho-JNK (P-JNK) blot shows the effect of the
JNK inhibitor on JNK phosphorylation status Quantifications are shown in Figure S12W–S12Y.

(F) Relative cell numbers (the number of Tam67-transfected cells divided by the number of mock-transfected cells) for MCC14.2 and MCC14.2 transduced with \(\text{eGFP}\) (lane 2) or \(\text{Atoh1-}\text{IRES-eGFP}\) (lanes 3 and 4) with dominant-negative c-jun (Tam67).

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ATOH1 Functions by RTK-Mediated JNK Activation

To test whether the RTKs may be functionally linked with
\(\text{ATOH1}\), we incubated the various MCC cell lines with K252a,
a narrow-specificity RTK inhibitor (NTRK, FGFR, PDGFR,
and IGFR) [35–37]. This results in a dose-dependent decrease
in doubling time of the MCC14.2-derived, \(\text{Atoh1}\)-expressing
cell lines, indicating that the \(\text{Atoh1}\)-induced change in
doubling time is RTK-dependent (Figure 7A). In addition,
the induction of apoptosis by \(\text{Atoh1}\) in the MCC14.2 and the
Ht29 cell line is blocked by RTK inhibition (Figure 7B and
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dependent decrease in the expression of \(\text{p21}^{\text{wt}}\) and \(\text{p-JNK}\)
(Figure 7D), suggesting that both \(\text{p21}^{\text{wt}}\) and \(\text{p-JNK}\) are
downstream effectors of \(\text{Atoh1}\)-induced RTK signaling. Next,
we asked whether \(\text{p-JNK}\) is required for \(\text{p21}^{\text{wt}}\) up-regulation
and apoptosis by treating MCC cells with SAPK Inhibitor II, a
specific JNK inhibitor [38]. We observed a decrease in the Atoh1-induced expression of p21<sup>wafl</sup> and cleaved caspase-3 (Figure 7E). Similarly, transfection of dominant-negative c-jun (TAM67) [39] leads to a significant increase in cell number compared to mock-transfected cells (t-test: \( p = 0.04 \)), specifically in the MCC14.2-Atoh1.2a, which has high Atoh1 expression levels (Figure 7F).

In summary, taken together, evidence from mutation analysis in human patients, as well as gain- and loss-of-function analysis in mouse and human cells, support a model (Figure 8) in which ATOH1 modulates JNK activity, possibly via co-option of context-specific RTK signaling, to induce apoptosis and up-regulate p21<sup>wafl</sup> expression, keeping tumor growth in check. Loss-of-function mutations in ATOH1 prevent JNK-mediated apoptosis and p21-mediated cell cycle arrest, leading to enhanced tumor progression.

**Discussion**

Our data support an evolutionarily conserved tumor suppressor role for ATOH1 in CRC and MCC. Loss of ATOH1 promotes tumor formation and progression, and mutations in the ATOH1 locus are found with relatively high frequency. Given the high deletion and methylation rate of ATOH1 in human tumor samples, loss of ATOH1 function is likely to be an early event in these tumors. We therefore propose that ATOH1 acts as a key switch regulating the transformation of pre-oncogenic epithelia to neoplastic and metastatic tumors.

Genetic analysis of the function of *Drosophila ato* in fly eye tumor suggests that its anti-oncogenic function is linked to its activity as a regulator of cell fate commitment and differentiation [7]. This is similar to what we observe in the different mouse models, where the adenomas and adenocarcinomas do not contain secretory cells (Figure 2) [3]. Interestingly, this also appears to be the case in human cancer, where the majority of human CRCs do not contain differentiated secretory cells. The loss of ATOH1 in most human CRC patients likely explains this observation. In addition, cell type-specific RTK differentiation genes are up-regulated upon overexpression of *Atoh1* in CRC and MCC cell lines. Importantly, these markers of differentiation are necessary for the anti-oncogenic effect of *ato*/ATOH1.

