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Running title: Expression of Tmem41b and MMP13 in osteosarcomas

Expression of Tmem41b and MMP13 associated with poor outcome in osteosarcomas

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Osteosarcoma (OS) is a malignant bone sarcoma characterized by a propensity for metastatic spread. Tmem41b is a multi-spanning membrane protein that acts as a novel autophagy-related (ATG) gene; however, its effect on the malignant phenotypes of tumor cells and the corresponding molecular details remains unknown. In the current study, RNA-sequencing, quantitative PCR (qPCR), and immunohistochemical analysis were conducted to prove Tmem41b upregulation in 103 OS tissue specimens and three OS cell lines (U-2OS, U87, and MG63). It was strongly correlated with tumor size ($P < 0.01$), metastases ($P < 0.05$), and recurrence ($P < 0.05$) as well as poor survival time in OS patients. Subsequently, gene set enrichment analysis (GSEA) of U-2OS cells with Tmem41b knockdown links cell receptor activation, proliferation, and invasion according to RNA-sequence, and PCNA, Cyclin D1, Cyclin E1, and MMP13 expression levels were decreased by western blotting assay. Furthermore, the suppressive effect of Tmem41b knockdown on cell proliferation and invasion was demonstrated in vitro and in vivo. Additionally, Tmem41b silencing could significantly inhibit the AKT and p38 signaling pathways. Last, MMP13 upregulation was positively correlated with Tmem41b expression and poor survival time in OS patients via analysis of immunohistochemical detection and bioinformatics. All together, these findings demonstrate the role of Tmem41b and MMP13 as a novel prognostic marker and an attractive therapeutic target for OS.

Key words: osteosarcoma; Tmem41b; MMP13; proliferation; invasion

Osteosarcoma (OS) represents a widespread malignant primary bone sarcoma in humans [1]. It accounts for approximately 20% of all bone tumors and 5% of pediatric tumors [2] and is characterized by high tumor cell proliferation, mortality, and metastatic rates [3]. This condition is most commonly seen in patients aged < 25 year (60%) and in 5% of pediatric tumors [4]. Most sub-types of OS are characterized by the production of osteoid bone by malignant cells with a

propensity for metastatic spread, particularly to the lungs [5]. OS patients have a poor prognosis with a 5-year survival rate of < 20% using the traditional methods of treatment, including chemotherapy, wide tumor resection, and amputation [6]. Thus, the pathogenesis of the growth and invasion of OS should be deeply understood to prevent, treat, and improve patient survival.

The development and tumorigenesis of OS is relative to alteration of numerous molecular, including gene sequence abnormalities, chromosomal rearrangements, epigenetic change and dysregulation of signaling pathways [4]. The inhibition of rubidium (Rb) and the p53 tumor suppression signal pathways [7] and the hyperactivation of receptor tyrosine kinases (RTKs) [8] and EGFR pathways [9], ERK/MAPK pathway [10], and PI3K/AKT pathway [11], which participated in tumor cell proliferation, migration, and invasion, have been found in OS, whereas the exact mechanisms involved in these processes remain unclear.

Transmembrane protein 41 (Tmem41b) is an evolutionarily conserved transmembrane protein first identified as an essential requirement for the development of normal neurotransmission in mammals [12]. Recently, numerous studies showed that Tmem41b is a novel regulator of autophagy and lipid mobilization and is involved in the formation of autophagosomes [13-16]. Furthermore, some studies have reported that autophagy signaling can affect tumor cell proliferation and invasion in pancreatic cancer [17], renal carcinoma [18], and OS [10]. Meanwhile, AKT and MAPK signaling pathway also induce autophagy to promote proliferation and increase anti-cancer drug resistance [19, 20]. Notably, Tmem41b is one of the most significantly mutated genes in the lung neuroendocrine tumor spectrum, which affects cellular metabolism, immune regulation, cell cycle, apoptosis, and cell death [21]. Additionally, abnormal Tmem41b expression has been observed in OS, according to the Gene Expression Omnibus (GEO) database [22], indicating that Tmem41b may be involved in tumorigenesis and the development of OS. Yet, surprisingly, to the best of our knowledge, studies on the role of Tmem41b in OS are still lacking.

Thus, this research intended to identify the relationship between Tmem41b expression and the clinicopathological features of OS, as well as to investigate the roles of Tmem41b in OS cell proliferation and invasion.

Patients and Methods

OS tissue specimens. Tumor tissue specimens and the corresponding para-cancer tissues (control)

were obtained from 103 patients diagnosed with OS from orthopedic operation at the Orthopedics department of the Tangshan Second Hospital between January 2014 and December 2019. The samples were divided into two parts: one was snap□frozen in liquid nitrogen, and the other was paraffin□embedded. The patients with OS were staged according to the Enneking staging system [23]. All subjects provided written informed consent. The study protocol was approved the Ethics Committee of the Tangshan Second Hospital.

