Am J Cancer Res 2022;12(2):713-728
www.ajcr.us /ISSN:2156-6976/ajcr0139726

Original Article

Semaphorin 4C promotes motility and immunosuppressive activity of cancer cells via CRMP3 and PD-L1

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Received October 10, 2021; Accepted December 22, 2021; Epub February 15, 2022; Published February 28, 2022

Abstract: Semaphorins (SEMAs) are membrane-bound or soluble proteins that participate in organ development and cancer progression, however, the detailed role of SEMAs in carcinogenesis is not fully elucidated yet. Our *in silico* analysis showed among the differentially expressed SEMAs in colon cancer tissues, patients with higher SEMA4C expression tumors had worse survival. The migration and invasion of the HCT116 and CT26 colon cancer cells were significantly suppressed by SEMA4C neutralizing antibody treatment; while enhanced by ectopic expression of SEMA4C. Subsequently, RNA sequencing study revealed microtubule polymerization- and nucleation-related genes are highly enriched in SEMA4C overexpression HCT116 cells. Western blotting showed the negative correlation between the levels of SEMA4C expression and tubulin acetylation. Mechanistic study showed SEMA4C interacted with and stabilized collapsin response mediator protein 3 (CRMP3), a novel deacetylase, to increase α-tubulin deacetylation and cell motility, which could be effectively attenuated after HDAC inhibitors treatment. We also found that a tumor-suppressive miRNA let-7b can target SEMA4C and act synergistically with SEMA4C neutralizing antibody to suppress the motility of colon cancer cells. In addition, blockade of SEMA4C could attenuate the expression of program death ligand 1 (PD-L1). Collectively, our results highlight that SEMA4C may promote colon cancer progression through modulating CRMP3-mediated tubulin deacetylation and PD-L1-mediated immunosuppression.

Keywords: SEMA4C, CRMP3, PD-L1, colon cancer

Introduction

Semaphorins (SEMAs), a family of proteins initially found as axon guidance factors, have been demonstrated to participate in diverse cellular processes and disease development [1-3]. In mammals, 20 SEMAs have been identified and are grouped into four classes (3, 4, 5 and 6) based on structure similarity [3]. Class 3 SEMAs are secretory proteins, while classes 4-6 are transmembrane proteins that may interact with other transmembrane proteins like plexins and neuropilins to activate bi-directional signals to modulate proliferation, migration, and cell-cell communication [4].

Among the SEMAs, the potential roles of class 3 SEMAs in tumorigenesis have been suggested. Because the main binding partners of class 3 SEMAs are neuropilin-1 and -2, two molecules that also function as the receptors of vascular endothelial growth factor A (VEGF-A), it has been hypothesized that class 3 SEMAs may modulate tumor angiogenesis. Indeed, six members of this class including SEMA 3A, 3B, 3C, 3D, 3E, and 3F have been shown to affect angiogenesis-dependent tumor growth [5-10]. In addition to angiogenesis, SEMA 3F and 3G have been reported to suppress lymphatic metastasis of various cancers by attenuating VEGF-C-promoted lymphangiogenesis [11, 12]. Recently, a new function of SEMA3E in immune regulation has been proposed. Study of clinical samples showed that the expression of SEMA3E was reduced in the airways of the patients with severe asthma [13]. Genetic de-

pletion of SEMA3E promoted allergic asthma in mice by promoting airway inflammation and hyperresponsiveness [14]. Mechanistically, the recruitment and function of dendritic cells and neutrophils to inflammatory sites were significantly affected after SEMA3E ablation. Interestingly, plexin D1, the receptor of SEMA3E, is also expressed in natural killer (NK) cells, suggesting SEMA3E may regulate the biological activity of NK cells [15]. These results suggested that class 3 SEMAs may modulate cancer formation by affecting cancer cell and tumor microenvironment.

Colon cancer is one of the most threating cancers worldwide. To clarify the function of SEMAs in colon cancer, we did systematic *in silico* analysis and found the alteration of several SEMAs in colon tumors. Among the altered SEMAs, the increase of SEMA4C raised our attention. SEMA4C has been reported to interact with postsynaptic density protein 95 (PSD-95) to regulate synaptic signaling [16]. Previous studies demonstrated that SEMA4C plays an important role in the regulation of migration of neural and immune cells [17, 18]. The main binding receptor of SEMA4C is plexin B2. Interestingly, inhibition of plexin B2 also impaired SEMA4C-controlled cerebellar development and breast cancer formation [17, 19]. Lines of evidences suggested that SEMA4C may exhibit tumor-promoting activity. Firstly, SEMA4C is overexpressed in a number of cancer types [19-22]. Secondly, SEMA4C increased lymphangiogenesis and angiogenesis to enhance tumor metastasis [19, 23]. Thirdly, SEMA4C has been shown to promote epithelial-mesenchymal transition (EMT) in cancer cells [20-22]. However, the underlying mechanisms by which SEMA4C enhanced tumorigenesis are not well characterized. In this study, we addressed how SEMA4C modulates the invasiveness and immune escape of colon cancer cells.

