The American Journal of Pathology, Vol. 191, No. 1, January 2021

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The American Journal of
PATHOLOGY
ajp.amjpathol.org

TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Transmembrane and Immunoglobulin Domain Containing 1, a Putative Tumor Suppressor, Induces G2/M Cell Cycle Checkpoint Arrest in Colon Cancer Cells

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Accepted for publication September 23, 2020.

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Colorectal cancer (CRC) is a leading nonfamilial cause of cancer mortality among men and women. Although various genetic and epigenetic mechanisms have been identified, the full molecular mechanisms deriving CRC tumorigenesis are not fully understood. This study demonstrates that cell adhesion molecule transmembrane and immunoglobulin domain containing 1 (TMIGD1) are highly expressed in mouse and human normal intestinal epithelial cells. TMIGD1 knockout mice were developed, and the loss of TMIGD1 in mice was shown to result in the development of adenomas in small intestine and colon. In addition, the loss of TMIGD1 significantly impaired intestinal epithelium brush border membrane, junctional polarity, and maturation. Mechanistically, TMIGD1 inhibits tumor cell proliferation and cell migration, arrests cell cycle at the G2/M phase, and induces expression of p^{21CIP1} (cyclin-dependent kinase inhibitor 1), and p^{27KIP1} (cyclin-dependent kinase inhibitor 1B) expression, key cell cycle inhibitor proteins involved in the regulation of the cell cycle. Moreover, TMIGD1 is shown to be progressively down-regulated in sporadic human CRC, and its downregulation correlates with poor overall survival. The findings herein identify TMIGD1 as a novel tumor suppressor gene and provide new insights into the pathogenesis of colorectal cancer and a novel potential therapeutic target. *(Am J Pathol 2021, 191: 157–167; https://doi.org/10.1016/j.ajpath.2020.09.015)*

Colorectal cancer (CRC) is the second most common malignant tumor in western countries.[1] CRC's high mortality is associated with tumor metastasis and poor response of patients to current standard-of-care drugs.[2–4] The development of CRC is complex and involves multiple molecular pathways characterized by numerous genetic and epigenetic lesions.[5] CRC can arise from hereditary and nonhereditary sporadic mutations, but >85% of CRCs are nonfamilial. The inactivation of the adenomatous polyposis coli (APC) or its downstream signaling components are common in hereditary and nonhereditary CRCs and are among the best understood pathways involved in the initiation of CRC.[4] However, other genetic and epigenetic alterations are required for the full development of CRC.[5] Loss of APC function results in the accumulation of β-catenin, resulting in the transcription of a large number of cancer-causing target genes,[6,7] alteration of cell-cell adhesion, and cell migration.[8] Despite these noticeable functions of APC, its functional loss alone in mice or humans is not sufficient to account for the full-blown development of CRC,[9] suggesting a significant involvement of other pathways in the tumorigenesis of CRC.

K.O.C.D.L.C., R.X.-Y.H. and R.A. contributed equally to this work.

Supported in part through NIH grants R21CA191970, R21CA193958, RO1CA175382, and R01 HL132325 (all to V.C.C.) and by Clinical and Translational Science Institute grant 1UL1TR001430 (N.R.). The work was also supported in part by the Malory Fund, Department of Pathology and Laboratory Medicine, Boston University.

Disclosures: None declared.

https://doi.org/10.1016/j.ajpath.2020.09.015

Transmembrane and immunoglobulin domain containing (TMIGD) family proteins are a newly identified class of immunoglobulin domain containing cell adhesion molecules, which include TMIGD1, immunoglobulin and proline rich receptor-1,[10–12] and TMIGD3,[13] which also acts as a tumor suppressor.[14] Although originally described as a protector of renal epithelial cells from oxidative cell injury,[15] TMIGD1 is down-regulated in human renal cancer, and its reexpression in renal tumor cells inhibits cell proliferation, migration, and tumor growth in mouse tumor xenograft.[16] More importantly, a recent whole genome sequencing of preinvasive colorectal tumor revealed that expression of TMIGD1 is progressively lost in colon cancer.[17] Although the change of TMIGD1 expression in colon cancer has been documented, the functional importance of loss of TMIGD1 in human colon cancer remains to be elucidated. This study demonstrates that the loss of TMIGD1 in mice results in the development of intestinal adenomas. The study provides evidence that TMIGD1 acts as a tumor suppressor by arresting cell cycle at G2/M. These findings provide novel insights into the pathogenesis of colorectal cancer and a possible new therapeutic target.

Materials and Methods

Antibodies, Plasmids, and Primers

Anti-TMIGD1 antibody was previously described.[15] The following antibodies was also used in this study. Proliferating cell nuclear antigen (PCNA) antibody (catalog number ab2426) was purchased from Abcam (Cambridge, MA). Zona occludens 1 (ZO1) antibody (catalog number 339,100) was purchased from Life Technologies (Carlsbad, CA). Actin (catalog number 4968S), β-catenin (catalog number 8480P), CDX2 (catalog number 3977S), and E-cadherin (catalog number 14472S) antibodies were purchased from Cell Signaling Technologies (Danvers, MA). Villin antibody (catalog number sc-58897) was purchased from Santa Cruz Biotechnologies (Dallas, TX). TMIGD1 was cloned into the retroviral vector pMSCV-puro as previously described.[15]

Cell Culture

HCT116, DLD1, HT29, and RKO cells were grown in RPMI 1640 medium plus 10% fetal bovine serum (FBS) and penicillin/streptomycin. Cells were purchased from ATCC (Manassas, VA). Normal human intestinal cell line (NCM460) was kindly provided by Arthur Stucchi (Department of Surgery, Boston University, Boston, MA) were grown in RPMI 1640 medium supplemented with 10% FBS penicillin/streptomycin.

Mouse Studies

TMIGD1 heterozygous mice were generated at the Nanjing BioMedical Research Institute of Nanjing University (Nanjing, China), on C57BL/6J background. Homozygous TMIGD1 mice were generated with subsequent breeding. All mice used in this study were bred and maintained at Boston University Medical Center (Boston, MA) after approval from the Institutional Animal Care and Use Committee. The following primers were used for genotyping of TMIGD1 mice: forward primer: 5′-CCCTATATCCTCAGGCTCTG-3′ and reverse primer: 5′-CGTTCAGCACTACTGTAACGGAC-3′.

Preparation of Mouse Intestine and Histologic Analysis

Mice were euthanized, and the intestinal tissues were harvested. A gavage needle was used to flush the colon and small intestine with ice-cold phosphate-buffered saline (PBS). Organs were then stretched across filter paper and opened longitudinally to fix in 10% formalin overnight at 4°C. Tissues were Swiss rolled with the distal end of the intestine closest to the center of the coil and the proximal end at the outside. The Swiss-rolled intestines were paraffin embedded and sectioned at 4 μm for histologic examination. Three sections from different segments of the block were stained with hematoxylin and eosin and graded by a veterinary pathologist and a surgical pathologist (Q.Z.) in a blind manner for detection of atypical hyperplasia, adenoma, or adenocarcinoma. Additional sections were used for immunohistochemistry or immunofluorescence staining with antibodies as described in Figure 1.