RNA-sequence analysis. Total RNA was extracted and quantified, as well as the mRNA profiles were detected in the three tumor tissue samples obtained from patients with OS and the corresponding para-cancer tissues (control) as well as from U-2OS cells transfected with si-Tmem41b or si-NC by Illumina HiSeq 2000. Libraries were sequenced using 1 × 58 bp single-end reads, with two indexed samples per lane, yielding about 32.5 million reads per sample. After alignment of the sequencing reads to the human genome, counts for each gene were computed for each sample by use of the HTSeq software (version v0.5.3p3).

Differentially expressed mRNAs were identified using the paired *t*-test. A gene set enrichment analysis (GSEA) was conducted for further analysis of the biological pathways involved in the pathogenesis of OS through the Tmem41b pathway according to RNA-sequencing data.

Immunohistochemistry (IHC). Tumor tissues were fixed in 10% formalin followed by paraffin embedding and sectioning at 4 μm, which were dewaxed, rehydrated and subjected to microwave-induced antigen retrieval. Subsequent to blocking endogenous peroxidase activity, the sections were incubated with the primary antibodies against Tmem41b (1:100) overnight at 4 °C. Immunohistochemistry was performed using SP method immunohistochemical kit (PH1699, Phygene, Life science). The slides were analyzed and images were captured using an Olympus BX60 microscope (Olympus Corporation, Tokyo, Japan). Sections that are known to stain positively were incubated in each batch and negative controls were also established by replacing the primary antibody with pre-immune serum.

The results of IHC staining were evaluated independently by two trained pathologists who were blind to the clinical data. The specimens were graded into three groups according to the extent of positivity as follows: negative, no positive stain; low, < 25% of the tumor cells showed positive stain; and high, > 25% of tumor cells showed positive stain. The slides were re-examined by the pathologists together, and an agreement was reached for the final evaluation.

104 **Bioinformatics analysis.** The overall survival analysis of OS patients was conducted from the
105 GEPIA dataset (Gene Expression Profiling Interactive Analysis) (http://gepia.cancer-pku.cn).
106 **Cell culture.** The human osteosarcoma cell lines (U-2OS, U87, and MG63) and human osteoblast
107 cell line Hob were obtained from American Type Cell Culture (ATCC). All three cell lines were
108 cultured in a Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine
109 serum and 100 U/ml penicillin/streptomycin (Gibco, Carlsbad, CA, USA) in a humidified
110 atmosphere with 5% CO_2 at 37 °C.
111 **Cell transfection.** Specific siRNA against Tmem41b and the indicated constructs were purchased
112 from GenePharma (Suzhou, China). The siRNA sequences of the Tmem41b duplex were as follows:
113 5′-CACATCTTCAGCACCGCTCGTCAGATGTGGAGAAACGTCAAGAGCTTTTTTG-3′ and
114 5′-GTCCAAAAAAAGCTTTTTGCAGCACATCCGTCTCAATGTGGCTGAAGAACAGC-3′.
115 The transfection was conducted through mixing of lipo2000 (Invitrogen, Carlsbad, CA, USA) with
116 the siRNA, and the mixture was added to the cells and incubated for 6 h. DMEM (10% FBS) was
117 then used for substitution and 24 h incubation, following subsequent cell treatment and collection.
118 qPCR and western blotting were used to validate the transfection efficiency of Tmem41b siRNA 24
119 h after transfection.
120 **Lentivirus infection.** The pLVX-AcGFP-Tmem41b lentiviral vector and the corresponding
121 pLVX-AcGFP-C1 lentiviral vector (NC) were constructed by GenePharma (Suzhou, China),
122 respectively. Lentivirus infection for 48 h in U-2OS cells inhibits Tmem41b expression. GFP
123 fluorescence expression confirms lentiviral infection efficiency. QPCR detection was performed to
124 verify the suppression of Tmem41b expression.
125 **Cell proliferation assay.** CCK-8 assay was used to detect the proliferation effects of Tmem41b on
126 U-2OS cells (Dojindo, Rockville, MD, USA) assay. Briefly, transfected U-2OS cells and control
127 were plated in 96-well plates (1×10^4 cells per well). CCK-8 solution (100 μl/well) was added at
128 different time point (0, 24, 48, and 72 h), and then were cultured for 1 h. Absorbance (450 nm) was
129 measured using the Multiskan EX plate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA)
130 for quantification of the reaction product.
131 **Cell invasion assay.** Analysis of cell invasion by Transwell assay was referenced by modified
132 Boyden chamber (BD, San Diego, CA, USA). Transfected U-2OS cells and control (1×10^5) were
133 plated in the top chamber of the insert pre-coated with Matrigel, the number of transmigrated cells

134 through the membrane was counted by hematoxylin-eosin staining (HE) staining and light
135 microscopic examination.