Materials and methods

Cell lines and reagents

Human colon cancer cell line HCT116 and mouse colon cancer cell line CT26 were kindly provided by Dr. Ming-Derg Lai (Department of Biochemistry and Molecular Biology, National Cheng Kung University) and cultured in 10% FCS (HyClone, Marlborough, MA, USA) and 1% penicillin-streptomycin-glutamine (PSG; Gibco, Waltham, MA, USA)-containing RPMI (HyClone) under 5% CO_2. Human colon cancer cell line SW480 was cultured in 10% FCS- and 1% PSG-containing DMEM (HyClone). BSA (A7906) was from Sigma (St. Louis, MO, USA). Rabbit control IgG (10201) was from Leadgene (Tainan, Taiwan). Antibodies for SEMA4C flow cytometry and blockage (GTX32865), acetyl lysine (GTX80693), total α-tubulin (GTX628-802), HDAC6 (GTX100722), PD-L1 (GTX10-4763), human c-MET (GTX631992), and pan-cadherin (GTX132646) were from GeneTex (Hsinchu, Taiwan). Antibody for Flag (F1804) was from Sigma. Antibody for acetyl α-tubulin (5335) was from Cell Signaling Technology (Danvers, MA, USA). Antibody for CRMP3 (ab128875) was from Abcam (Cambridge, MA, USA). Antibody for GAPDH (MAB374) was from Millipore (Burlington, MA, USA). Antibodies for human SEMA4C (AF6125) or mouse SEMA4C (AF6120) Western Blotting were from R&D (Minneapolis, MN, USA). Antibodies for mouse c-MET (AF527) and human/mouse phospho-c-MET (Y1234/Y1235) (AF2480) were also from R&D. EasyBlot (GTX628906-01) secondary antibody for immunoprecipitation-Western Blotting was from GeneTex.

Cell treatment and transfection

For antibody blockage, cells were treated with 2 μg/ml rabbit control IgG or SEMA4C antibody for 24 h. For reagent treatment, cells were treated with histone deacetylase inhibitor Vorinostat (SAHA; MedChemExpress, Monmouth Junction, NJ, USA) at indicated concentrations for 24 h. For plasmid transfection, cells were transfected with 2 μg plasmid together with 3 μl HyFect transfection reagent (Leadgene) for 48 h. For miRNA transfection, cells were transfected with 200 nM negative control miRNA or let-7b mimic (GenePharma, Shanghai, China) together with 3 μl HyFect reagent for 48 h. SEMA4C-overexpressing plasmid (HG11955-CF), control vector (CV020), CRMP3-overexpressing plasmid (HG22668-UT), and untag vector (CV011) were from Sino Biological (Beijing, China). shRNAs were from RNAi core of Academia Sinica (Taipei, Taiwan) and the sequence was listed as below: sh-h-CRMP3, CCGGGAAGTTTCTCGAAGGTGCTTGCTCGAGCAAGCACCTTCGAGAAACTTCTTTTTG.

Flow cytometry

Cells were fixed with 4% paraformaldehyde (Sigma) for 10 min at 4°C, stained with primary antibody in 1% BSA for 1 h, and then stained with fluorescent secondary antibody (Molecular Probes, Waltham, MA, USA) for another 1 h. Signal intensity of stained cells were analyzed with FACSCalibur (BD, San Jose, CA, USA) and FlowJo software (BD).

Antibody internalization assay

Antibody internalization assay was performed as previously described [24, 25] with minor modifications. Briefly, cells were seeded in 96-well plates at a density of 50,000 cells/ml. On the next day, cells were treated with control IgG or SEMA4C antibody for 1 h at 37°C. Cells were then permeabilized with 0.05% Tween-20-PBS for 10 min and probed with peroxidase-polymer secondary antibody Histofine Simple Stain MAX PO (NICHIREI, Tokyo, Japan) for 10 min. Bound antibody was detected with 3,3',5,5'-tetramethylbenzidine (Sigma), and reaction was stopped by 2N H_2SO_4. Reaction product was measured at O.D. 450 with spectrometer FlexStation 3 (Molecular Probes).

RNA sequencing

Sequencing library was constructed with TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA) following manufacturer's instructions. mRNA was purified from 1 μg RNA by oligo(dT)-coupled magnetic beads. First-strand cDNA was synthesized using random primers and reverse transcriptase. After generation of double-strand cDNA and adenylation on 3' ends of DNA fragments, adaptors were ligated and purified with AMPure XP system (Beckman Coulter, Beverly, MA, USA). Quality of libraries was assessed on Agilent Bioanalyzer 2100 system and real-time PCR. Qualified libraries were sequenced using Illumina NovaSeq 6000 platform with 150 bp paired-end reads generated by Genomics (New Taipei City, Taiwan). Filtered reads were aligned to reference genomes with Bowtie2 (version 2.3.4.1). Transcript quantification was performed with RSEM (version 1.2.28). Identification of differentially expressed genes was performed with EBSeq (version 1.16.0). Pathway enrichment was performed with (1) gene ontology and KEGG implemented in R package clusterProfiler (version 3.6.0), (2) gene ontology database AmiGO [26] and (3) geneset enrichment analysis (GSEA) [27].