Cell Cycle Analysis

HCT116 and RKO cells expressing empty vector (EV) and TMIGD1 were plated onto 10-cm tissue culture plates at 70% to 80% confluence. Cells from each cell line were starved for 0 and 72 hours. For harvesting, cells were collected by trypsinization and washed with PBS. Cells were then fixed with 70% ethanol and stored at 4°C for at least 30 minutes. After fixation, cells were washed twice with PBS, and 1×10^6 cells per group were resuspended in PBS supplemented with 50 μL of RNase (100 μg/mL of stock). Ten minutes before flow cytometry analysis is performed, propidium iodide (5 μL per group, 1 mg/mL of stock) is added to the samples and briefly vortexed. Flow cytometry was performed by BD LSRII (BD Biosciences, San Jose, CA) and analyzed with FlowJo software (Beckton, Dickson and Co., Franklin Lakes, NJ).

Immunopaired Antibody Detection Analysis

The ActivSignal assay examines phosphorylation or expression of 70 different human protein targets, which covers 20 major signaling pathways.[16] RKO cells expressing EV or TMIGD1 were plated in 96-well plates in triplicates and subjected to ActiveSignal Assay analysis as described in Figure 2.

In Vitro Cell Migration Assay and Mouse Tail Vein Metastasis Assay

In vitro cell migration assay was performed using the Boyden chamber assay (Corning Transwell, purchased from Thermo Fisher Scientific, Waltham, MA). Briefly, cells (2×10^4 cells per well, triplicate per group) were plated on the Matrigel-coated transwells (Corning Transwell, purchased from Thermo Fisher Scientific) in which the upper chamber contained 1% FBS and the lower chamber contained 10% FBS medium. After 6 hours, the nonmigrated cells from the upper side of the membrane were removed by Q-tip, cells were fixed and stained with crystalline blue, and the number of migrated cells were counted under a light microscope. To evaluate the capacity of the CRC cell line HCT116 cell—expressing green fluorescent protein (GFP) alone or expressing TMIGD1/GFP to extravasate and grow, equal numbers of cells (5×10^5 cells per/mouse) were injected via the tail vein and metastasis to lung was evaluated after 2 weeks. Specifically, mice were sacrificed, lungs were removed, and slides of frozen tissues were prepared and viewed under a fluorescence microscope.

Immunofluorescence Microscopy

Mice intestinal tissues were sectioned to 4 μm and baked for 1 hour. Tissue slides were then deparaffinized using decreasing alcohol gradients. Slides were then submerged into sodium citrate buffer for antigen unmasking. Tissue sections were then permeabilized using 0.3% Triton X-100. Slides were blocked using 5% bovine serum albumin in tris-buffered saline and Tween 20. Primary antibody was then added to tissue sections, and sections were incubated. Fluorescent antibody was added to the tissue sections and was incubated in the dark. Slides were then mounted using VectaShield Antifade Mounting Medium (Vector Laboraties, Burlingame, CA) with DAPI.

Immunohistochemistry

Two human colorectal cancer tissue microarray slides (catalog numbers BC05012a and BC05118a; US Biomax, Derwood, MD) consisting of 172 tissue samples (72 on BC05012a and 100 on BC05118a) were stained with anti-TMIGD1 antibody. The patient age, tumor grade, tumor stage, and TNM status were provided for each sample on both microarrays. Six samples were excluded from the final analysis because of poor tissue quality secondary to artifact or complete absence of CRC tissue in the specimen. Immunohistochemistry was performed on both microarray slides using validate TMIGD1 antibody[15,16] and polymer—horseradish peroxidase secondary antibody (Abcam). The staining intensity of each specimen was rated independently by two pathologists (Q.Z.). Each specimen was rated from 0 to 3, with 0 representing no TMIGD1 staining and 3 representing the highest intensity of TMIGD1 staining. The staining pattern was described for each specimen as granular, perinuclear, or diffuse.

Statistical Analysis

The mean TMIGD1 staining intensity was stratified based on patient age, tumor grade, tumor stage, and T rating in the TNM staging system. The mean intensity values of each subgroup were compared for statistical significance via analysis of variance with the Tukey post-hoc test. The α value for significance was set at $P < 0.05$.

Results

Loss of TMIGD1 in Mice Causes Intestinal Adenoma

First, the expression of TMIGD1 in normal human and mouse intestinal tissues was examined via immunofluorescence staining using a previously validated anti-TMIGD1 antibody.[15,16] TMIGD1 was detected at the membranous regions of intestinal epithelial cells in human (Supplemental Figure S1, B and C). Consistent with its previously described characteristic as a cell adhesion molecule, TMIGD1 co-localized with E-cadherin in human intestinal epithelial cells, a well-characterized marker of epithelial cell adherens junctions (Supplemental Figure S1, D—F), but not with the tight junction protein ZO-1 (Supplemental Figure S2, A—C). Similar to its expression in human intestinal tissue, TMIGD1 is also expressed in mouse intestinal epithelial cells (Supplemental Figure S3B). Next, overall TMIGD1 expression in human intestinal tissue was examined via the Genevestigator data set (HS_mRNA-Seq_Human_GL-0; Genevestigator, Nebion AG, Zurich Switzerland).[18] This analysis revealed that TMIGD1 mRNA is highly expressed in human colonic tissues, including cecum, small intestine, large intestine, and jejunum (Supplemental Figure S4A). In addition, analysis of the RNA sequence of 19 human fetus tissues (NIH Roadmap Epigenomics Mapping Consortium via EBI, *https://www.ebi.ac.uk/gxa/home*, last accessed November 20, 2019) similarly demonstrated that TMIGD1 mRNA (*http://www.ncbi.nlm.nih.gov*; GenBank accession number NM_206,832.3) is highest in the intestinal tissues followed by the kidney (Supplemental Figure S4B). The TMIGD1 mRNA in other organs, including heart, stomach, muscle, adrenal gland, and placenta, were not expressed or expressed at very low levels (Supplemental Figure S4B). To investigate the importance of TMIGD1 in the intestinal function *in vivo,* homozygous TMIGD1 knockout (KO) mice were generated via CRISPR/Cas9 system (Figure 1A). CRISPR/Cas9-mediated loss of TMIGD1 was confirmed by real-time quantitative PCR (Figure 1B). Although TMIGD1$^{-/-}$ mice are viable and fertile and pups display no apparent abnormality, as they get older ($\geq$4 months), they develop intestinal hyperplasia (Figure 1C). Analysis of intestines of TMIGD1$^{-/-}$ mice revealed that 80% (8 of 10) of TMIGD1$^{-/-}$ mice develop polyps in small intestine, large intestine, and rectum (Figure 1C). The mean number of polyps observed was 7

