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# Systemic TM4SF5 overexpression in *Apc*$^{Min/+}$ mice promotes hepatic portal hypertension associated with fibrosis

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**Mutation of the gene for adenomatous polyposis coli (APC), as seen in *Apc*$^{Min/+}$ mice, leads to intestinal adenomas and carcinomas via stabilization of β-catenin. Transmembrane 4 L six family member 5 (TM4SF5) is involved in the development of non-alcoholic fatty liver disease, fibrosis, and cancer. However, the functional linkage between TM4SF5 and APC or β-catenin has not been investigated for pathological outcomes. After interbreeding *Apc*$^{Min/+}$ with TM4SF5-overexpressing transgenic (Tg$^{TM4SF5}$) mice, we explored pathological outcomes in the intestines and livers of the offspring. The intestines of 26-week-old dual-transgenic mice (*Apc*$^{Min/+}$:Tg$^{TM4SF5}$) had intramucosal adenocarcinomas beyond the single-crypt adenomas in *Apc*$^{Min/+}$ mice. Additional TM4SF5 overexpression increased the stabilization of β-catenin via reduced glycogen synthase kinase 3β (GSK3β) phosphorylation on Ser9. Additionally, the livers of the dual-transgenic mice showed distinct sinusoidal dilatation and features of hepatic portal hypertension associated with fibrosis, more than did the relatively normal livers in *Apc*$^{Min/+}$ mice. Interestingly, TM4SF5 overexpression in the liver was positively linked to increased GSK3β phosphorylation (opposite to that seen in the colon), β-catenin level, and extracellular matrix (ECM) protein expression, indicating fibrotic phenotypes. Consistent with these results, 78-week-old Tg$^{TM4SF5}$ mice similarly had sinusoidal dilatation, immune cell infiltration, and fibrosis. Altogether, systemic overexpression of TM4SF5 aggravates pathological abnormalities in both the colon and the liver. [BMB Reports 2022; 55(12): 609-614]**

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## INTRODUCTION

Pathological malignancies, including cancers in the colon and the liver, are life-threatening because of their high occurrence and lower survival rates. Whereas the five-year (2015-2019) survival rate in Korean cancer patients was 74.3% for colon cancer and 37.7% for liver cancer, the occurrence in 2019 was 279,717 (13% out of the total 2,147,503 occurrences) for colon cancer and 75,261 (3.5%) for liver cancer, as reported (December 2021) by the Korea Central Cancer Registry at the National Cancer Center of Korea. Therefore, both cancer types, with either a high occurrence rate (for colon cancer) or a low survival rate (for liver cancer), can extensively influence the health and economic situations of the population.

Identification of molecular biomarkers for these cancer types would provide clinical benefits, such as developing strategies for earlier diagnosis and therapeutic approaches. Accumulations or mutations of certain oncogenes and tumor-suppressor genes for colorectal cancer are well-known in cases of adenomatous polyposis coli (APC), KRAS proto-oncogene, serine/threonine-protein kinase B-Raf, phosphatidylinositol 3-kinase (PI3K), SMAD family member 4, transforming growth factor β, and tumor protein p53 (TP53) (1). In cases of hepatocellular carcinoma, the protein biomarkers include α-fetoprotein, β-catenin, TP53, phosphatase and tensin homolog, axin 1 (AXIN1), and retinoblastoma transcriptional corepressor 1 (2). Thus, both cancer types commonly involve β-catenin and TP53. Moreover, we reported that transmembrane 4 L six family member 5 (TM4SF5) is highly expressed in colon and liver cancer in humans and in mouse models (3, 4). In addition, overexpression of TM4SF5 in hepatocytes or liver can promote non-alcoholic steatohepatitis (NASH) (5) and fibrosis (6). Systemically TM4SF5-overexpressing Tg$^{TM4SF5}$ C57BL/6 mice reveal nonalcoholic steatosis at 1 year old and NASH associated with fibrosis at 1.5 years old (5). Further, the *Tm4sf5*$^{-/-}$ KO mice fed high-fat diets gained less body weight than did wildtype mice (7). Therefore, TM4SF5 expression may be related to metabolic liver disease, although its functional relevance to APC, important for colon carcinogenesis (8), has not been explored. TM4SF5 is an *N*-glycosylated membrane