L1000CDS^2 analysis

To identify potential therapeutics for SEMA4C-high colon cancer, gene-drug interaction search engine L1000CDS^2 [28] based on gene-drug interaction databases Connectivity Map and L1000 [29] was applied. Co-expressed gene signature was uploaded and predicted therapeutics were presented according to frequency. Therapeutics with the highest frequency in prediction was applied for *in vitro* validation.

Western blotting

Cells were lyzed in CelLytic M (Sigma) with 1 mM Na_3VO_4, 5 μg/ml aprotinin, and 1 mM PMSF for 20 min at 4°C, and centrifuged at 12,000 rpm for 15 min at 4°C. Supernatant was harvested and protein concentration was quantified with Bradford assay (Bio-Rad, Hercules, CA, USA). Equal amount of protein from each sample was mixed with 5× sample buffer (0.1% bromophenol blue, 8% dithiothreitol, 10% SDS, 50% glycerol, 250 mM Tris-HCl pH 6.8), and heated for 5 min at 95°C. The samples were subjected to electrophoresis, and transferred at 100V for 90 min to PVDF membrane (Millipore). The membranes were blocked with 5% BSA for 1 h, and incubated with primary antibody overnight at 4°C. After extensively washing, the membranes were incubated with secondary antibody (Jackson Immuno Research, West Grove, PA, USA) for 1 h, and signals were developed by ECL reagent (PerkinElmer, Waltham, MA, USA) and analyzed with UVP (ANALYTIKJENA, Jena, Germany).

Immunoprecipitation

Immunoprecipitation was performed as previously described [30, 31] with minor modifications. Briefly, cells were lyzed and protein concentration was quantified as described in Western Blotting. 20% sample was kept as input. Remaining samples were mixed with 0.5 μg control IgG or CRMP3 antibody, as well as 100 μl Protein G Mag Sepharose Xtra (GE28-9670-66, Sigma), and agitated overnight at 4°C. After washing, protein-antibody mix was resuspended in 5× sample buffer and heated

for 5 min at 95°C to elute CRMP3-interacting proteins. The eluted proteins were subjected to electrophoresis.

Immunofluorescence

Cells were seeded onto 6-well plates containing cover glass pre-coated with 0.1% gelatin (Sigma). After treatment, cells were fixed with 4% paraformaldehyde for 10 min, blocked with 2% FCS-PBS for 10 min, and incubated with primary antibody overnight at 4°C. After washing, cells were stained with fluorescent secondary antibody for 1 h and 0.5 μg/ml DAPI (Sigma) for 5 min. The images were analyzed under fluorescent microscope (IX71; OLYMPUS, Tokyo, Japan) and the signal intensity was analyzed with ImageJ.

Proliferation assay

Cell proliferation was analyzed with trypan blue exclusion assay as previously described [32].

Adhesion assay

96-well plates were pre-coated with 0.1% gelatin overnight at 4°C, and the wells were blocked with 1% BSA-PBS for 1 h at 37°C. Cells were seeded and allowed to adhere for 30 min at 37°C under 5% CO_2. After washing, cells were fixed with 4% paraformaldehyde for 10 min, stained with 0.5% methyl blue for 10 min, and de-stained with ddH_2O. Stained cells were counted under microscope.

Migration and invasion assay

Cells were resuspended in FCS-free medium, treated with indicated reagents, and seeded in the upper chamber of transwell. The lower chambers were filled with 10% FCS medium. Cells were allowed to migrate for 24 h, fixed with methanol, stained with 0.5% methyl blue, and de-stained with ddH_2O. Migrated cells were counted under microscope. For invasion assay, 0.1% gelatin coating onto transwell was performed.

Bioinformatic analysis

TCGA database analyses regarding expression change, prognostic power, and expression correlation on target genes in colon cancer dataset were performed with cancer multiomics databases UALCAN [33], DriverDBv3 [34], and GEPIA [35]. Colon cancer proteomic analysis on SEMA4C was performed with the result from previous study [36] regarding mass spectrometry survey in colon cancer. miRNA analysis on SEMA4C targeting was performed with databases miRDB [34], TargetScan [37], and miRWalk [38]. Overlapped miRNAs were further analyzed with miRNA cancer prognosis database KM plotter [39].

Statistical analysis

Statistical analysis was performed with SPSS Statistics 17.0 (IBM, Armonk, NY, USA). Statistical difference between control and experimental groups was calculated with Student's *t*-test, and $P<0.05$ was considered statistically significant.