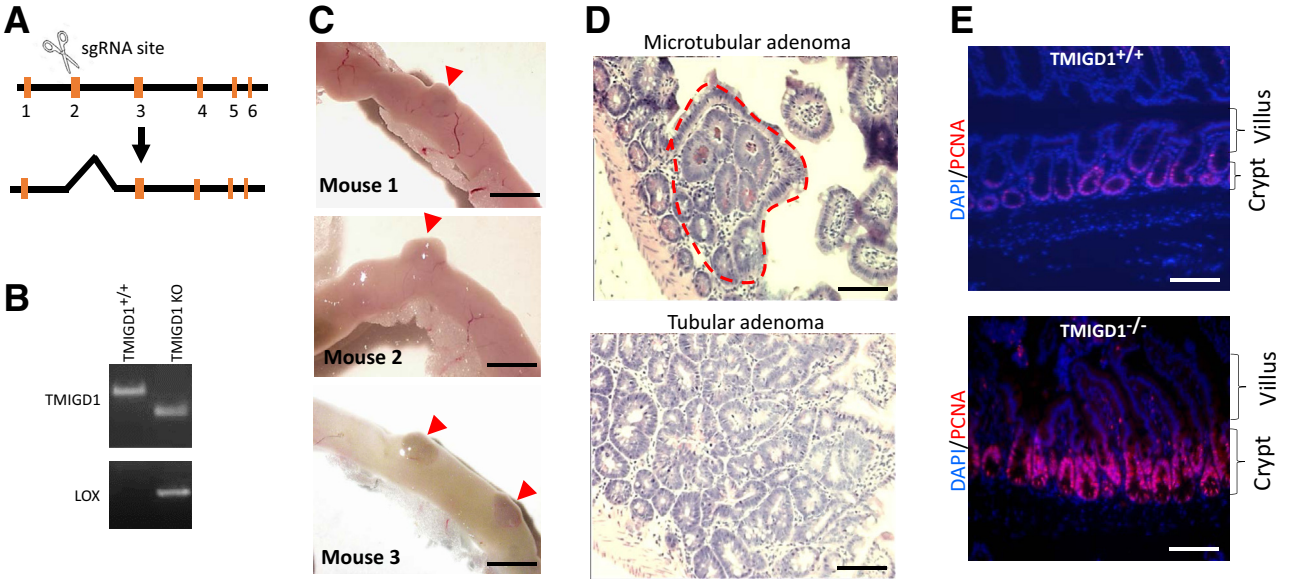

Figure 1 Loss of transmembrane and immunoglobulin domain containing 1 (TMIGD1) in mice causes intestinal adenoma. **A:** Schematic of single guide RNA (sgRNA)—mediated knockout of TMIGD1. **B:** Real-time quantitative PCR analysis of wild-type and TMIGD1 knockout (KO) mice. **C:** Representative images of formation of intestinal adenoma in TMIGD1 KO mice. The **red arrowheads** indicate intestinal adenomas. **D:** Hematoxylin and eosin staining of intestinal tissue of TMIGD1 KO. The **red dashed line** indicates the microtubular adenoma region. **E:** Immunofluorescence staining of wild-type and TMIGD1 KO mice intestines with proliferating cell nuclear antigen (PCNA) antibody (red, PCNA; blue, DAPI). Scale bars = 50 μm (**C**—**E**). Original magnification, ×40 (**C** and **D**). LOX, locus of x-over.

to 12 per mouse. Furthermore, hematoxylin and eosin staining demonstrated the development of microtubular adenoma and tubular adenoma in TMIGD1$^{-/-}$ mice (Figure 1D).

It is highly likely that development of adenoma in TMIGD1$^{-/-}$ mice is associated with aberrant cell proliferation. Therefore, intestinal tissues of wild-type and TMIGD1 KO mice were examined with PCNA, a marker for cell proliferation. Immunostaining of the intestinal tissues of TMIGD1$^{+/+}$ and TMIGD1$^{-/-}$ mice for PCNA showed that loss of TMIGD1 in mice results in the hyperproliferation of the intestinal epithelial cells (Figure 1E). Although there was weak staining for PCNA in the intestinal epithelium (in the crypt region) of TMIGD1$^{+/+}$ mice, there was strong staining for PCNA in the intestinal epithelium of TMIGD1$^{-/-}$ mice (Figure 1E). Furthermore, Ki-67 staining, another marker for *in vivo* cell proliferation, showed similar result (data not shown). TMIGD1$^{-/-}$ mice are currently being monitored for potential adenocarcinoma development and other potential pathologies as they get older. Taken together, loss of TMIGD1 in mice is shown to cause intestinal adenoma, with a significant potential role in human colorectal cancer.

Loss of TMIGD1 in Mice Alters Apicobasal Organization and Maturation of Intestinal Epithelial Cells

Examination of intestinal tissues of TMIGD1$^{-/-}$ mice further demonstrated that, in addition to development of adenoma, overall organization of the intestinal epithelium in TMIGD1$^{-/-}$ mice was abnormal. Specifically, hematoxylin and eosin staining revealed that brush border, an actin-based membrane protrusion known as microvilli, in TMIGD1$^{-/-}$ mice was distinctively reduced. Although brush border in the wild-type mouse intestine was clearly evident and well-organized, the brush border in the intestinal tissues of TMIGD1$^{-/-}$ mice was mostly absent (Figure 3A). To further investigate the apparent loss of brush border in TMIGD1$^{-/-}$ mice, the intestinal tissues of wild-type and TMIGD1$^{-/-}$ mice were stained with actin and villin, which are common markers for the brush border. Immunofluorescence staining demonstrated that both actin and villin are uniformly present at the brush border of wild-type mice (Figure 3, B and C). However, in TMIGD1$^{-/-}$ mice actin localization at the brush border was significantly reduced and was aberrantly localized at the crypt and villus regions (Figure 3B). Interestingly, villin localization to brush border was significantly reduced or mislocalized in TMIGD1$^{-/-}$ mice (Figure 3C). To obtain additional insight into the brush border impairment in the intestinal epithelial cells in TMIGD1 KO mice, the intestinal tissues of wild-type and the TMIGD1 KO mice were stained with atypical protein kinase C, a marker for apicobasal polarity. The data demonstrated that atypical protein kinase C is uniformly present at the brush border in the wild-type mice but not in the TMIGD1 KO mice (Figure 3D).