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protein that, like the tetraspanins, has four transmembrane domains, intracellular *N*- and *C*-termini, an intracellular loop, and two extracellular loops (one short and one long) (9). TM4SF5 can bind to many different membrane proteins, receptors, and cytosolic proteins to stabilize or translocate them, eventually altering their expression levels and signaling activities (10). Furthermore, diethylnitrosamine-treated C57BL/6 mice or TM4SF5-overexpressing FVB/N mice show tumor development when 10-12 months old (7). Therefore, it is likely that additional gene modification accelerates the development of pathological stages in TM4SF5 transgenic mice.

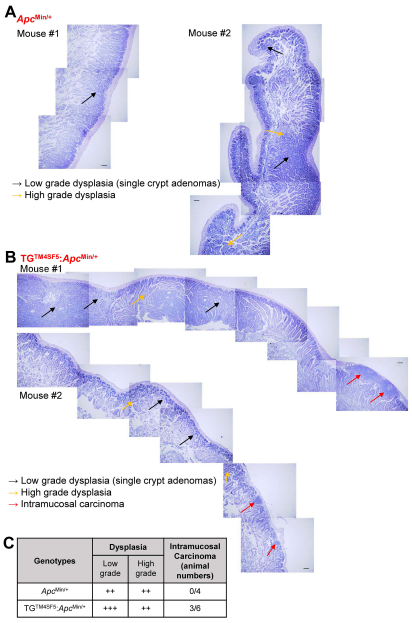
The loss of APC function by mutations and deletion is well-known for its pivotal role in colorectal carcinogenesis (11). Small dysplastic crypts are evident as pretumoral lesions or adenomas in the intestine of *Apc*$^{Min/+}$ mice (12). *Apc* mutation in *Apc*$^{Min/+}$ mice was detected as a nonsense mutation that emerged in the 850th codon of the two chains in the *Apc* gene, resulting in a truncated protein (8). Although all tumors in *Apc*$^{Min/+}$ mice are benign adenomas initially distributed in the small intestine, colon adenomas and adenocarcinomas can be observed in some older animals (13). APC forms the β-catenin destruction complex in association with casein kinase (CK1), AXIN1, and GSK3β and interacts with β-catenin (13). GSK3β-mediated β-catenin phosphorylation and the interaction between GSK3β and β-catenin supported by AXIN1 and APC can regulate the ubiquitination and/or stabilization of β-catenin, leading to its nuclear translocation, where it activates transcription (14).

In this study, we investigated whether a genetic transgenic mouse model with TM4SF5 overexpression together with *Apc* mutation (in *Apc*$^{Min/+}$ mice) may lead to more aggravated pathologies in either intestine/colon, liver, or both organs. Using an approach to interbreeding *Apc*$^{Min/+}$ mice with the systemic TM4SF5-overexpressing transgenic mice (Tg$^{TM4SF5}$), we examined the intestine/colon and liver tissues for pathological features. We also examined in vitro cell systems to find the molecular linkages, which were that TM4SF5 overexpression in the *Apc*$^{Min/+}$ mice caused intramucosal adenocarcinomas in the intestine/colon and portal hypertension with fibrotic features in the liver, suggesting that TM4SF5 is a promising therapeutic target against colon and liver malignancy.