Results

SEMA4C is upregulated in colon cancer and is associated with decreased survival

To investigate the importance of SEMAs during colon tumorigenesis, we applied UALCAN and DriverDBv3 cancer multiomics databases to analyze TCGA COAD dataset for target expression and survival association. Our results showed that a number of SEMAs displayed altered expression in colon tumor tissues. Downregulated SEMAs are 3B (8.31E-05), 3D (4.60E-12), 3E (6.63E-09), 3G (5.88E-09), 4B (5.44E-03), 4G (1.42E-10), 6A (<1E-12), 6C (1.91E-02), and 6D (6.66E-15), while SEMA 3F (<1E-12), 4C (5.57E-04), 4D (1.04E-11), 4F (1.62E-12), 5B (1.64E-11), 7A (2.13E-07) are upregulated (**Figure 1**). Among the altered SEMAs, only SEMA4C showed predictive power in both overall survival (hazard ratio =1.58, log-rank P=0.0221) and disease-free survival (hazard ratio =2.63, log-rank P=0.022) in colon cancer patients (**Figure 2A** and **2B**). In addition to mRNA level, we found that SEMA4C protein level in colon cancer is much higher than that in normal tissue (**Figure 2C**), based on previous mass spectrometry analysis [36]. Therefore, we focused on addressing the oncogenic function of SEMA4C.

SEMA4C influences cell motility but not cell proliferation

To identify the effect of SEMA4C on colon cancer behavior, we used human colon cancer cell

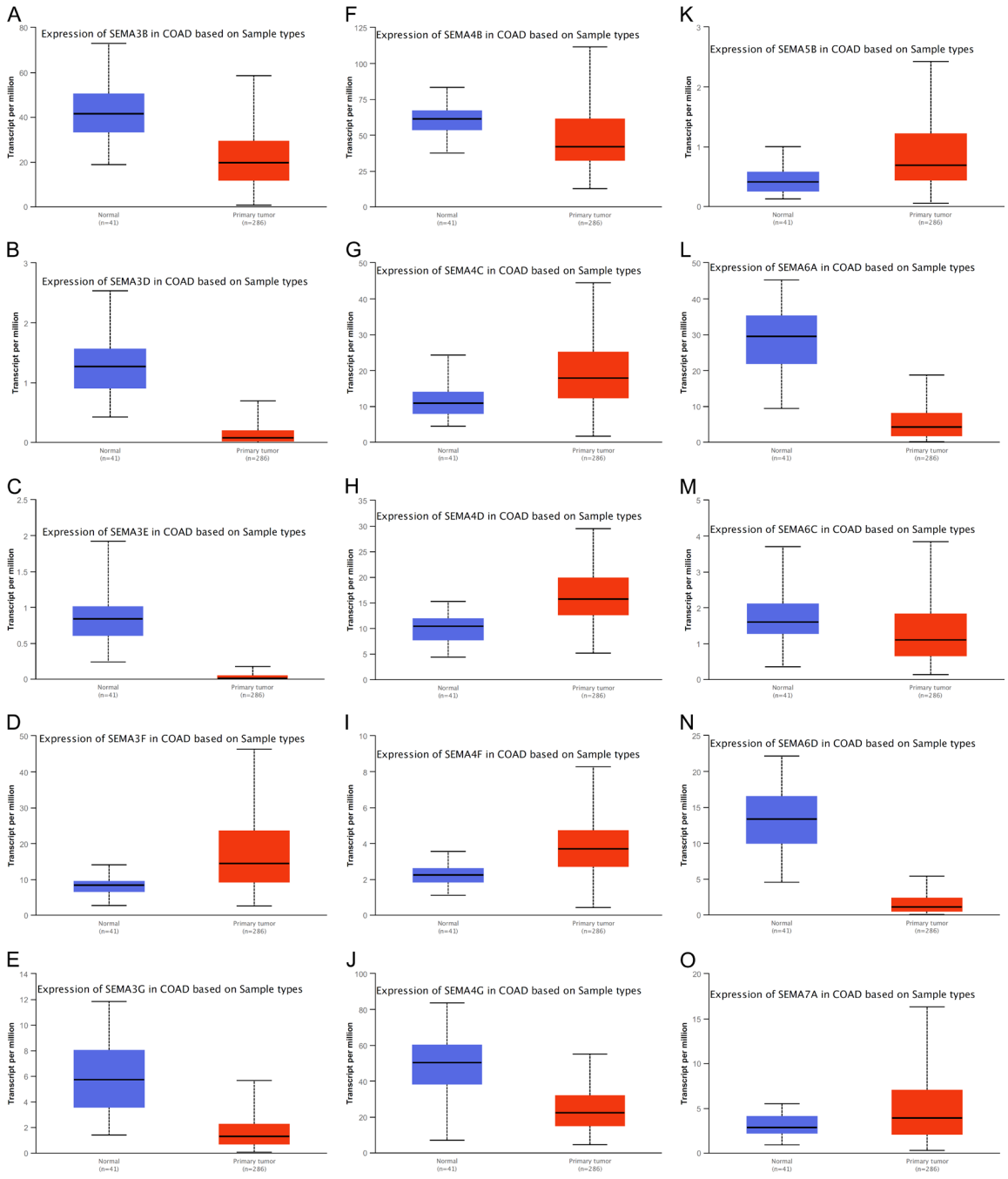

Figure 1. Alteration of expressions of SEMAs in TCGA colon cancer dataset. Expression levels of SEMAs in TCGA COAD was analyzed with cancer multiomics database UALCAN and statistical significance was tested.