Staining with the E-cadherin antibody demonstrated that in the wild-type mice, E-cadherin was strongly positive at the crypt and microvilli of epithelium (Figure 4A), which indicates a normal cellular junctions of epithelium. Similarly, β-catenin staining displayed a similar pattern (Figure 4B). However, although the crypt epithelial cells were positive for E-cadherin in TMIGD1$^{-/-}$ mice, it was diffusely present at the cell junctions, prominently detected at the apical membrane (Figure 4A), and mostly absent in

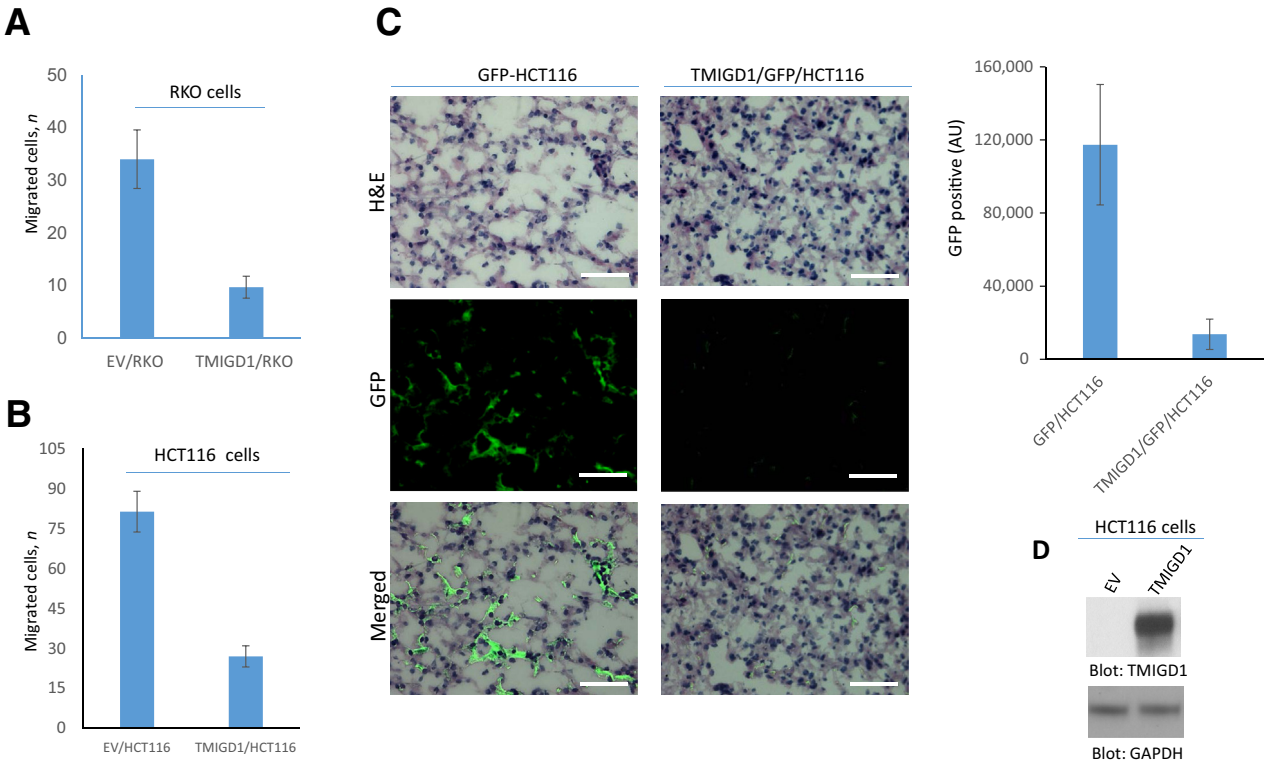

Figure 2 Re-expression of transmembrane and immunoglobulin domain containing 1 (TMIGD1) in colorectal cancer cells inhibits cell migration and metastasis. **A** and **B:** Migration of RKO and HCT116 cells expressing empty vector (EV) or TMIGD1 was determined in a Boyden chamber coated with Matrigel. RKO cell. **C:** Tail vein mouse metastasis assay of HCT116 cells expressing green fluorescent protein (GFP) or TMIGD1/GFP. Metastasis of cells to mouse lung is shown. ImageJ software version 1.52o (NIH, Bethesda, MD; *http://imagej.nih.gov/ij*) was used to quantify GFP-positive cells in mouse lung. **D:** Western blot analysis of TMIGD1 expression in HCT116 cells. $n = 4$ mice per group. Data are expressed as means ± SEM (**A; B;** and **C, second panel**). $P = 0.00187$ (**A** and **B**, RKO cells), $P = 0.0241$ (**A** and **B**, HCT116 cells), and $P = 0.0034$ (**C**). Scale bars = 20 μm (**C**). AU, arbitrary units; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin and eosin.

the brush border (Figure 4A). Moreover, β-catenin was diffusely present at the intestinal cell junctions in TMIGD1$^{-/-}$ mice (Figure 4B). E-cadherin is known to localize to the lateral membrane of differentiated epithelial cells, providing the structural foundation for adherens junctions, which also promotes epithelial apical-basal polarization.[19] Though E-cadherin is required for maturation of intestinal Paneth cells,[20] β-catenin is thought to play a central role in maturation and morphogenesis of crypt and villus.[21,22] Additional staining with the ZO1 protein, a marker for tight junction that is also required for tight junction formation of epithelial cells,[23] demonstrated that in TMIGD1$^{-/-}$ mice, ZO1 is largely absent at the junctions of intestinal epithelial cells (Figure 4C).

To gain further insights into a possible mechanism of aberrant junctional development of intestinal epithelium in TMIGD1 KO mice, whether loss of TMIGD1 in mice alters the intestinal epithelial cell maturation was examined. To this end, the TMIGD1$^{-/-}$ mouse intestinal tissue was stained with CDX2, a marker for maturation of intestinal epithelial cells, including goblet and Paneth cells.[24–26] Although the CDX2-positive epithelial cells were uniformly present at the crypt and villus of the wild-type mice, in TMIGD1$^{-/-}$ mice, CDX2-positive cells were detected only at the lower part of the crypts, albeit weakly and inconsistently (Figure 4D). Altogether, these data demonstrate that the loss of TMIGD1 in mice impairs intestinal epithelial cell maturation and intercellular junctions.

TMIGD1 Arrests Cell Cycle at the G2/M Phase and Inhibits Cell Migration and Metastasis

Considering that loss of TMIGD1 in mice induced intestinal adenomas and increased cell proliferation (Figure 1), whether the effect of ectopic expression of TMIGD1 in colon cancer cells can inhibit cell proliferation was investigated. TMIGD1 or EV was initially expressed through a retroviral expression system in RKO cells. Expression of TMIGD1 in RKO cells was confirmed by Western blot analysis using anti-TMIGD1 antibody (Figure 5A). Overexpression of TMIGD1 in RKO cells significantly inhibited cell proliferation (Figure 5B). Although proliferation of RKO cells expressing EV (EV/RKO) increased in a time dependent manner, proliferation of RKO cells expressing TMIGD1 (TMIGD1/RKO) was reduced by 53% at day 4 ($P = 0.0033$, number of cell for EV/RKO cells was 66.5×10^4 versus 30.8×10^4 for TMIGD1/RKO cells) (Figure 5B). It was postulated that TMIGD1 could inhibit cell proliferation by a mechanism that