## RESULTS

### TM4SF5 overexpression in *Apc*$^{Min/+}$ mice promoted intestinal adenocarcinomas

To investigate the influence of systemic TM4SF5 overexpression in *Apc*$^{Min/+}$ mice, we bred TM4SF5-overexpressing Tg$^{TM4SF5}$ mice with *Apc*$^{Min/+}$ mice, and investigated the dual-transgenic offspring at 26 weeks of age for pathological features in the intestine and liver. In intestinal tissues, *Apc*$^{Min/+}$ mice had distinct single-crypt adenomas (Fig. 1A), whereas the dual-transgenic (*Apc*$^{Min/+}$:Tg$^{TM4SF5}$) mice had further advanced phenotypes, such as intramucosal adenocarcinomas and different grades of dysplasia (Fig. 1B). These observations suggest that TM4SF5 might be functionally linked to *Apc* mutation, resulting in the pathological aggravation of the mouse intestine.




C

| Genotypes | Dysplasia | | Intramucosal Carcinoma (animal numbers) |
|---|---|---|---|
| | Low grade | High grade | |
| *Apc*$^{Min/+}$ | ++ | ++ | 0/4 |
| TG$^{TM4SF5}$;*Apc*$^{Min/+}$ | +++ | ++ | 3/6 |

**Fig. 1.** Additional systemic overexpression of TM4SF5 in *Apc*$^{Min/+}$ mice led to intramucosal adenocarcinomas in the intestines. We analyzed the intestines of *Apc*$^{Min/+}$ ($n$ = 4) or *Apc*$^{Min/+}$:Tg$^{TM4SF5}$ ($n$ = 6) mice at 26 weeks old using hematoxylin and eosin (H&E) staining. Tissues from two representative animals are shown separately in combined images (A, B). We quantified the pathological conditions (C).

### TM4SF5 overexpression mediated the intestinal stabilization of β-catenin

Immunohistochemical staining of β-catenin in the tissues revealed that overexpression of TM4SF5 led to the invasive accumulation and increased expression levels of β-catenin in the intestinal tissues of *Apc*$^{Min/+}$ mice (Fig. 2A). The TOPFlash luciferase reporter assay in HT29 cells without or with TM4SF5 suppression, using control or shTM4SF5 construct transfection, respectively, revealed that the Wnt-3a-mediated increase in transcriptional activation of β-catenin was partially inhibited by TM4SF5 suppression (Fig. 2B), indicating that TM4SF5 mediated β-catenin activation upon Wnt-3a treatment in HT29 cells. Overexpression of wild-type (WT) APC (APC-full) reduced the expression of TM4SF5, β-catenin, and c-Myc and decreased the phosphorylation of β-catenin at Tyr142 (pY$^{142}$β-catenin), but increased GSK3β phosphorylation at Ser9 (pS$^{9}$GSK3β) (Fig. 2C). Additionally, TM4SF5 suppression in HT29 cells reduced β-catenin levels and abolished Wnt-3a-promoted effects, such as STAT3 and Akt phosphorylation, whereas pS$^{9}$GSK3β was increased (Fig. 2D). Moreover, overexpression of TM4SF5 in HCT116 cells increased STAT3 and Akt phosphorylation, β-catenin expression and Y142 phosphorylation, and c-Myc expres-