It is tempting to speculate that in other tissues, similar loss of differentiation factors is involved in oncogenesis. In this sense, the loss of differentiation factors has already been implicated in late stages of tumor progression as with GATA-3 (ENSG00000107485) in breast cancer [40]. Although loss of GATA-3 in early tumor leads to an inhibition of tumor formation, loss of GATA-3 in later stages leads to the acquisition of metastatic potential. We, therefore, wonder whether loss of other differentiation factors, perhaps bHLH proteins, might play a role in earlier stages of breast cancer development.

Tumor suppressor genes are defined by the fact that (1) loss-of-function mutations make the cells more prone to malignant transformation, (2) overexpression leads to inhibition of the malignant phenotype, and (3) spontaneous somatic mutations are found in patients with cancer. “Classical tumor suppressor genes,” such as *p53* (ENSG00000141510), are special in the sense that when mutated, the cell is more prone to accumulate additional mutations, and thus actively drive malignant progression as opposed to just “taking away the

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**Figure 8.** Schematic Representation of the Potential Mechanism of ATOH1’s Function as a Tumor Suppressor

In preneoplastic tissue, Atoh1 keeps malignant transformation in check in a JNK-dependent mechanism by the induction of apoptosis and the inhibition of cell cycle progression. When Atoh1 is lost due to deletion or methylation, these brakes on oncogenesis fail, and malignant transformation can progress.

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brakes” [41]. It is notable that ato1/Atoh1 shows most of the hallmarks of a tumor suppressor gene. Because silencing of ato1/Atoh1 is not sufficient to drive oncogenesis, we suggest that ato1/Atoh1, and similar genes, are important brakes on malignant transformation. Therefore, the role that differentiation factors might play as key switches in malignant transformation in different tissues is not different from the classical definition of tumor suppressor in a functionally relevant sense.

The RTK and JNK signaling pathways, which are essential for Atoh1’s tumor suppressor activity, have been suggested as context-dependent oncogenes or tumor suppressors [42]. Our data indicate that Atoh1 is important in deciding this context by the up-regulation of cell type–specific RTKs. The activation and co-option of RTK and JNK signaling by Atoh1 in this context suggests that the status of differentiation of the tumor-initiating cell may be the key determinant of the specific role of various signaling pathways in cancer. This may have important clinical implications: treatment of CRC or MCC patients with RTK or JNK inhibitors might have an adverse effect on tumors where ATOH1 is still expressed.

In this medical context, our data suggest that screening for ATOH1 expression, deletion, and methylation may be a useful diagnostic tool for early detection and treatment decision of MCC and CRC. Similarly, treatment of CRC and MCC patients whose tumors show epigenetic silencing of ATOH1 with DNA methyltransferase inhibitors might prove a powerful avenue for therapy, because it appears to be sufficient to restore ATOH1 expression and induce cancer cell death. Furthermore, such treatment in combination with Notch inhibitors may enhance re-expression of the ATOH1-driven differentiation program [43,44] and synergistically inhibit cancer growth. Therefore, elucidation of the basic mechanisms of ato1/Atoh1 function, as well as their target genes and interacting proteins, might offer potential avenues for future therapeutic intervention.

Materials and Methods

Cloning of ATOH1ERD expression construct. The ATOH1 open reading frame was PCR amplified and fused in frame with the engressed repressor domain after XhoI restriction digest. This fusion product was then blunt ligated in the MSCV-ires-GFP vector (Clontech), giving rise to the MSCV-ATOH1ERD-ires-GFP construct.

Mouse models and treatments. Atoh1floX/ and Atoh1floX mice are a conditional deletion of Atoh1 and are described in more detail elsewhere [3,45]. These mice were generated using the loxPcre system in which Cre-mediated deletion of Atoh1 is mosaic and is restricted to the distal ileum and large intestine (80%–90% deletion). Atoh1fllox mice were purchased from The Jackson Laboratory and mated with Atoh1fllox mice to generate Atoh1fllox and Atoh1fllox mice. Eight-week-old male Atoh1fllox and Atoh1fllox littermates were injected intraperitoneally with AOM (Midwest Research Institute of the National Cancer Institute’s chemical carcinogen repository) at 10 mg/kg body weight. AOM was injected weekly for a total of six injections, and the mice were sacrificed and the colon was isolated and flushed with ice-cold PBS, and then fixed in 10%