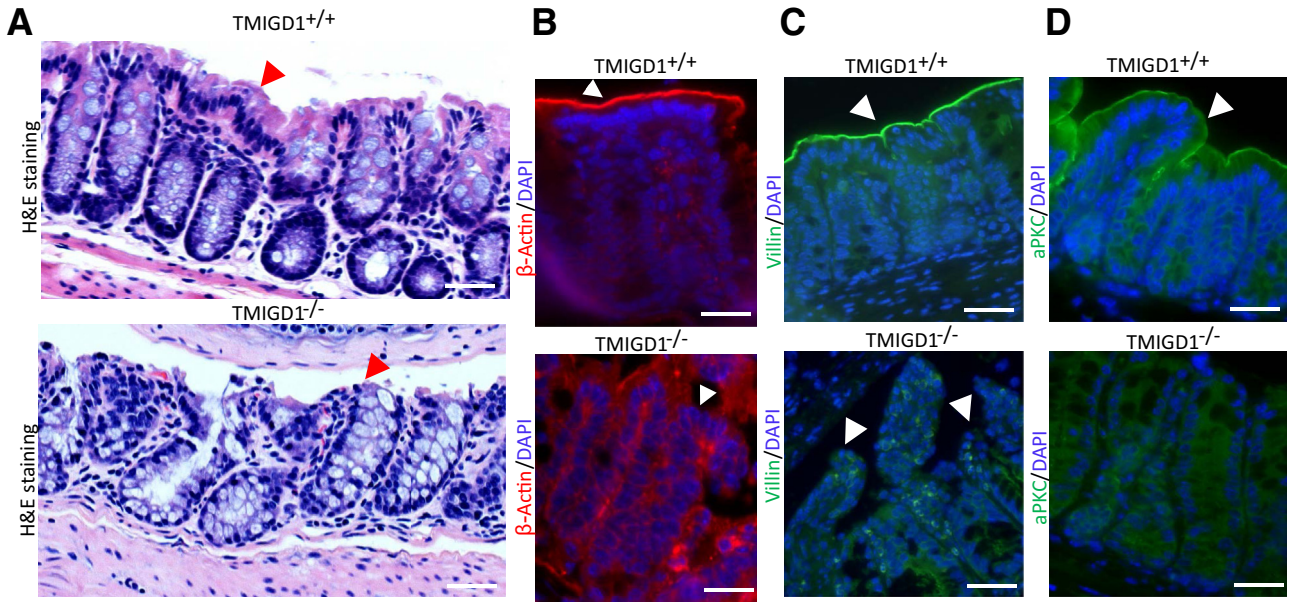

Figure 3 Loss of transmembrane and immunoglobulin domain containing 1 (TMIGD1) in mice impairs brush border development. **A:** Hematoxylin and eosin (H&E) staining of wild-type and TMIGD1 mice. **Red arrowheads** point to the brush border region of intestine from wild-type and TMIGD1 knockout (KO) mice. **B:** Immunofluorescence staining of intestine from wild-type and TMIGD1 KO mice with β-actin antibody. **White arrowheads** point to brush border regions in wild-type and TMIGD1 KO mice. **C:** Immunofluorescence staining of intestine from wild-type and TMIGD1 KO mice with villin antibody (green, villin; blue, DAPI). **White arrowheads** point to brush border regions in wild-type and TMIGD1 KO mice. **D:** Immunofluorescence staining of intestine from wild-type and TMIGD1 KO mice with atypical protein kinase C (aPKC) antibody (green; blue, DAPI). **White arrowhead** points to the expression of aPKC in the brush border region. Scale bars = 50 μm.

modulates the cell cycle. Therefore, the cell cycle distribution of EV/RKO and TMIGD1/RKO cells was examined by FACS (LSRII) analysis via propidium iodide staining. TMIGD1-expressing RKO cells showed a significant cell cycle arrest at the G2/M phase (25.4% versus 9.3%) in the presence of 10% FBS (Figure 5, C and D) and serum-starved (72 hours) 14.1% versus 7.2% (Figure 5, E and F) G2/M phase arrest in the cell cycle of TMIGD1/RKO cells followed by a markedly decreased G0/G1 phase of the cell cycle (Figure 5 C–F). The effect of ectopic expression of TMIGD1 on cell cycle was not limited to RKO cells because its ectopic expression in HCT116 cells (Supplemental Figure S5A) similarly resulted in the cell cycle arrest at the G2/M phase (Supplemental Figure S5B). In a complementary approach, TMIGD1 was knocked down by shRNA in normal colon epithelial cells, NMC460 (Supplemental Figure S6A), and the cell cycle profile was examined. The result showed that the knockdown of TMIGD1 in NMC460 cells reduced the serum starvation–induced cell cycle arrest at the G2/M phase (17.5% versus 10.2%) (Supplemental Figure S6B). Taken together, these data demonstrate that reintroduction of TMIGD1 in CRC cells (RKO and HCT116) promotes cell cycle arrest at the G2/M phase, and knockdown of TMIGD1 in normal intestinal epithelial cells, NMC460, decreases the serum starvation–induced cell cycle arrest at the G2/M phase.

To investigate the potential mechanism by which TMIGD1 affects cell cycle, RKO cells expressing TMIGD1 for activation of 20 major cancer pathways consisting of 70 individual proteins were analyzed via an immunopaired antibody detection system (ActiveSignal Assay) analysis platform.[16] Among the major pathways that were affected by TMIGD1 in RKO cells were the proteins known to inhibit cell cycle and cell proliferation. Specifically, TMIGD1 up-regulated expressions of $p21^{CIP1}$ (cyclin-dependent kinase inhibitor 1) and $p27^{KIP1}$ (cyclin-dependent kinase inhibitor 1B) (Supplemental Figure S7A), proteins whose expression is critically important for negative regulation of the cell cycle progression.[27] Furthermore, TMIGD1 reduced phosphorylation of CyclinD1 and Cyclin-dependent kinase 1 (CDK1/Cdc2), and increased phosphorylation of retinoblastoma protein (Rb), and p38MAPK (MAPK14) (Supplemental Figure S7A). The effect of TMIGD1 on the phosphorylation of p38, phosphorylation of Rb, and induction of $p21^{CIP1}$ and $p27^{KIP1}$ was further confirmed by Western blot analysis (Supplemental Figure S7B). Taken together, the data suggest that TMIGD1 induces growth suppression via cell cycle arrest at the G2/M phase, and loss of TMIGD1 expression in CRC could contribute to hyperproliferation of cancer cells.

To examine the effect of TMIGD1 in tumorigenesis beyond cell proliferation and cell cycle, the effect of TMIGD1 in cell migration in RKO and HCT116 cells was measured. Expression of TMIGD1 in RKO and HCT116 cells significantly inhibited cell migration (Figure 2, A