lines HCT116 and SW480, as well as mouse colon cancer cell line CT26, in the present study. Because SEMA4C is a transmembrane protein, we performed antibody blockage to suppress SEMA4C function in cancer cells. Flow cytometric analysis confirmed the specific binding of SEMA4C neutralizing antibody (**Figure 2D** and **2I**). After 24-h incubation, antibody induced SEMA4C internalization into cells to terminate its signaling (**Figure 2E** and **2J**; Supplementary Figure 1) [40]. Our data showed that SEMA4C blockage had minor effect on proliferation (**Figure 2F** and **2K**). However, migration and invasion of HCT116 and CT26 colon cancer cells were significantly reduced (**Figure 2G**, **2H**, **2L**, **2M**). To verify the results,

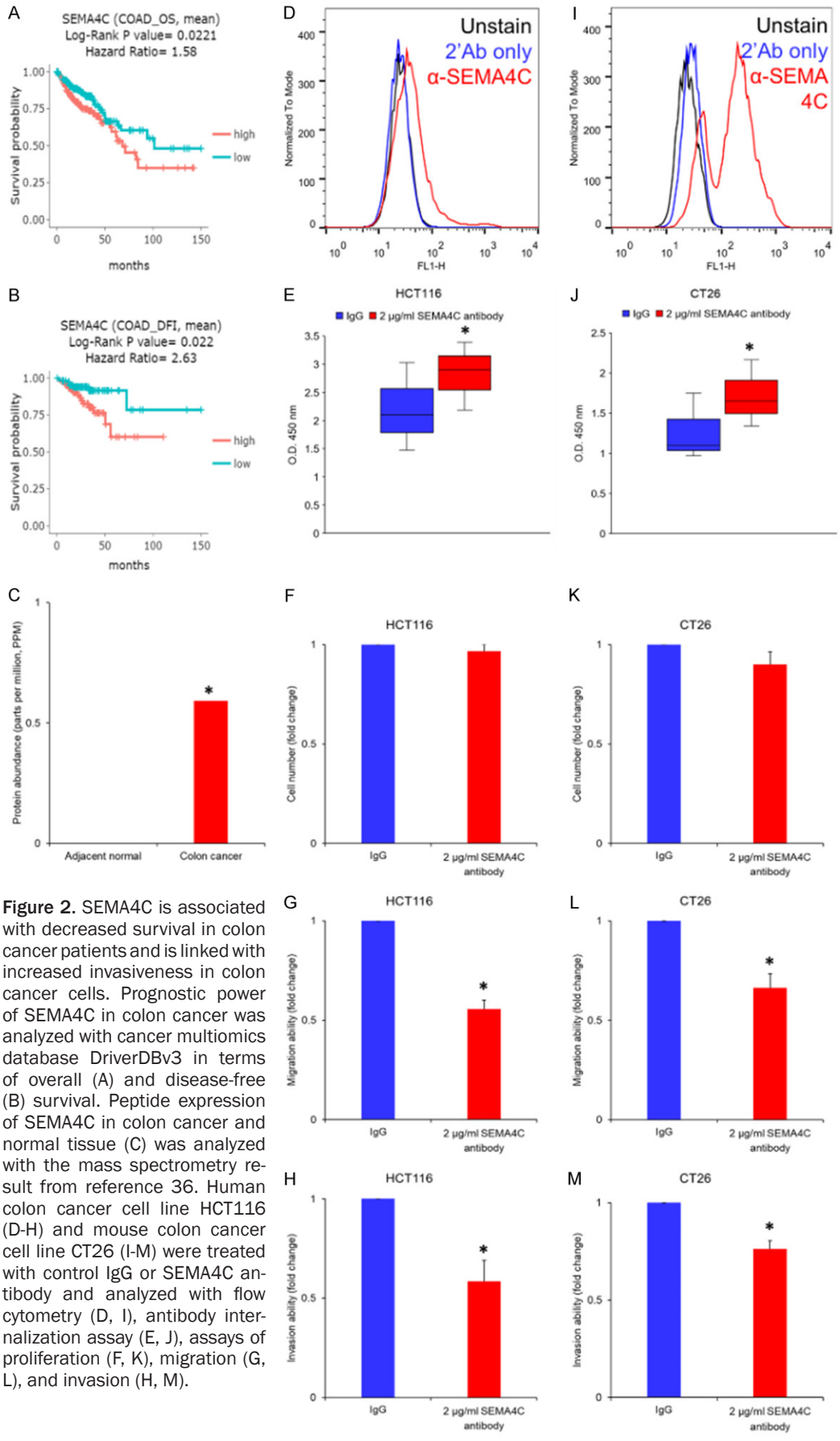