Immunohistochemistry and microscopic analysis. The colons were imbedded in paraffin blocks and sectioned for hematoxylin and eosin staining by the University of Cincinnati pathology core facility. Tumor phenotypes were determined as in Boivin et al. [2003] [46]. Paraffin-embedded colons of AOM-treated mice were sectioned at 5 μm and used for BrdU and cleaved caspase-3 staining. Mouse anti-BrdU antibodies were purchased from the Hybridoma Bank maintained by the Department of Biological Sciences of the University of Iowa. The sections were de-paraffinized, rehydrated, and antigen retrieval was performed in citric acid buffer (pH = 6) using a microwave. Endogenous peroxidase activity was blocked with hydrogen peroxide/methanol solution, and Avidin/Biotin block was performed according to the manufacturer’s recommendations (Vector Laboratories). Endogenous immunoglobulins block was primary and secondary antibody incubations were performed using the M.O.M. kit following the manufacturer’s recommendations (Vector Laboratories). Tissue sections were incubated for 16 h at 4°C in a humidified chamber. Color was developed using the DAB peroxidase substrate kit (Vector Laboratories) followed with hematoxylin staining and dehydration of the tissues. Nuclei from well-oriented crypts in Atoh1fllox and Atoh1fllox colons were counted at 40× magnification, followed by counts of BrdU-positive cells. The percent of BrdU-positive Atoh1fllox or Atoh1null cells was determined for each animal (at least 1,000 cells per genotype were counted for each animal). Student two-tailed t-test was used to measure significance. Cleaved caspase-3 and counting were performed similarly to BrdU staining and analysis. Polyclonal rabbit anti-cleaved caspase-3 antibodies (1:100) were obtained from Cell Signaling Technology. The Rabbit IgG VECTASTAIN ABC kit was used according to the manufacturer’s recommendations for blocking, antibody incubations, and signal amplification (Vector Laboratories).

Crypt prep. Crypts from wild-type and Atoh1fllox mice were isolated using a modification of the Evans method [47,48]. Mice were sacrificed and the colons removed and flushed with ice-cold PBS, opened flat, and then placed in cold PBS containing protease and phosphatase inhibitors. After a brief vortexing, the colon was cut into four pieces and placed into a 15-ml tube containing shaking solution (1.5 mM KCL, 96 mM NaCl, 27 mM Na Citrate, 8 mM KH2PO4, 5.6 mM Na2HPO4, 15 mM EDTA, and 1 mM dithiothreitol supplemented with protease and phosphatase inhibitors). The tubes were agitated at 4°C on a vortexer holding the tubes at 180°C until the solution appeared cloudy (5–8 min). The colon pieces were transferred into new tubes with shaking buffer and agitated until most of the crypts were released (enrichment was assessed by phase contrast microscopy at different time points). The solution containing the crypts was filtered through a 100-μm cell strainer to isolate the crypts, and sorbitol (2% final concentration) was slowly added and mixed immediately. The solution was centrifuged at 160g for 8 min at 4°C. The supernatant (containing single cells) was removed, and the pellet (containing purified crypts) was snap frozen in liquid nitrogen, to be used for protein and RNA analyses.

Quantitative reverse-transcriptase polymerase chain reaction. RNA was purified from colon crypts isolated from wild-type and Atoh1fllox mice using Trizol (Invitrogen) according to the manufacturer’s recommendations. Trizol-purified RNA (100 μg) was subjected to DNase digestion and further purification (RNaseasy Mini; Qiagen); 2 μg of total RNA was reverse transcribed (Superscript III, Invitrogen), and cDNA equivalent to 100 ng of RNA used for SYBR Green-based real-time PCR using an Mx3005 (Stratagene). For each gene assessed, colon crypt RNA from nine wild-type and eight Atoh1fllox mice was used for relative quantification. All gene expression levels were normalized to the expression of GAPDH. t-Tests measured significant differences between the average normalized expression levels in wild-type versus Atoh1fllox mice.