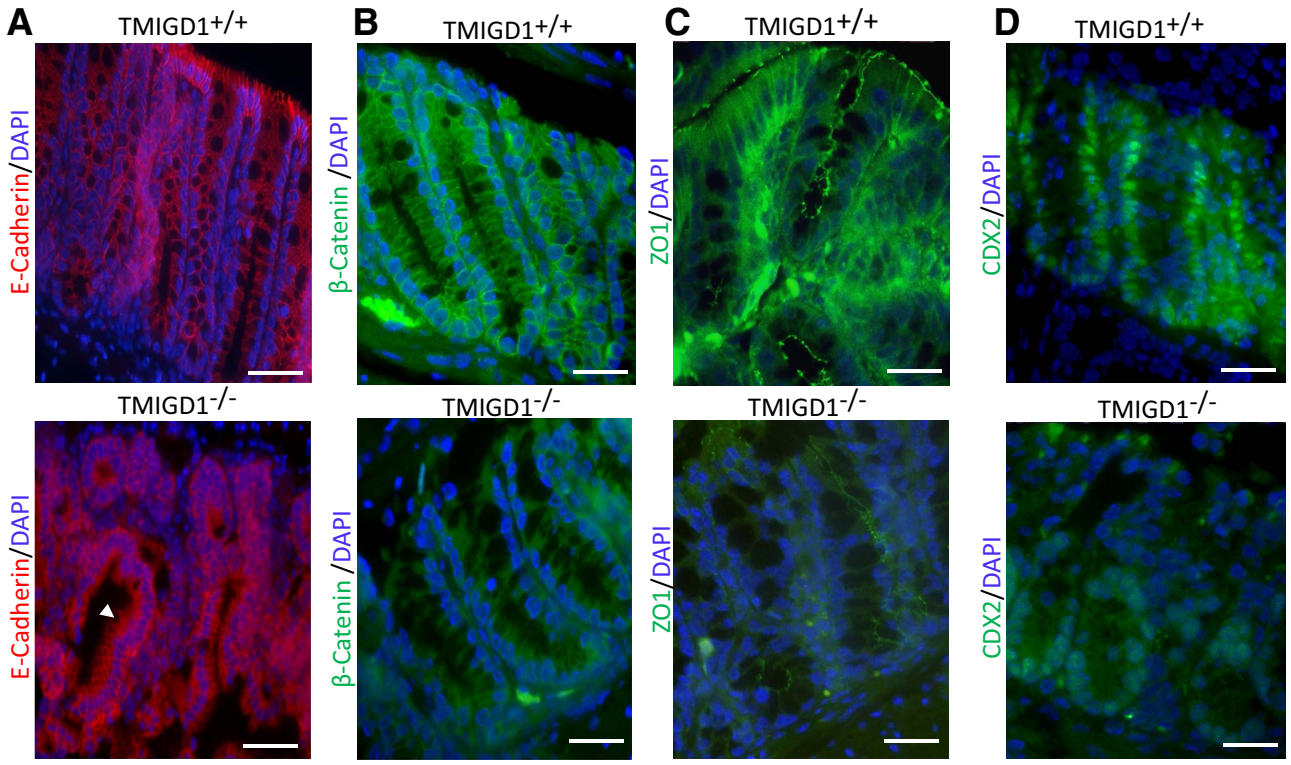

Figure 4 Loss of transmembrane and immunoglobulin domain containing 1 (TMIGD1) alters intestinal epithelium organization and maturation. **A:** Immunofluorescence staining of intestines of wild-type and TMIGD1 knockout (KO) mice with E-cadherin antibody (red, E-cadherin; blue, DAPI). **White arrowhead** points to mislocalization of E-cadherin in the intestinal epithelium in a TMIGD1 KO mouse. **B:** Immunofluorescence staining of intestines of wild-type and TMIGD1 KO mice with E-cadherin–associated protein β-catenin antibody (green, β-catenin; blue, DAPI). **C:** Immunofluorescence staining of intestines of wild-type and TMIGD1 KO mice with zona occludens 1 (ZO1) antibody, a tight junction marker protein (green, ZO1; blue, DAPI). **D:** Immunofluorescence staining of intestines of wild-type and TMIGD1 KO mice with CDX2 antibody, a marker for intestinal epithelium marker (green, CDX2; blue, DAPI). Scale bars = 50 μm.

and B). To further investigate the inhibitory effect of TMIGD1 in colon cancer cell migration, GFP/HCT116 cells expressing TMIGD1 or EV were generated, and their metastatic potentials in the experimental metastasis mouse model was assessed via tail vein injection. Mice injected with EV/GFP/HCT116 cells had significant metastasis to lung as determined by the presence of GFP-positive cells in lung. All the mice ($n = 5$, $P = 0.0034$) injected with TMIGD1/GFP/HCT116 cells had metastasis in lung. However, the metastasis of TMIGD1/GFP/HCT116 cells ($n = 5$) was significantly less (Figure 2C). Given that the ectopic expression of TMIGD1 in these cells also inhibits cell proliferation, it is possible that the reduced tumor metastasis in mice, in part, is associated with the reduced proliferation rate of HCT116 cells expressing TMIGD1. However, TMIGD1 expression in RKO and HCT116 cells inhibited cell migration within a period of 6 hours (Figure 6, A and B), indicating that the effect of TMIGD1 in cell migration cannot be fully attributable to the reduced cell proliferation rate of HCT116 cells by TMIGD1. Ectopic expression of TMIGD1 in HCT116 cells is shown in Figure 2D. Altogether the data demonstrate that restoring the expression of TMIGD1 in CRC tumor cells inhibits tumor cell proliferation, migration, and metastasis.

TMIGD1 Is Down-Regulated in Colon Cancer Which Correlates with Poor Survival

Considering that the loss of TMIGD1 in mice resulted in the development of adenoma, altered epithelial phenotype, and increased cell proliferation, whether TMIGD1 expression is down-regulated in human cancers was investigated. An initial analysis of TMIGD1 mRNA across 23 major human cancer types from The Cancer Genome Atlas via the TIMER online site (*https://cistrome.shinyapps.io/timer*, last accessed March 21, 2020)[28,29] demonstrated that TMIGD1 mRNA (*http://www.ncbi.nlm.nih.gov*; GenBank accession number NM_206,832.3) is significantly reduced in human colon, rectum, and renal cancers compared with their corresponding normal tissues (Supplemental Figure S8A). TMIGD1 expression in other tissues, both normal and cancer, was significantly low (Supplemental Figure S8A). Consistent with the down-regulation of TMIGD1 in human colon cancer, analysis of 46 human colon cancer cell lines via The Cancer Cell Line Encyclopedia[30] also revealed that TMIGD1 mRNA was significantly down-regulated in colon cancer cell lines (data not shown).Next, TMIGD1 protein expression levels on the human CRC tissue microarray slides ($n = 166$) were examined via immunohistochemical analysis, and TMIGD1 levels were semi-quantitatively analyzed. The normal adjacent tissue ($n =$