136 **Quantitative real time PCR.** The extraction of total RNA and qRT-PCR assay were conducted as
137 described previously [22]. The primers sequences used were as follows: Tmem41b,
138 5'-ATGGCGAAAGGCAGAGTCG-3' and 5'-ACCCAGGATTTTTCCTTCTGG-3'; MMP13,
139 5'-CTTGACCACTCCAAGGACCC-3' and 5'-CAGGCGCCAGAAGAATCTGT-3'; Sp2,
140 5'-GTCGTAATGAGCGATCCACAG-3' and 5'-AAGCAGGGCTAAGGGAGATG-3'; Klk11,
141 5'-AACCCAGCCTACCTGCTGT-3' and 5'-CCCCAGGTTCCTAGGCTCT-3'; Fkbp11,
142 5'-AGAGACGTTGCTCTTCCGTG-3' and 5'-ATCTACCAAGCTTCCCGCTG-3'; Slc22a17,
143 5'-TACCCCGCAGACAGATTTGG-3' and 5'-CGGTGATCATTCGGCATCAG-3'; Nr4a2,
144 5'-GGTCCCTTTTGCCTGTCCA-3' and 5'- GCCTGTCCAATCTCCTCCTTAG-3'; Dtx2,
145 5'-GAGTTTCACTCTGGCTGCCT-3' and 5'- GCTGAGGAGGACAAGAGGAC-3';
146 Hist1h2af, 5'- GCACCGTCTGCTTTACAAGG-3' and 5'- GCTCCTCATCGTTGCGAATG-3';
147 Crtap, 5'-GCTCAGTGGTTGCTGTTTGG-3' and 5'- GCAGTGGCCACAAGGAGTAT-3';
148 GAPDH: 5'-CACCCACTCCTCCACCTTTG-3' and 5'-CCACCACCCTGTTGCTGTAG-3'.

149 **Western blotting assay.** Total proteins were extracted from treated U-2OS cells and control as well
150 as tumor tissues via RIPA lysis buffer (Beyotime, Beijing, China) with protease inhibitors, and the
151 protein concentrations were measured by BCA kit. SDS-PAGE (10%) was used to separate proteins,
152 which were transfected to PVDF membrane. After blocking in 5% BSA, it was subjected to
153 incubation using primary antibodies against PCNA (1:1000, 10205-2-AP, Proteintech), Cyclin D1
154 (1:1000, 26939-1-AP, Proteintech), Cyclin E1 (1:1000, 11554-1-AP, Proteintech), MMP13 (1:1000,
155 18165-1-AP, olProteintech), Tmem41b (1:1000, PA553257, Thermo), LC3B (1:2000, #2775, Cell
156 Signaling), p62 (1:2000, #5114, Cell Signaling), AKT (1:1000, 60203-2-Ig, Proteintech), p-AKT
157 (1:1000, 66444-1-Ig, Proteintech), ERK (1:1000, 11257-1-AP, Proteintech), p-ERK (1:1000,
158 28733-1-AP, Proteintech), p38 (1:1000, 14064-1-AP, Proteintech), p-p38 (1:500, bs-0636R-2,
159 Bioss), and anti-β-actin (1:5000, 20536-1-AP, Proteintech) overnight at 4 °C. After washed in TBST,
160 membranes were then incubated with the HRP-conjugated secondary antibody (1:8000, Rockland)
161 for 1 h at room temperature. All images were obtained with the ECL detection system (Cell
162 Biosciences, Santa Clara, CA.).

163 **In vivo experiments.** In this study, 24 male nude mice (6-week-old) were randomly grouped into

two groups (12 mice/group), and U-2OS cells (2×10^8) infected with pLV-Vector or pLV-Tmem41b for 48 h and then was injected subcutaneously into the right flank of mice to establish the osteosarcoma xenograft model. Four weeks after injection, the isolated tumor volume was measured and then was weighed. The Experimental Animal Care Commission of Hebei Medical University approved all the experiments.

Statistical analysis. All data are mean determinations of three independent experiments. The statistical analysis was conducted using GraphPad Prism 8.0 software (San Diego, CA, USA) including ANOVA post-hoc tests (Bonferroni), Chi-square test and Student's t-test for cell proliferation, cell number, band density, gene expression. Overall survival regarding OS patients was evaluated using the Kaplan-Meier survival curve and the log-rank non-parametric test. A two-sided p-value < 0.05 was considered statistic significant for all tests.