Whole-tissue/polyp preparations. Cecal and colonic tissues from wild-type and Atoh1fllox mice were homogenized in lysis buffer (1× Phosphate Buffered Saline, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.7 mM EDTA supplemented with protease and phosphatase inhibitors), sonicated, and the lysates used for quantitative western blots. Similarly, lysates of colons and polyps from Atoh1fllox and Atoh1fllox double-mutant mice were used for whole shave preparation and western analysis. The following antibodies were used for western blot analysis: p21 (Cell Signaling) monoclonal antibody (1:500, cat #556431; BD Pharmpingen), actin (1:500, cat #11410; Thermo Scientific), IgM antibody (1:1000; JLA24, Hybridoma Bank), mouse monoclonal p27kip antibody (1:250, cat #610241; BD Transduction Laboratories), goat anti-p57 polyclonal antibodies (1:100, cat # sc-1039; Santa Cruz Biotechnology), rabbit
polyclonal c-fun antibodies (1:1000, cat # sc-1694; Santa Cruz Biotechnology), and pJNK antibody (1:1000, cat #55039; EMD-Calbiochem).

\[ \frac{\ln(2)}{\text{time in culture}} \]

Formalin-fixed tissues from AOM-treated mice were used in the experiments. The procedure has been approved by the ethical committee of the University of Leuven. Experiments performed on normal human keratinocytes were approved by the ethical committee of the University of Leuven. Experiments performed on normal human keratinocytes were approved by the ethical committee of the University of Leuven.

**Cell culture.** MCC cell lines were cultured in RPMI medium supplemented with 15% FCS (Pernix). The H29 cell lines (obtained from the culture Sammlung von Mikroorganismen und Zellkulturen [DSMZ]) were cultured in McCoy medium supplemented with 10% FCS. Primary human keratinocytes were isolated and pooled from foreskins of three different donors (less than 6 y). Fourth passage cells were used in the experiments. The procedure has been approved by the ethical committee of the University of Leuven. Experiments performed on keratinocytes isolated from foreskins of three different donors were approved by the ethical committee of the University of Leuven.

Keratinocytes were seeded in serum-free and growth factor–containing medium (medium and centrifuging the medium. The pellet was resuspended in lysis buffer containing PBS with 1 mL EDTA, 1 mM EGTA, 50 mM NaF, 1% TritonX, 5 mM Na3VO4, 20 mM PAO, and Complete Protease Inhibitor. The crude extract was separated by SDS-PAGE in a 4%–12% NuPAGE bis-tris gel (Invitrogen) and electroblotted onto Hybond-ECL membrane (Amersham). Antibodies were diluted in the appropriate concentrations in 5% BSA in TBS-tween20. The antibodies used are beta-actin (clone AC-15; Sigma), anti–cleaved caspase-3 (Asp-175; Cell Signaling Technology), polyclonal antibody to caspase-9 (Active) (ALEXIS), chromogranin A-B (Abcam), cyclin A-S (Santa Cruz Biotechnology), and Cleaved Notch (Val174; Cell Signaling Technology). All western blot analyses are quantified in Figure S11.

**Tyrosine kinase inhibitor, SAPK inhibitor II, and \( \gamma \)-secretase inhibitor.** K252a diluted in DMSO, from VWR, and DMSO as control were used. Final concentrations ranged from 0.0 μM to 0.5 μM; when no concentration was mentioned, 0.5 μM was used. K252a from Calbiochem, was used at a concentration of 10 μM. We used inhibitor X as a \( \gamma \)-secretase inhibitor (Calbiochem). Concentrations used are mentioned in the figure legend.