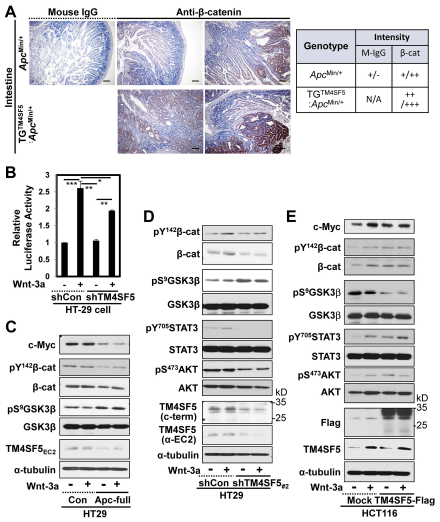



**Fig. 2.** Additional systemic overexpression of TM4SF5 in $Apc^{Min/+}$ mice increased β-catenin stabilization and transcriptional activity. (A) We analyzed the intestines of $Apc^{Min/+}$ ($n$ = 4) or $Apc^{Min/+}$:$Tg^{TM4SF5}$ ($n$ = 6) mice at 26 weeks old using immunohistochemistry (IHC) with mouse IgG or anti-β-catenin antibody. β-catenin immunostaining intensities were categorized as explained in the Materials and Methods section or quantitative comparison between conditions. (B) We analyzed HT29 cells via luciferase reporter assay following transfection with the β-catenin-responsive LEF/TCF-1 reporter pTOP-FLASH vector with shRNA plasmids (shControl or shTM4SF5) for 24 h; cells were treated with (+) or without (−) Wnt-3a for 12 h before the analysis. *, **, *** depict $P < 0.05$, 0.01, and 0.005, respectively. (C-E) HT29 (C, D) or HT116 (E) cells were independently transfected with control or Apc-full (C), shControl (shCon) or shTM4SF5 (D), or Mock-Flag or TM4SF5-Flag (E) plasmids for 24 h, and the cells were treated with recombinant Wnt-3a for 12 h, as explained in (B), prior to whole-cell extract preparation for standard Western blots for the indicated molecules. The data represent three independent experiments.

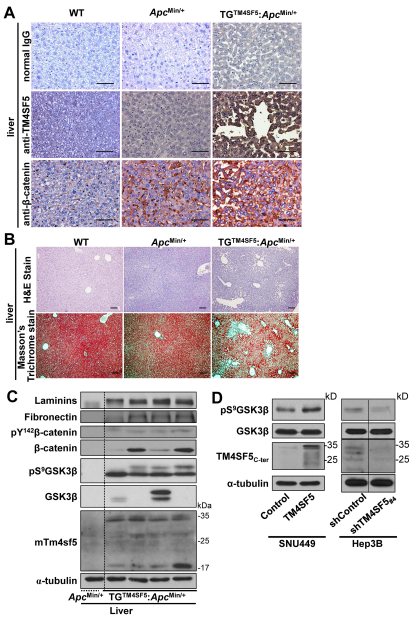



**Fig. 3.** $Apc^{Min/+}$:$Tg^{TM4SF5}$ mice showed sinusoidal dilatation, portal hypertension, and extracellular matrix (ECM) deposits in the liver. (A-C) We analyzed liver tissues from wild-type (WT, $n$ = 4), $Apc^{Min/+}$ ($n$ = 4), or $Apc^{Min/+}$:$Tg^{TM4SF5}$ ($n$ = 6) mice at 26 weeks old using IHC with normal IgG, anti-TM4SF5, or anti-β-catenin antibody (A) and H&E staining or Masson's trichrome staining (B). The liver tissues were also immunoblotted for the indicated molecules (C). (D) TM4SF5-null SNU449 or endogenously TM4SF5-expressing Hep3B hepatocytes were transfected with control, TM4SF5 WT, shControl, or $shTM4SF5_{\#4}$ plasmids for 48 h, before whole cell lysate preparation for immunoblots for the indicated molecules. The data represent three independent experiments.

sion, but decreased $pS^9GSK3\beta$ (Fig. 2E). Therefore, we suggest that the co-expression of APC-full with TM4SF5 reduces TM4SF5-mediated stabilization of β-catenin and Wnt-3a-mediated transcriptional activation.