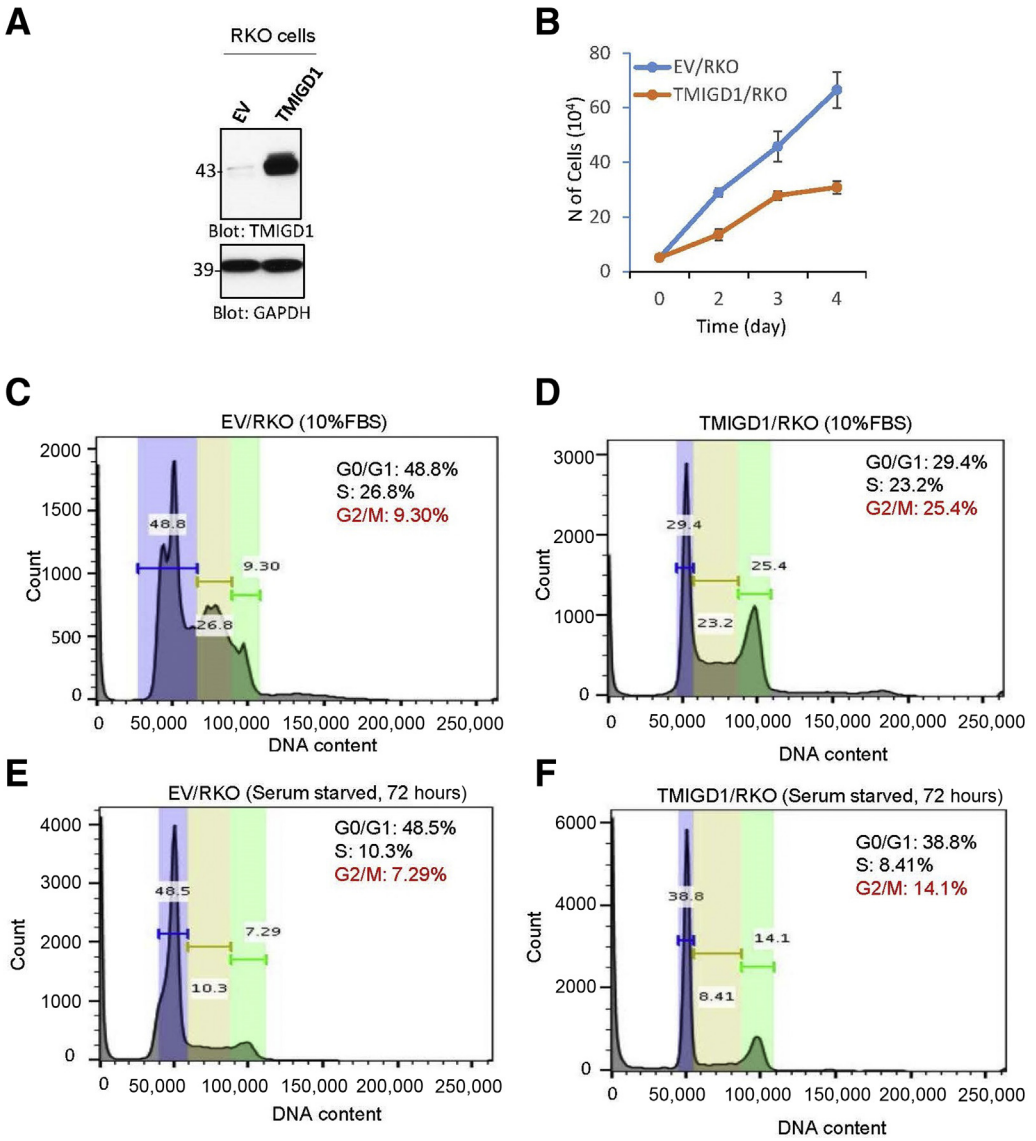

Figure 5 Re-expression of transmembrane and immunoglobulin domain containing 1 (TMIGD1) in RKO cells inhibits cell proliferation and results in cell cycle arrest at the G2/M phase. **A:** Western blot analysis of TMIGD1 expression in RKO cells. **B:** Proliferation of RKO cells expressing empty vector (EV) or TMIGD1 was determined by direct counting of cells by hemocytometer. **C**–**F:** Equal number of RKO cells expressing EV and TMIGD1 at approximately 70% to 80% confluence were kept in 10% fetal bovine serum (FBS) or starved for 72 hours. Cells were fixed with 70% ethanol, stained with propidium iodide, and analyzed by BD LSRII. Graphs were generated with FlowJo software. **B**–**F:** All experiments were repeated at least three times and repeated in triplicate. $P < 0.05$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

52) had a mean TMIGD1 staining score of 1.87, and grade 1 ($n = 21$), grade 2 ($n = 62$), and grade 3 ($n = 29$) tumors had means of 2.24, 1.52, and 1.14, respectively (Figure 6, A and B). Tumor differentiation was graded morphologically (grades 1, 2, and 3 as well, moderately, and poorly differentiated, respectively). The level of differentiation of each tumor was also subjectively assessed based on the number of glands, gland architecture, and cell morphology; samples were rated along a spectrum of well, moderately, or poorly differentiated. Interestingly, TMIGD1 expression was higher in well-differentiated tumors compared with adjacent normal tissue, but the differences were not statistically significant (Figure 6, A and C). However, expression of TMIGD1 was progressively down-regulated with tumor progression as the lowest level of TMIGD1 expression was observed in the poorly differentiated tumors (Figure 6, A and B). No significant differences were observed when TMIGD1 staining intensity was stratified by patient age or tumor stage (data not shown). Furthermore, TMIGD1 expression in multiple colon cancer cell lines (HT29, HCT116, RKO, and DLD1) and renal cancer cell lines (786 to 0, CAKI-1, A498, and TK10) as determined by Western blot analysis showed that TMIGD1 protein levels in colon cancer and renal cancer cell lines was very low or undetectable (Figure 6D). However, in two CRC cell lines (HCT116 and HT29), a protein band at the lower molecular weight was detected (Figure 6D), which may correspond to partially