**RT-PCR and qPCR.** mRNA was amplified using the Superscript II One-Step RT-PCR system with Platinum Taq-100 reactions (Invitrogen) according to the manufacturer’s protocol. Quantitative RT-PCR was performed on a ABI prism 7000 (Applied Biosystems). The primers were designed using Primer Express (Applied Biosystems). The primers used are ATOH1: CAGGCGTCTGAAGGAAAG and GAAAAATCTCCCGTCCCTTC, and the Hes1 primers GGA- CATTGGAATGAACTGGAAGG and AGGCCAGGCTGTCATCTGC, according to the manufacturer’s protocol. Quantitative RT-PCR was performed on a ABI prism 7000 (Applied Biosystems). The primers were designed using Primer Express (Applied Biosystems). The primers used are ATOH1: CAGCGACGGAAGGAAAG and GAAAATCTCCCGTCGCTTC, and the Atoh1 locus: ATOH1 locus set 1: GCCGGGGAATCTGTG and TCTGGTGTGGTTGAGG. To check copy number of the ATOH1 locus, ATOH1 locus set 1: TCTGGGAGC- GAGCATTAG and GCCGGAATCGATGACATGAAAGA and ATOH1 locus set 2: GCCGAGCCAGAGAGAAAAA and GAAAATCTCCCGTCGCTTC. Control locus set 1: TCTGGGAGC- GAGCATTAG and GCCGGAATCGATGACATGAAAGA and ATOH1 locus set 2: GCCGAGCCAGAGAGAAAAA and GAAAATCTCCCGTCGCTTC. Control locus set 2: GCCGAGCCAGAGAGAAAAA and GCCGGAATCGATGACATGAAAGA.

**Western blotting.** Methylation of the DNA was detected using Apat enzyme. DNA (2 μg) was dissolved in 60 μL of the appropriate buffer and 10 μL of Apat, and incubated at 30 °C overnight. A mirror control was done in which the Apat was exchanged by glycerol as a control for unspecific degradation. The resulting DNA was analyzed using two primer sets: one set spanning the restriction site (AAATAGACGTTGCAAGAGG and TCGCAGGACGAAAAT- TAAAGGCTG) and another set next to the restriction site (CCCGGGGACATGGCAAGGA and TGCCGAGCACCAAAAT- TAAAGGCTG).

Pull-down of methylated DNA fragments (restricted using EcoRI) was performed using the MethylCollector kit (Active Motif), and bisulfite modification of DNA was done using the EZ DNA Methylation-Gold kit (Zymo Research) according to the manufacturer’s protocols.

**Array CGH.** We carried out array CGH using Code Linked Slides (AP Biotech) containing the 3,527 BAC clones from the Wellcome Trust Sanger Institute 1 Mb Clone Set, a gift from N. P. Carter (The Wellcome Trust Sanger Institute). Array CGH was performed as described [17].

**Sequencing.** The ATOH1 open reading frame was amplified with PCR using AATGAGACGTGTAGAAGAG and TCGCAGGACGAAAAT- TAAAGGCTG and AmpliTag Gold DNA polymerase under the following conditions (900 μM AmpliTaq Gold DNA polymerase under the following conditions (900 μM AmpliTaq Gold DNA polymerase and the GeneAmp PCR System 2400 (Applied Biosystems)) were purified and sequenced in both directions on the ABI Prism BigDye (Terminator Cycle Sequencing Kit version 1.1) on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems).

**APOPTOSIS DETECTION.** Annexin-V staining was performed using Annexin-V-biotin (Roche) and streptavidin-PerP (BD biosciences) as described by the manufacturer. Cells were imaged with the Leica TCS Sp2 confocal microscope, and images were analyzed and quantified using LCS software.
Supporting Information

Figure S1. Gut-Associated Lymphoid Tissues in AOM-Treated Atoh1wt Mice
The monoclonal AOM-treated Atoh1wt mice showed large GALT that were macroscopically counted as polyps; shown is 5X magnification of representative colonic GALTs. GALT indicated by an arrow.

Found at doi:10.1371/journal.pbio.1000039.sg001 (726 KB PDF).

Figure S2. Histology of Polyps in APCmin Background
(A) Polyps in the APCmin Background still have goblet cells, indicating that Atoh1 is still active.
(B) The polyps in the APCmin, Atoh1null mice originate in Atoh1 mutant tissue as seen by the absence of goblet cells.