Figure 2. SEMA4C is associated with decreased survival in colon cancer patients and is linked with increased invasiveness in colon cancer cells. Prognostic power of SEMA4C in colon cancer was analyzed with cancer multiomics database DriverDBv3 in terms of overall (A) and disease-free (B) survival. Peptide expression of SEMA4C in colon cancer and normal tissue (C) was analyzed with the mass spectrometry result from reference 36. Human colon cancer cell line HCT116 (D-H) and mouse colon cancer cell line CT26 (I-M) were treated with control IgG or SEMA4C antibody and analyzed with flow cytometry (D, I), antibody internalization assay (E, J), assays of proliferation (F, K), migration (G, L), and invasion (H, M).

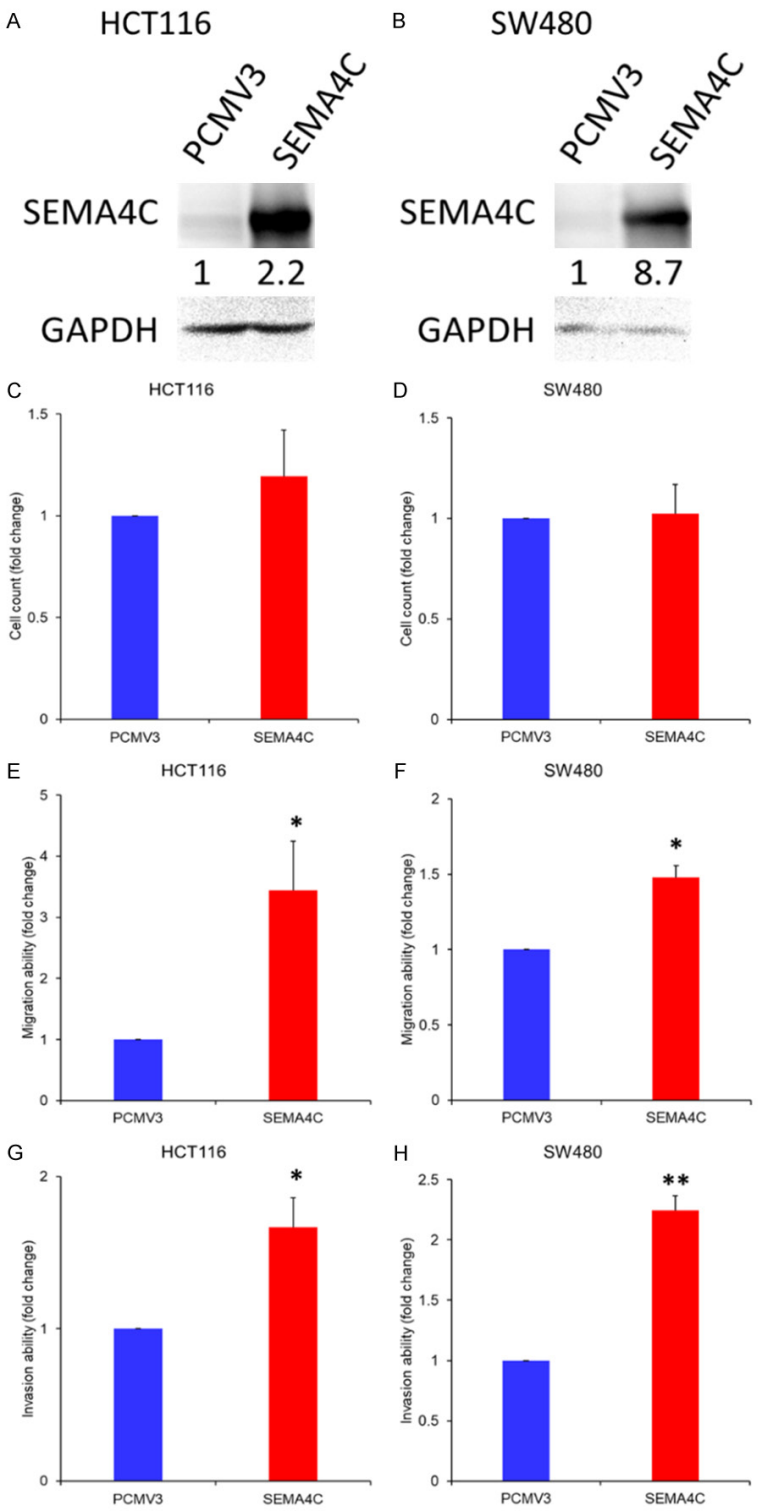

Figure 3. Ectopically expressed SEMA4C increases colon cancer motility. HCT116 (A, C, E, G) and SW480 (B, D, F, H) were transfected with control vector or SEMA4C-overexpressing plasmid, and analyzed for expression (A, B), proliferation (C, D), migration (E, F), and invasion (G, H).