### *APC* mutation in $Tg^{TM4SF5}$ mice led to hepatic portal hypertension associated with fibrosis

We next explored how dual expression of TM4SF5 and $Apc^{Min/+}$ affects the pathological features in the liver. Compared to the relatively normal livers of $Apc^{Min/+}$ mice, the dual-transgenic mice had distinct sinusoidal dilatations and abnormal portal vessels, in addition to increased TM4SF5 and β-catenin expression (Fig. 3A). Furthermore, the livers of the dual-transgenic mice showed abnormal portal vesicular channels for portal hypertension with distinct collagen I deposits (Fig. 3B). Contrasting with its overexpression in the intestines, TM4SF5 overexpression in the livers of the dual-transgenic mice related positively with $pS^9GSK3\beta$ and the expression of β-catenin, laminins, and fibronectin (Fig. 3C). Furthermore, ectopic overexpression or suppression of TM4SF5 in TM4SF5-null SNU449 or endogenously TM4SF5-expressing Hep3B hepatocytes, respectively, revealed a TM4SF5-mediated increase in $pS^9GSK3\beta$ (Fig. 3D). Thus, we suggest that the expression of the APC mutant and TM4SF5 in the liver involves positive relationships between $pS^9GSK3\beta$, β-catenin, and extracellular matrix (ECM) proteins.

### TM4SF5 overexpression without *Apc* mutation in older mice led to non-alcoholic steatohepatitis, portal hypertension, and fibrosis

Next, we hypothesized that TM4SF5 overexpression without *Apc* mutation for a longer period may affect the mouse liver in a way similar to that seen with TM4SF5 overexpression with *Apc* mutation for a shorter time. We thus examined the liver tissue of $Tg^{TM4SF5}$ mice that were about 1.5 years old, compared with that of the dual-transgenic ($Apc^{Min/+}$:$Tg^{TM4SF5}$) mice at 26 weeks (i.e., 0.5 years). Interestingly, compared to age-matched WT mice, 1.5-year-old $Tg^{TM4SF5}$ mice had livers with sinusoidal dilatation, portal and lobular inflammation, collagen I accumulation, and fat-droplet deposits (Fig. 4). Given that β-catenin might be induced via TM4SF5 overexpression, TM4SF5 overexpression without any contribution from *Apc* mutation for a

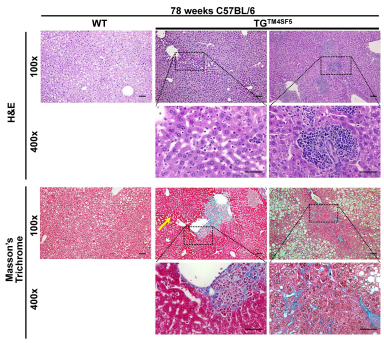



**Fig. 4.** $Tg^{TM4SF5}$ mice at 1.5 years old showed the phenotypes of portal hypertension associated with steatohepatitis and ECM deposits. We collected and analyzed liver tissues from 1.5-year-old C57BL/6 WT or $Tg^{TM4SF5}$ male mice (n = 7) using H&E staining and Masson's trichrome staining. Random representative images are shown. Image magnification is shown at 100× or 400×.

longer period appeared to result in non-alcoholic steatohepatitis, portal hypertension, and fibrosis/cirrhosis.

## DISCUSSION

We found that systemic TM4SF5 overexpression in $Apc^{Min/+}$ mice aggravated pathological features in the colon and liver, leading to intestinal adenocarcinoma and hepatic portal hypertension associated with fibrosis/cirrhosis. Further, TM4SF5 overexpression led to hepatic sinusoidal dilatations with ECM deposits (this study), in addition to our recent report that knockout of the *TM4SF5* gene in mice results in blood pressure lower than that of WT mice (15). Hepatic sinusoids are small blood vessels that constitute the liver microcirculation. Therefore, blockade of hepatic sinusoids via scar tissues in cirrhosis and the resulting increase in hepatic vascular resistance to portal flow is the primary cause of portal hypertension. A thrombus (blood clot) that develops in the portal vein can also lead to portal hypertension (16). For such phenotypes to develop, TM4SF5 overexpression-mediated β-catenin stabilization and transcriptional activation might be caused by decreased $pS^9$GSK3β in the intestines but increased $pS^9$GSK3β in the livers of $Apc^{Min/+}$ mice. The results of immunoblotting for $pS^9$GSK3β and β-catenin performed in colon HT29 cells and liver tissue extracts showed a similar opposite relationship. Indeed, $pS^9$GSK3β in the development of cancerous phenotypes can be either tumor-promoting or -suppressing (17). Active GSK3β with lower $pS^9$GSK3β functions as a tumor promoter in colorectal (18), renal (19), and pancreatic (20) carcinomas, whereas inactive GSK3β with higher $pS^9$GSK3β has been observed in skin cancer (21), mammary tumors (22), lung cancer (23), and liver cancer models (17). TM4SF5 expression may be functionally linked to the biomarkers or signaling pathways already well-known for cancer development in the liver or colon. We observed that TM4SF5-mediated GSK3β phosphorylation at Ser9 appeared differently regulated, being consistently significant in cancer developments in both organs as above, which might alternatively be effects secondary to the signaling molecules (or pathways) and/or pathological conditions in each organ. Understanding the different effects of $pS^9$GSK3β on β-catenin-mediated cell proliferation that depend on organ types would thus be interesting as the basis for future projects.