Found at doi:10.1371/journal.pbio.1000039.sg002 (4.70 MB PDF).

Figure S3. Representative Normal- Appearing Crypts in AOM-Treated Atoh1wt and Atoh1null Colon

AOM-treated colon sections with well-oriented crypts were used for BrdU and cleaved caspase-3 (c-Caspase-3) staining. The genotypes of the representative slides are indicated on the left side of the figure. The specific stain is identified at the top part of the figure. Atoh1wt (WT) crypts were distinguished from Atoh1null (null) crypts by the lack of the secretory goblet cells in the null crypts.

(A–C) Hematoxylin and eosin (H&E) staining of Atoh1wt crypts in Atoh1wt mice (A), and nondeleted Atoh1wt (B) and Atoh1null (C) in Atoh1null mice.

(D–F) Representative BrdU staining of normal-appearing crypts in Atoh1wt mice (D) and nondeleted Atoh1wt (E) and Atoh1null (F) in Atoh1null mice.

(G–I) Representative cleaved caspase-3 staining of normal-appearing crypts in Atoh1wt mice (G); and nondeleted Atoh1wt (H) and Atoh1null (I) in Atoh1null mice. Arrows point to positive cells at the surface of the crypts. The images were captured at 20X magnification.

Found at doi:10.1371/journal.pbio.1000039.sg003 (4.96 MB PDF).

Figure S4. ATOH1 Expression Correlates with Population Doubling Time
(A) ATOH1 mRNA transcripts in five independent MCC cell lines. The name of the cell line is indicated above each lane. RT-PCR was done with 100 ng of RNA under nonsaturation conditions.

(B) Doubling times in hours of the five MCC cell lines. Error bars indicate the standard deviation.

(C) RT-qPCR for ATOH1 in MCC1 and MCC14.2 cell lines. ATOH1 mRNA levels standardized to GAPDH mRNA levels.

Found at doi:10.1371/journal.pbio.1000039.sg004 (760 KB PDF).

Figure S5. Summary Table of All Patient Data
First column indicates patient numbers. Next, the relative ratios for ATOH1 genomic DNA (gDNA) over control locus is presented for each of the two primer sets as analyzed by qPCR. The next two columns indicate the classification of the deletion/duplication status of the ATOH1 locus. The mRNA expression is shown relative to control colon samples, with next to it, the classification of the expression. Clinical data are given in the third panel, namely cancer stage and metastasis. In the last panel, methylation of the ATOH1 locus is shown using three different methods.

Found at doi:10.1371/journal.pbio.1000039.sg005 (760 KB PDF).

Figure S6. Array CGH Analysis of Three Patient Samples, Demonstrating No Aberration of the ATOH1 Locus
Each bar represents the log2 of the value for affected individuals (ind) versus a control sample (reference) for each probe, ordered based on the probes’ chromosomal location. The region between 131 Mb and 141 Mb is shaded. The location of the ATOH1 locus is shown with an arrow. The abnormalities were confirmed by dye swap experiments.


Figure S7. Methylation of the ATOH1 Locus
(A) Schematic representation of ATOH1 locus. The white box indicates the ATOH1 ORF, and the gray box the position of the CpG island. The primers are indicated as arrows.

(B) Detection of methylation at the ATOH1 locus using the Aпал methylating-sensitive restriction enzyme. PCR fragments generated using primers spanning the Aпал restriction site from Aпал restricted genomic DNA; presence of a band indicates methylation of the ATOH1 CpG island.

(C) Detection of methylation using methylation-sensitive PCR: upon bisulfite modification, presence of a band indicates methylation of the ATOH1 CpG island.

Found at doi:10.1371/journal.pbio.1000039.sg007 (159 KB PDF).

Figure S8. Atoh1 Binds the Dnmt Proteins and Associates to a DNA Methyltransferase Activity
(A) GST Pull-down assays using Atoh1 protein fused to GST (GST-Atoh1) and in vitro translated Dnmts (IVT-Dnmt1, IVT-Dnmt3a, or IVT-Dnmt3b).