Results

Up-regulation of Tmem41b expression is associated with poor survival in human osteosarcomas tissues. RNA-sequence was conducted to detect the mRNA expression profiles in the three tumor tissue samples from the patients with OS and the corresponding para-cancer tissues (control). As shown in Figure 1A, the top 50 mRNAs with significant differential expression patterns are listed according to a cut-off value of 2.0-fold. Furthermore, RT-qPCR validation showed that Tmem41b (15.3-fold) and MMP13 (18.7-fold) were most up-regulated in the human OS tissues (Figure 1B). In the IHC analysis, high levels of Tmem41b expression were observed in 74 OS tissues, whereas 21 samples presented with low expression levels; negative expression was observed in eight tissue samples (Figure 1C).

Table 1 lists the clinical pathological details of the 103 OS patients, whom were grouped through the mean level of Tmem41b expression. Correlation analysis indicated that its expression was positively correlated with tumor size, metastases as well as recurrence. Furthermore, the overall survival time in OS patients with low Tmem41b expression was significantly higher than that in patients with high Tmem41b expression (p=0.038, Figure 1D). Similarly, the overall survival time of 262 OS patients from the GEPIA dataset verified that patients with high Tmem41b expression have low survival rates (p=0.002, Figure 1E). The above results showed that Tmem41b may be a promising prognostic factor in OS patients.

194 **Tmem41b knockdown affects the proliferation and invasion of OS cells.** To further validate
195 Tmem41b up-regulation is associated with OS, we examined Tmem41b expression in three cell
196 lines (U-2OS, U87, and MG63) and in human osteoblast cell line Hob (control). The Western
197 blotting assay showed that Tmem41b expression was significantly higher in OS cells than in control,
198 and the highest expression was observed in the U-2OS cells (Figure 2A). Tmem41b expression was
199 knocked down by transfecting the OS cells with Tmem41b-specific siRNA (si-Tmem41b) or
200 nonspecific siRNA (si-NC). The results of the qPCR and Western blot demonstrated the marked
201 attenuation of Tmem41b expression in the si-Tmem41b-transfected cells compared with the
202 si-NC-transfected cells (Figures 2B, 2C). Meanwhile, expression of cell autophage-associated
203 protein LC3II and p62 was also significantly down-regulation (Figure 2C). Furthermore, GSEA
204 analysis indicated knockdown of Tmem41b in U-2OS cells mainly affected the genes related to
205 receptor activity, cyclin-dependent protein, and metastasis process (Figure 2D). Consequently, the
206 down-regulation of the proliferation- and invasion-associated proteins was verified (Figure 2E).
207 Collectively, the above results suggest that Tmem41b expression promotes OS cell proliferation and
208 invasion.

209 **Tmem41b knockdown suppresses cell proliferation and invasion.** Subsequently, it was found
210 that the si-Tmem41b-transfected U-2OS cells exhibited decreased proliferation (Figure 3A) and
211 invasion (Figure 3B) by CCK8 and Transwell assays, respectively. Additionally, these findings were
212 further confirmed in the xenograft nude mice model. As shown in Figures 3C-3F, xenograft nude
213 mice induced by a subcutaneous injection of U-2OS cell infected with pLV-Tmem41b or
214 pLV-Vector after 4 weeks demonstrated a significant decrease in tumor volume (Figures 3D, 3E)
215 and weight (Figure 3F) in pLV-Tmem41b group compared with that from pLV-Vector, and
216 accompanied by a decreased Tmem41b expression (Figure 3C). These results indicated the effects
217 of Tmem41b on the proliferation and invasion of OS cells.

218 **Tmem41b knockdown suppressed the AKT and p38 signaling pathways and MMP13**
219 **expression.** Further experiments using Tmem41b-knockdown U-2OS cell lines were conducted to
220 confirm whether Tmem41b involved in the proliferation and invasion of OS cells associated with
221 the activation of the ERK, AKT, and p38 signaling pathways. As shown in Figure 4A, the
222 knockdown of Tmem41b markedly decreased phosphorylated AKT (p-AKT) and p38 levels in the
223 U-2OS cells, indicating that Tmem41b-induced cyclin D/E and MMP13 expression positively

224 regulates p-AKT and p-p38 signaling. Furthermore, IHC analysis proved that MMP13 expression
225 was positive correlated with Tmem41b expression in the OS tissues (Figure 4B). Simultaneously,
226 the overall survival time of patients with low MMP13 expression was notably higher than that of
227 patients with high MMP13 expression levels ($p < 0.01$; n=103; Figure 4C). Similarly, the overall
228 survival time of 262 OS patients from the GEPIA dataset indicated that patients with high MMP13
229 expression had a shorter overall survival time (Figure 4D). Conclusively, Tmem41b knockdown
230 attenuated the proliferation and invasion of OS cells through the inhibition of cyclin D/E and
231 MMP13 expression via the AKT and p38 signaling pathways.
232