Mutation of *Apc* is a well-known cause of intestinal adenomas and carcinomas in murine models (8), an initiating factor in roughly 80% of all colorectal cancers, and increasingly being investigated in other cancers (24). TM4SF5 is involved in non-alcoholic steatohepatitis (5), fibrosis (6), and cancerous cell growth (3). Furthermore, TM4SF5 expression in liver cancer cells can promote metastatic potentials, such as angiogenesis (25), migration (26), invasion (27), self-renewal, and tumor-circulating capacity (28). Indeed, angiogenesis and fibrosis are involved in hepatic portal hypertension (16). However, the correlation between *Apc* mutation and TM4SF5 has not been explored for any colon and/or liver pathologies, although APC-mediated regulation of β-catenin stabilization and activation have been much explored (8). In the absence of a Wnt signal (i.e., inactive Wnt/β-catenin signaling pathway), APC forms a "destruction complex" with AXIN1, CK1, and GSK3β that leads to hyperphosphorylation of β-catenin and consequently ubiquitin-mediated proteolysis. Protein truncation generated by *Apc* gene mutation or decreased levels of APC protein activate the canonical Wnt signaling pathway. When the destruction complex disassembles, β-catenin fails to be degraded and accumulates in the cytoplasm, translocates into the nucleus, and binds to the TCF/LEF transcription factor family, causing the abnormal expression of downstream genes, such those for c-Myc, cyclin D1, and VEGF (29).

The relationship between TM4SF5 and β-catenin in the liver or hepatocytes appears to be in a bidirectional positive linkage. Further, as we have shown in this study, the role of $pS^9$GSK3β in β-catenin stabilization in the intestine/colon was different from that in the liver. TM4SF5 mediates the induction of β-catenin in the liver and intestines (this study), and a previous report revealed that β-catenin mediates TM4SF5 induction in hepatocytes (17). TM4SF5 overexpression in hepatocytes promotes β-catenin transcriptional activity for the growth of a three-dimensional sphere in aqueous conditions; a bidirectional positive cross-talk between CD133 and TM4SF5 transduces a signal to GSK3β/β-catenin activity to induce TM4SF5 (17). For this bidirectional positive cross-talk, TM4SF5 in hepatocytes binds CD133, a well-known biomarker for cancer stem cells (30), including those involved in hepatocellular carcinoma (31). Further, CD133 can trigger Akt activation, $pS^9$GSK3β and β-catenin stabilization, and transcriptional activation of *CD44* and *TM4SF5*. The translational product TM4SF5 is located at membranes and activates c-Src (32), which phosphorylates STAT3 at Tyr705 (33) or CD133 at Tyr828/Tyr858 (34). TM4SF5 (3) and phosphorylated CD133-mediated PI3K activity allows Akt1 to be activated for $pS^9$GSK3β (35). The signal trans-

duction emanating from TM4SF5 can, thus, activate Akt/GSK3β for β-catenin stabilization.