we ectopically expressed SEMA4C in human colon cancer cell lines HCT116 and SW480 (**Figure 3A** and **3B**). Consistent with the results of antibody blockage assay, cell proliferation was not affected (**Figure 3C** and **3D**). However, cell migration and invasion were dramatically enhanced by SEMA4C (**Figure 3E-H**). These results suggested that SEMA4C enhanced colon cancer motility *in vitro*. Our finding is in accordance with previous reports [21, 39]. However, the mechanism underlying this phenomenon has yet to be clarified.

RNA sequencing reveals the alteration of cell adhesion pathway in SEMA4C-overexpressing colon cancer cells

To elucidate the molecular mechanism by which SEMA4C enhanced cancer cell motility, we analyzed TCGA colon cancer dataset and found the enrichment in the pathways of diverse biological processes, from vasculature development to electron transport chain, as well as cell adhesion and movement (**Figure 4A**). We compared the expression profile of parental and SEMA4C-overexpressing HCT-116 cells by RNA sequencing. As shown in **Figure 4B**, gene ontology study revealed that microtubule polymerization- and nucleation-related genes are highly enriched by SEMA-4C overexpression. Gene set enrichment analysis (GSEA) showed the enhancement of focal adhesion and cell adhesion molecules in the RNA seq results from TCGA database and our study (**Figure 4C** and **4D**), implying the control of cell movement by SEMA4C. *In vitro* adhesion assay confirmed that SEMA4C blockage by neutralizing antibody decreased colon cancer adhesion in human and mouse colon can-