233 **Discussion**

234 In this study, we present the strong evidence of the important role of Tmem41b in the proliferation
235 and invasion of OS cells. Tmem41b expression in OS patients was higher than that in patients with
236 bone cysts. OS patients with high expression levels of Tmem41b and MMP13 had significantly
237 shorter survival periods. Furthermore, Tmem41b down-regulation inhibited OS cell proliferation
238 and invasion by suppressing the AKT and p38 signaling pathway and the expression of cyclin
239 D1/E1 and MMP13. The bioinformatics analysis further validated that Tmem41b expression may be
240 a diagnostic biomarker for OS.
241 Tmem41b is an integral endoplasmic reticulum membrane protein and functions as a novel
242 regulator of autophagosome biogenesis and lipid droplet dynamics [13-15]. Recently, two
243 documents have reported that Tmem41b was the most significantly mutated gene in neuroendocrine
244 prostate cancer [24] and pulmonary carcinoid tumors; additionally, it was found to affect the
245 cancer-relevant pathways and biological processes, including the MAPK/ERK pathway [19]. The
246 proliferation of NSCLC cells can be effectively inhibited by targeting the autophagy-related 2B
247 (ATG2B) expression [25]. Hence, we presumed that the knockdown of the autophagy-related
248 Tmem41b expression could have a similar effect on cell proliferation and invasion. Expectedly, the
249 results of the correlation analysis in this study showed that Tmem41b up-regulation was strongly
250 related to tumor size ($p < 0.01$), metastases ($p < 0.05$), and recurrence ($p < 0.05$).
251 Furthermore, Tmem41b expression levels in both human OS tissues and cultured OS cells were
252 significantly higher than those in the corresponding controls; the highest levels were observed in
253 U-2OS cell lines. Therefore, U-2OS cells were used in the subsequent experiments using RNA

knockdown to investigate the roles of Tmem41b in OS. The results of the GSEA analysis of RNA-seq detection indicated that the related gene enrichments for receptor activity, cyclin-dependent protein, and metastasis process were decreased in si-Tmem41b group, which guided us to further explore whether Tmem41b down-regulation involved in the inactivation of the biological pathway-associated proteins. As expected, Tmem41b knockdown tremendously reduced the levels of the cell cycle-associated proteins (PCNA, cyclin D1, and cyclin E) and the invasion-associated protein MMP13 [26], suggesting that Tmem41b may be an essential upstream molecule in the proliferation and invasion-associated signal pathways involved in the tumorigenesis and progression of OS.

Numerous studies have reported that the regulation of the ERK [27], AKT [28], and p38 [29] signaling pathways can significantly attenuate the growth and invasion of OS. Consistently, the down-regulation of Tmem41b decreased cyclin D/E and MMP13 expression levels in the U-2OS cells and suppressed cell growth and invasion via the inhibition of the AKT and p38 signaling pathways in the present study. Additionally, the inhibition of Tmem41b expression suppressed tumor growth in vivo, demonstrating that it will become a potential therapeutic target for the OS.

To conclude, our findings demonstrated that Tmem41b is of great significance in the tumorigenesis, progression, and prognosis of OS in humans. Tmem41b expression is significantly enhanced in human OS tissues. The dismal prognosis of OS patients can be predicted by the high expression levels of Tmem41b. Additionally, the proliferation and invasion of U-2OS cells were inhibited following the down-regulation of Tmem41b expression in vitro and in vivo. Thus, Tmem41b may prove valuable as an independent potential prognostic biomarker for patients suffering from OS.