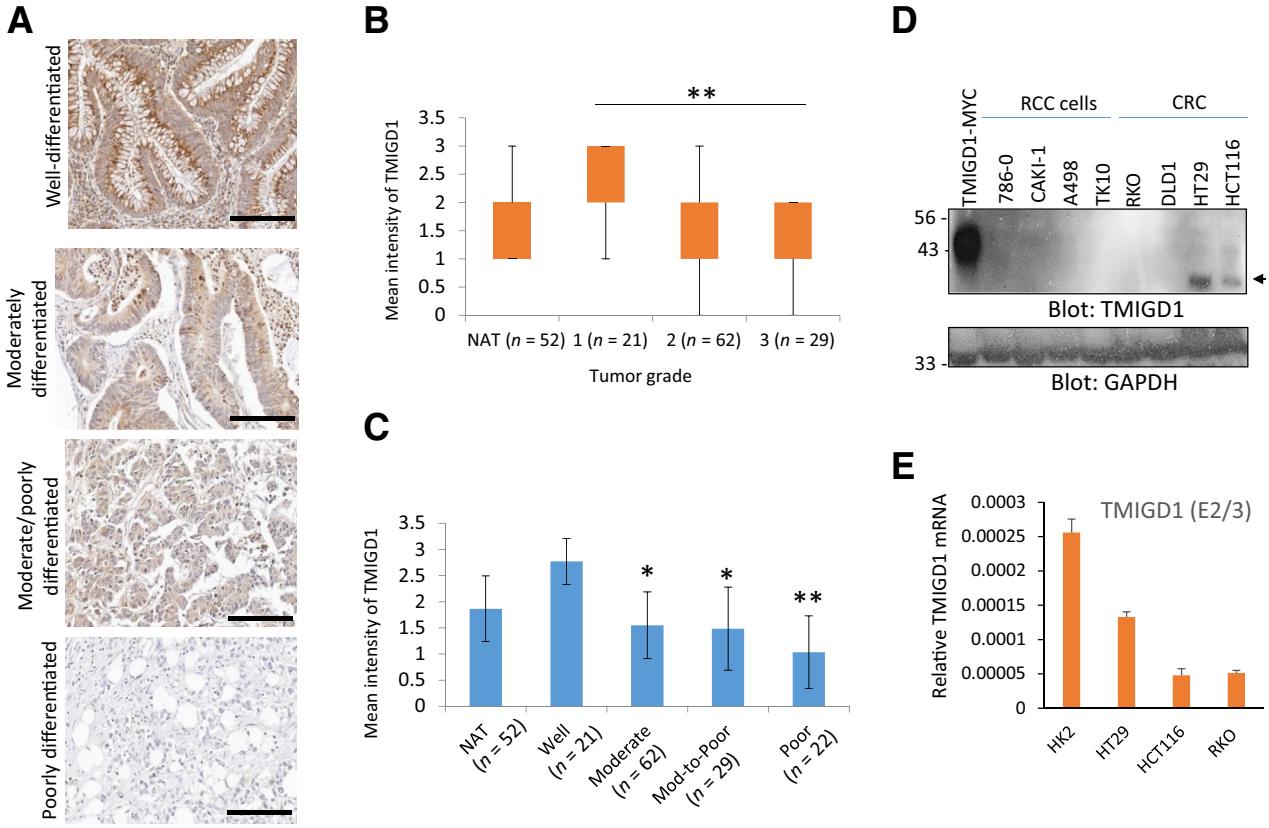

Figure 6 Transmembrane and immunoglobulin domain containing 1 (TMIGD1) is down-regulated in advanced human colon cancer. **A:** Two human colorectal cancer (CRC) tissue microarray slides consisting of 172 tissue samples were stained with anti-TMIGD1 antibody. Staining was scored as 0 (negative, <5% cells positive), 1+ (6% to 25% cells positive), 2+ (26 to 50% cells positive), and 3+ (>50% cells positive). The tumor differentiation was graded morphologically (grades 1, 2, and 3 as well, moderately, and poorly differentiated, respectively). **B:** The mean staining intensities were compared using analysis of covariance (ANOVA) with Tukey post hoc test per tumor grade. **C:** The mean staining intensities were compared using ANOVA with Tukey post hoc test per tumor differentiation. **D:** Western blot analysis of TMIGD1 expression in human renal cancer cell (RCC) and CRC cell lines. **Arrow** points to proteins with a low molecular weight. **E:** Real-time quantitative PCR analysis of TMIGD1 expression in CRC cell lines (HT29, HCT116, and RKO). Human kidney epithelial cells (HK2) was used as a positive control. $*P \leq 0.05$, $**P \leq 0.01$. Scale bar = 50 μm. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NAT, normal adjacent tissue.

glycosylated or unglycosylated TMIGD1 or alternatively spliced variant of TMIGD1 (Figure 6D). In addition, real-time quantitative PCR analysis revealed that TMIGD1 mRNA is expressed at low levels in HT29, HCT116, and RKO cells compared with that at normal human kidney epithelial cells (Figure 6E). The observed down-regulation of TMIGD1 in CRC prompted us to question whether TMIGD1 expression status correlates with the survival of patients with CRC. To answer this question, a Kaplan-Meier survival analysis of the publicly available CRC data set from The Cancer Genome Atlas was performed. The result revealed that patients with CRC and high TMIGD1-expressing tumors had significantly better median overall survival compared with those with low TMIGD1-expressing tumors (Supplemental Figure S8B). However, a large cohort of patients with CRC is needed to fully establish the potential link between the down-regulation of TMIGD1 and CRC survival.

Discussion

This study found that TMIGD1 is expressed in the intestinal epithelial cells, prominently in the brush border membranes. Targeting TMIGD1 in mice resulted in brush border impairment and adenoma development. TMIGD1 is down-regulated in CRC, and its downregulation correlates with poor survival. Ectopic expression of TMIGD1 in CRC cell lines markedly decreased cell proliferation, induced G2/M cell cycle checkpoint arrest, and inhibited cell migration and invasion. Silencing TMIGD1 by shRNA in non–tumor cell line NCM460 cells increased cell proliferation. These findings, coupled with previous observations on the role of TMIGD1 in renal cancer,[16] suggest a significant function of the TMIGD1 pathway in the regulation of renal and intestinal cancers. Mechanistically, TMIGD1 appears to modulate epithelial cell proliferation via regulation of the G2/M cell cycle checkpoint, which involves induction of p^{21CIP1} and p^{27KIP1}. $P21^{CIP1}$ and p^{27KIP1} are key cell cycle inhibitor proteins involved in the regulation of the cell cycle, which may account for the effect of TMIGD1 in cell cycle and tumorigenesis. However, future work is needed to establish the role of TMIGD1 pathway in modulating the G2/M cell cycle checkpoint.

CRC is the second leading cause of cancer morbidity and mortality among men and women. Although only

approximately 5% to 10% of all CRC are genetic, >85% of CRC are sporadic.[1] The *APC* tumor suppressor gene is mutated in most familial and sporadic CRCs. However, despite its prevalence, mutations in the *APC* gene are thought to be an early, if not an initiating, event in multistep processes of CRC progression.[4] More importantly, although the mechanism of APC inactivation is fairly well documented, the process by which APC inactivation leads to tumorigenesis remains poorly understood. Results from the current study show that the loss of TMIGD1 in mice increases intestinal epithelial cell proliferation and induces adenoma. TMIGD1 was demonstrated to be down-regulated in human colon cancer, and its downregulation is associated with poor survival. Interestingly, a recent study also demonstrated that TMIGD1 is significantly down-regulated in patients with Crohn disease, underscoring its role in the regulation of intestinal epithelial cell function beyond cancer.[31]

Intestinal epithelium displays strong apical-basal polarity with a highly developed brush border in the apical plasma membrane of absorptive epithelial cells and a similarly highly polarized organization of the other cell types that populate the intestine. Another interesting and novel aspect of the current study is the observation of TMIGD1 being prominently present in the brush border membranes and its loss in mice significantly impairing the brush border development and the integrity of intestinal epithelium as was evident by almost nonexistence actin and villin at the brush border and altered E-cadherin and ZO1 cellular distribution. This theme was further echoed by the observation that loss of TMIGD1 in mice interfered with the maturation of intestinal epithelium, especially given that the CDX2-positive cells were only sparsely and inconsistently present in the intestinal epithelium.

In summary, the current study provides several lines of evidence supporting that *TMIGD1* is a tumor suppressor gene that plays a central role in the integrity of intestinal epithelium and proliferation and that loss of TMIGD1 in mice results in hyperplasia. Therefore, studies aimed to uncover the molecular basis of down-regulation of TMIGD1 in CRC, and signal transduction mechanisms used by TMIGD1 to regulate epithelial cell function could lead to the development of novel therapeutic modalities in CRC.

Acknowledgment

We thank Mostafa Belghasem (Department of Pathology, Boston University, Boston, MA) for his help with the histopathology and animal work.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.ajpath.2020.09.015*.

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