(B) A GST-fused Atoh1 protein was used to purify DNA methyltransferase activity from nuclear extracts. After incubation, the beads were washed with DNA and assayed for DNA methyltransferase activity read as c.p.m. of S-adenosyl-[methyl-3H] methionine incorporated into an oligonucleotide substrate. GST-tagged embryonic ectoderm development protein (GST-EDD) was used as a positive control.

(C) Coimmunoprecipitation experiments shows that Atoh1 associated with Dnmt1. The 295T cells were transiently transduced in culture dishes (10-cm diameter) with 3 μg of HA-Atoh1 plasmid.

(D) Mapping of Atoh1 binding to Dnmt1. GST Pull-downs were performed with Dnmt1 fragments fused to GST and in vitro-translated Atoh1. The upper part is a schematic representation of the Dnmt1 sequences used.

Found at doi:10.1371/journal.pbio.1000039.sg008 (1.04 MB PDF).

Figure S9. Immunohistochemical Analysis of Phosphorylated JNK in Atoh1null and Atoh1wt Mice
(A) Representative pJNK1/2 staining in Atoh1null crypt (dashed white line). The arrows indicate pJNK-positive cells.

(B) pJNK-positive cells in wild-type (dashed gray line) and Atoh1null (dashed black line) crypts in Atoh1null mice.

(C) Bar graph showing the percentage of pJNK-positive cells in wild-type (white); and wild-type (gray) and Atoh1null crypts in Atoh1null mice. Error bars indicate the standard error of the mean.

Found at doi:10.1371/journal.pbio.1000039.sg009 (4.18 MB PDF).

Figure S10. ATOH1 Interacts with the Cell Cycle and Apoptosis
(A) Cell cycle distribution of MCC14.2-derived cell lines without (MCC14.2 and MCC14.2-GFP) and with Atoh1 expression (MCC14.2-Atoh1.1a and MCC14.2-Atoh1.2a). No significant change in distribution throughout the cell cycle can be observed.

(B) Maximal projection image of AnnexinV staining (red) on cells transduced with lentiviral vectors expressing GFP (left panel) or Atoh1-ires-GFP (right panel). GFP is in green.

(C) Western blot analysis for CyclinA1, PCNA, p27kip, c-myc, phospho-H3 and p27 (F), c-myc, phospho-histoneH3. (H) Representative blots are shown in (C).

Found at doi:10.1371/journal.pbio.1000039.sg010 (2.90 MB PDF).

Figure S11. ATOH1 Acts Independently of Notch but Modulates RTK Expression
(A) RT-PCR for target of Notch signaling HES1 on mRNA isolated from MCC14.2 cells, MCC14.2 cells transduced with GFP, and two MCC14.2 cell lines transduced with Atoh1-ires-GFP (MCC14.2-Atoh1.1a and MCC14.2-Atoh1.2a). GAPDH loading control is shown below.

(B) Western blot analysis for cleaved intracellular Notch (NICD). Different concentrations of presinilin inhibitor X were used; a plus sign (+) indicates a positive control for cleaved NICD.

(C) Different concentrations of γ-secretase inhibitor (inhibitor X) on MCC14.2 do not influence the proliferation rate. First lane: 10 μM inhibitor X, second lane: DMSO control of previous lane, third lane: 1 μM inhibitor X, fourth lane: DMSO control of previous lane, and fifth lane: untreated.

(D) RT-PCR for expression of 90 tyrosine kinases scored from undetectable (white) over orange (expression) to red (strong expression) on mRNA or on untransduced MCC14.2 cells (lane 1), GFP transduced MCC14.2 cell line (lane 2), and two independent MCC14.2 different cell lines transduced with Atoh1-ires-GFP activity from lane 3: MCC14.2-Atoh1.2a) and on untransfected HT29 cells (lane 14: HT29), GFP-transfected HT29 cells (lane 15: HT29-GFP), and HT29 cells transfected with the Atoh1-ires-GFP construct (lane 6: HT29-Atoh1).

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