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Figure Legends

376 **Figure 1.** Tmem41b is highly expressed in human osteosarcoma (OS) tissues. A) A subset of the
377 differentially expressed mRNAs detected in the OS tissues (n=3) and control (n=3) using
378 RNA-sequence. B) Ten mRNAs showing differential expression in the OS and normal aortic tissues
379 were validated by the real-time quantitative polymerase chain reaction (RT-qPCR). Data represent
380 mean±SD (n=3), *p < 0.05 vs. control. C) Representative images of immunohistochemical staining
381 for Tmem41b in OS tissues demonstrating negative, low, and high expressions. D) Overall survival
382 curve of 103 osteosarcoma patients from the Second Hospital of Tangshan based on the Tmem41b
383 mRNA level. E) The overall survival time in 262 osteosarcoma patients from the GEPIA (Gene
384 Expression Profiling Interactive Analysis) dataset.
385
386 **Figure 2.** Involvement of Tmem41b expression in the biological pathways of OS. A) Tmem41b
387 expression levels in different OS cell lines (U87, U-2OS, and MG63) and human osteoblast cell line
388 Hob (Normal) were evaluated by Western blot. The band intensities relative to β-actin are presented
389 as mean±standard deviation (n=3; *p < 0.05 vs. normal). B) QPCR verification of the knockdown
390 efficiency of si-Tmem41b-transfected cells. Data represent mean±SD (n=3), ***p < 0.05 vs. si-NC.
391 NC: negative control. n=3. C) U-2OS cells were transfected with si-NS or si-Tmem41b for 24 h,
392 and total cell lysates were analyzed by Western blotting assay using the anti-Tmem41b, anti-LC3B
393 and anti-p62 antibody. E) Enrichment plots from the gene set enrichment analysis (GSEA) of
394 RNA-seq results in U-2OS cells were transfected with si-NC or si-Tmem41b. Several signaling
395 pathways, including receptor activity, cell cycle, and metastasis was significantly decreased in
396 si-Tmem41b group. F) U-2OS was transfected with si-NS or si-Tmem41b for 24 h and the levels of
397 PCNA, Cyclin D1, Cyclin E1, and MMP13 were analyzed by Western blot. The band intensities
398 relative to β-actin are presented as mean±standard deviation; n=3; *p < 0.05 and **p < 0.01 vs.
399 si-NC
400
401 **Figure 3.** Tmem41b down-regulation suppressed proliferation and migration of OS cells in vitro
402 and in vivo. A) U-2OS cells were transfected with si-Tmem41b or si-NC RNA for 24 h, and a
403 CCK8 assay was performed to determine the cell proliferation at different time points. *p < 0.05
404 and **p < 0.01 vs. si-NC at corresponding time points. B) U-2OS cells were treated as described in
405 (A), and a Transwell assay was conducted to detect the migration of the cells. Right: quantitative

406 analysis of cells that traversed the filter to the other side and were stained by hematoxylin and eosin.
407 Data are expressed as mean±SEM from three independent experiments. **$p < 0.01$ vs. si-NC. C)
408 Knockdown of Tmem41b expression was measured by Western blotting. D) Tmem41b knockdown
409 inhibited tumor growth in a nude mice xenograft model in vivo. E) Tumor volume and F) weight
410 were measured after knockdown of Tmem41b expression. ***$p < 0.001$ compared with pLV-Vector
411 group
412
413 **Figure 4.** Down-regulation of Tmem41b suppressed MMP13 expression via AKT and p38 signaling
414 pathways. A) Western blotting assay for signaling alteration of proliferation and invasion correlated
415 with the p-AKT, p-ERK, and p-p38 pathway after Tmem41b knockdown in the U-2OS cells. Right:
416 Band intensities were measured and normalized to β-actin and are shown below. *$p < 0.05$ and ***p
417 < 0.001, vs. total AKT, ERK, and p38 as well as β-actin (n=3). B) Representative images of
418 immunohistochemical staining for Tmem41b in the OS tissues. Survival analysis showed that
419 tumors with low Tmem41b expression have a favorable prognosis compared with those with high
420 Tmem41b expression. C) The overall survival time of 150 osteosarcoma patients from the Second
421 Hospital of Tangshan, based on the MMP13 protein level. D) The overall survival time of 259
422 osteosarcoma patients, based on the MMP13 mRNA level from the GEPIA dataset.
423

424 **Table 1.** Association between Tmem41b expression and clinical characteristics in OS patients.

Parameter		Tmem41b expression (n=103)		p-value
		High (n=74)	Low (n=29)	
Age (years; mean±SEM)		23.8±6.2	25.4±7.7	0.323
Gender	Male	41	17	0.767
	Female	33	12	
Tumor size (cm)		5.96±0.48	6.25±0.29	0.004
Enneking's stage	□	9	5	0.737
	□A	27	12	
	□B	28	8	
	□	10	4	
Tumor location	Femur	32	11	0.813
	Fibula	14	7	
	Tibia	17	5	
	Humerus	5	2	
	Others	6	4	
Metastases	Lung	37	6	0.023
	Other	11	8	
	No	26	15	
Recurrence	Yes	45	11	0.036
	No	29	18	