In conclusion, it is likely that TM4SF5 overexpression in the presence of *Apc* mutation and/or β-catenin stabilization aggravates carcinogenic processes in mouse intestine/colon and liver. We suggest that TM4SF5 is a promising target for the treatment of colon and liver malignancy. However, further clarification of the mechanisms underlying the different (positive or negative) stabilization of β-catenin via $pS^{9}$GSK3β in mouse intestine/colon versus the liver and whether these different effects are observed in humans would also be beneficial in the development of clinical therapies for the two prevalent cancer types. Meanwhile, strategies to block TM4SF5 expression and function are promising therapeutic approaches against colon and liver cancers (36).

## MATERIALS AND METHODS

### Cells

Human HT29 and HCT116 (Korean Cell Bank, Seoul National Univ., Korea) were cultured in Roswell Park Memorial Institute 1640 or Dulbecco's Modified Eagle's Medium (Welgene Inc., Daegu, Republic of Korea) containing 10% fetal bovine serum (GenDEPOT) and 1% penicillin/streptomycin (GenDEPOT, Barker, TX, USA) at 37°C in 5% $CO_2$. Lipofectamine RNAiMAX or Lipofectamine 3000 was used for the transfections following the manufacturer's protocols (Thermo Fisher Scientific, Waltham, MA, USA). Cells were checked for mycoplasma every other month, and their identities were confirmed upon receipt from the Korean Cell Bank.

### Mice

TM4SF5 transgenic C57BL/6 mice were generated (Macrogen, Seoul, Korea), and TM4SF5 expression was confirmed (37). The pcDNA3-hTM4SF5-FLAG plasmid (digested with *NruI* and *DraIII*), consisting of the cytomegalovirus promoter, whole human TM4SF5-FLAG sequence, and bovine growth hormone polyadenylation region, was used for the generation of C57BL/6 mice according to standard procedures, as explained previously (37). $Apc^{Min/+}$ mice were kindly gifted by Dr. Young-Yun Kong (Department of Biological Sciences, Seoul National University, Seoul, Republic of Korea). $Apc^{Min/+}$ mice were interbred with $Tg^{TM4SF5}$ to generate $Apc^{Min/+}$:$Tg^{TM4SF5}$ offspring. All animal experiments were performed in accordance with the guidelines of the Seoul National University Institutional Animal Care and Use Committee (SNU-130225-9-2).

### Western blots

Colon (HT29 or HCT116) or liver (SNU449 or Hep3B) cancer cells were grown in culture plates or dishes and harvested at 80% confluency before preparation of whole-cell lysates with modified radioimmunoprecipitation assay buffer (38). Primary antibodies used for immunoblots were as follows: anti-laminins (Abcam, Cambridge, UK), anti-β-catenin, anti-$pS^{473}$Akt, anti-Akt, anti-c-Myc, (Santa Cruz, Biotechnology, Santa Cruz, CA, USA), anti-Flag, anti-$pS^{9}$GSK3β, anti-GSK3β, (Cell Signaling Technology. Danvers, MA, USA), anti-$pY^{142}$β-catenin (ECM Biosciences, Versailles, KY, USA), anti-α-tubulin (Sigma), anti-signal transducer and activator of transcription 3 (STAT3, Chemicon, Rolling Meadows, IL, USA), anti-$pY^{705}$STAT3 (Millipore, Billerica, MA), anti-HA (BioLegend, San Diego, CA, USA), and anti-fibronectin (Dako Diagnostics, Glostrup, Denmark). Antibodies that detect the human TM4SF5 EC2 (long extracellular loop) sequence or C-terminus sequence (39) or the sequence ($^{117}$CLID NKWDYHFQETEGAYLRND138) in mouse TM4SF5 were custom designed (Pro-Sci, Poway, CA, USA).

### Statistical methods

A one-way ANOVA with Dunnett's or Turkey's multiple comparison test was performed to determine the significance of differences between two groups. A P-value less than 0.05 was considered significant.

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## CONFLICTS OF INTEREST

The authors have no conflicting interests.

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