425

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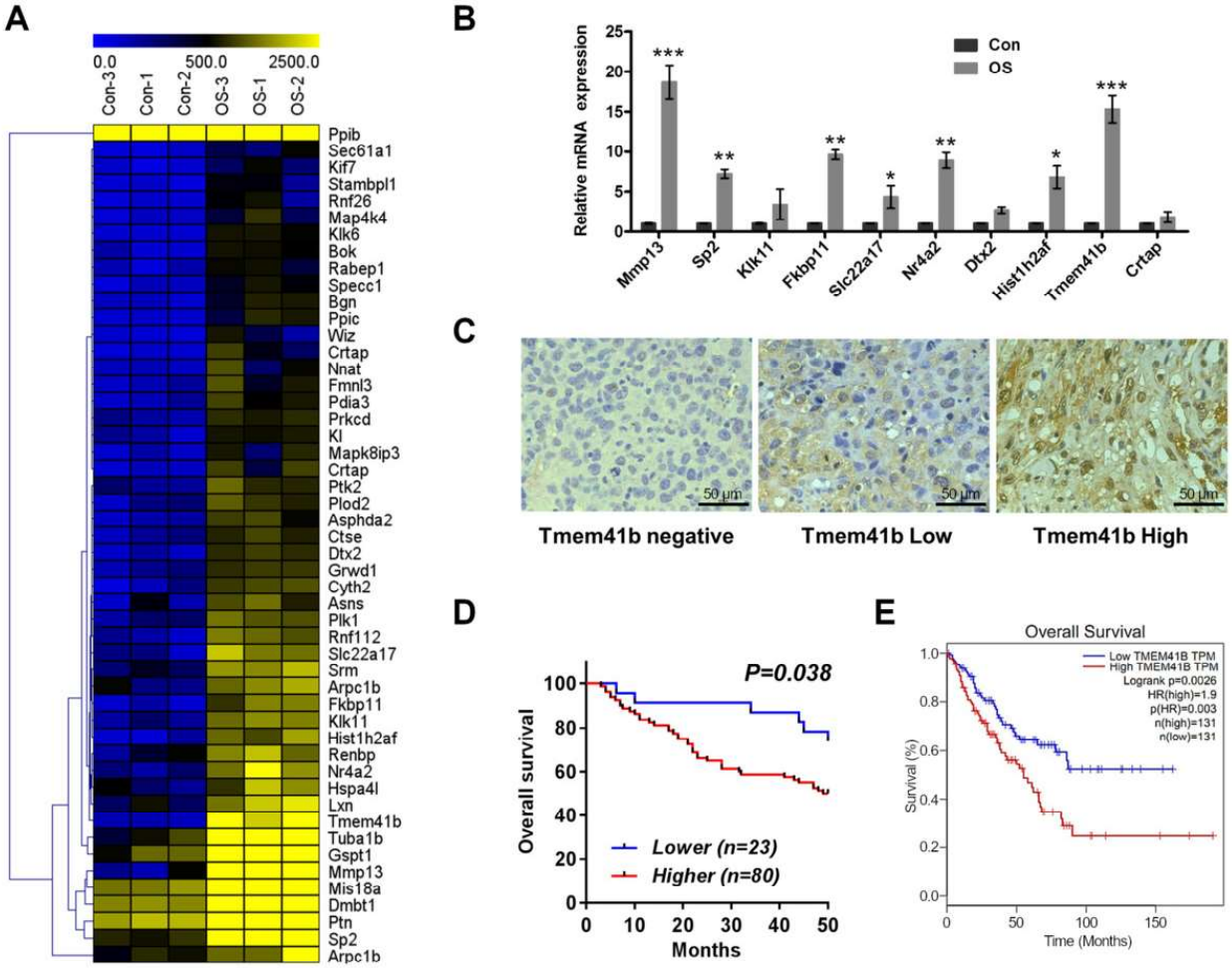

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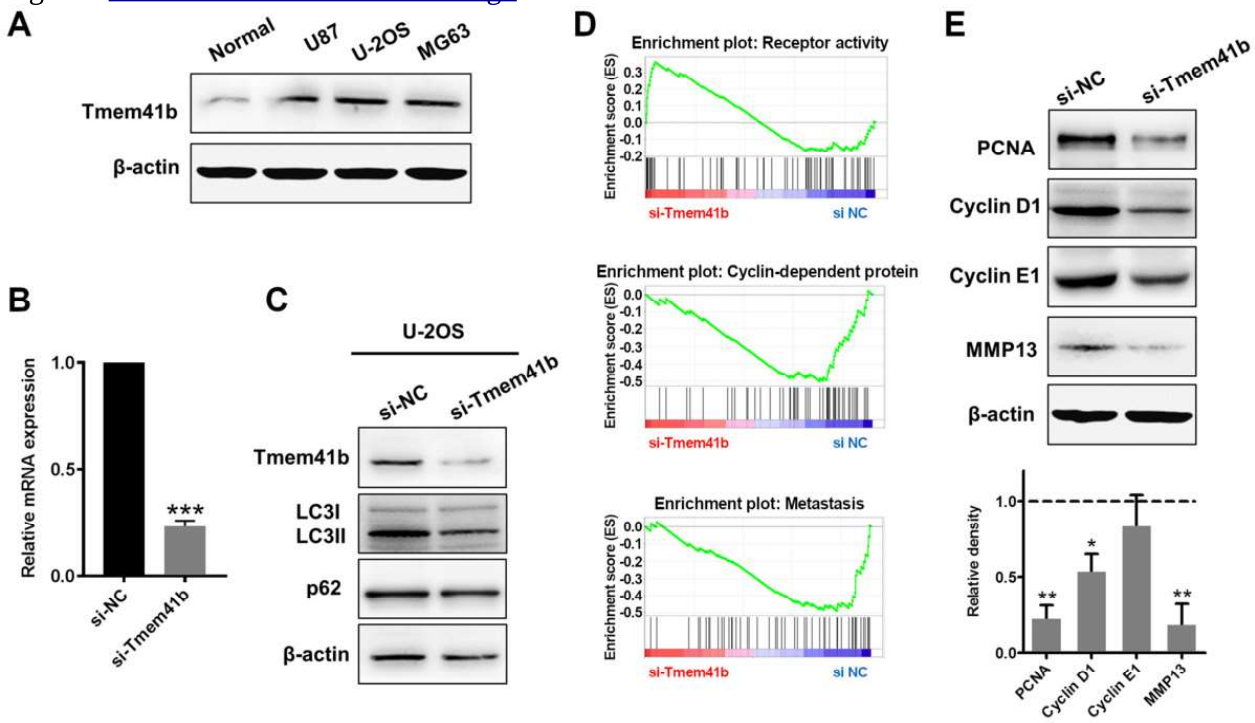

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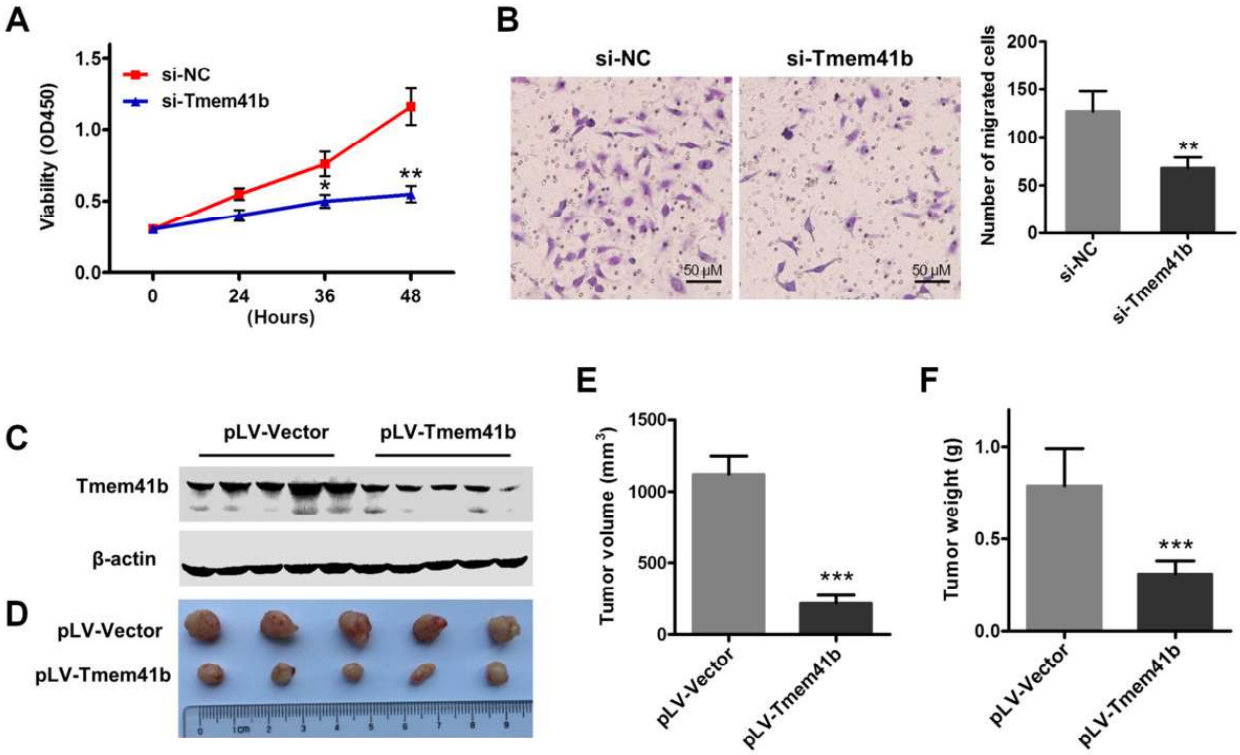

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