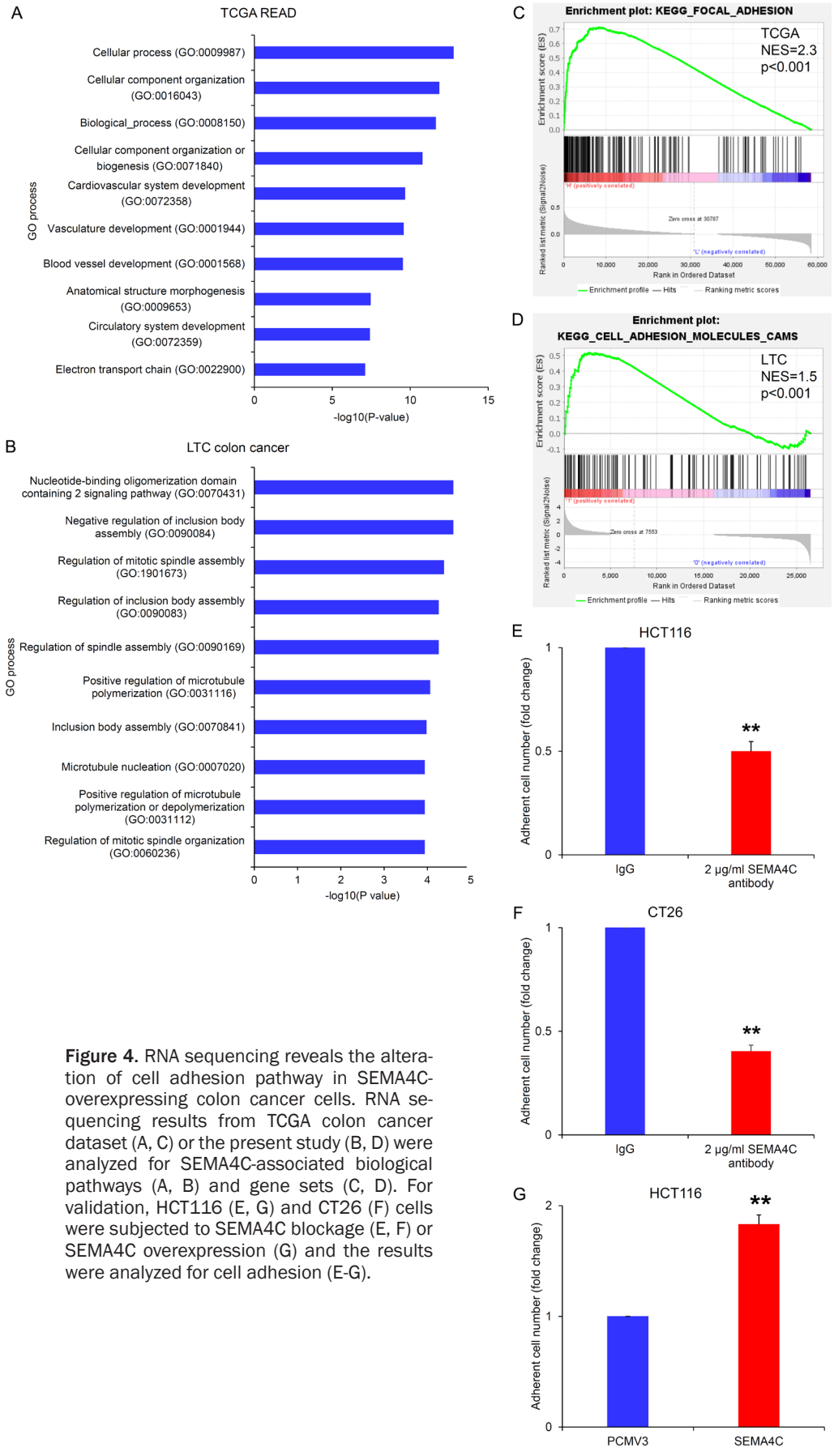

Figure 4. RNA sequencing reveals the alteration of cell adhesion pathway in SEMA4C-overexpressing colon cancer cells. RNA sequencing results from TCGA colon cancer dataset (A, C) or the present study (B, D) were analyzed for SEMA4C-associated biological pathways (A, B) and gene sets (C, D). For validation, HCT116 (E, G) and CT26 (F) cells were subjected to SEMA4C blockage (E, F) or SEMA4C overexpression (G) and the results were analyzed for cell adhesion (E-G).

cer cells (**Figure 4E** and **4F**). On the contrary, overexpression of SEMA4C promoted cell adhesion ability (**Figure 4G**).

SEMA4C affects tubulin acetylation via CRMP3 but not HDAC6

Microtubule polymerization and nucleation pathways identified in our RNA sequencing data are mainly controlled by the assembly and disassembly of tubulin, which is heavily dependent on acetylation of tubulin [39, 41]. Inhibition of SEMA4C by neutralizing antibody increased total protein acetylation and tubulin acetylation in HCT116 and CT26 cells (**Figure 5A** and **5B**). Conversely, overexpression of SEMA4C reduced tubulin acetylation (**Figure 5C**). Potential deacetylases associating with SEMA4C-regulated cell behavior may be HDAC6 and CRMP3 [42, 43]. Our results demonstrated that SEMA4C blockage decreased the protein level of CRMP3 but not HDAC6 in both HCT116 and CT26 cells (**Figure 5A**, **5B** and Supplementary Figure 2). On the contrary, SEMA4C overexpression increased CRMP3 level by 2.6-fold (**Figure 5C**). Immunoprecipitation-Western Blotting assay confirmed that endogenous SEMA4C interacted with CRMP3 in HCT116 and CT26 cells (**Figure 5D** and **5E**). In consistent with our hypothesis, overexpression of CRMP3 rescued SEMA4C blockage-decreased colon cancer motility (**Figure 5F** and **5G**). Conversely, knockdown of CRMP3 by shRNA suppressed SEMA4C-enhanced migration and invasion in HCT116 cells (**Figure 5H** and **5I**). These data suggested that CRMP3 is a downstream mediator for SEMA4C to modulate tubulin acetylation.

HDAC inhibitor Vorinostat inhibits SEMA4C-increased cell motility

We next searched for potential drugs that may suppress the enhancement of cell motility by SEMA4C via gene-drug interaction search engine L1000CDS[2]. Three classes of drugs including histone deacetylase inhibitors, calcium channel blockers and protein tyrosine kinase inhibitors were identified as possible candidates (**Figure 6A**). Because we have identified the deacetylase CRMP3 as a SEMA4C downstream mediator, we firstly tested the effect of Vorinostat, a clinically approved HDAC inhibitor. As shown in **Figure 6B**, Vorinostat at the concentrations between 1 to 10 μM (10 μM as predicted *in silico*; 1 μM as steady-state concentration in patients) did not significantly inhibit the proliferation of HCT116 cells (**Figure 6B**). However, basal and SEMA4C-increased migration and invasion were dramatically suppressed (**Figure 6C** and **6D**). Therefore, HDAC inhibitors have the potential to suppress SEMA4C-promoted colon cancer.

Dual targeting of SEMA4C by neutralizing antibody and miRNA synergistically suppresses cell invasiveness

Although neutralizing antibody could block the SEMA4C signaling, it only showed partial inhibition on cell motility. Therefore, we proposed a dual targeting strategy by combining antibody and miRNA, which may inhibit SEMA4C function and expression simultaneously. Three miRNA databases miRDB, TargetScan, and miRWalk were overlapped to identify miRNAs that target SEMA4C and several candidates including let-7b, miR-3064, miR-410, and miR-6895 were identified. Analysis of the TCGA dataset showed that high expression of let-7b is associated with better survival in colon cancer patients, suggesting the anti-cancer role of let-7b in colon cancer (**Figure 7A**). Because let-7b is a well-known tumor suppressor, we focused our study on this miRNA. The sequence of let-7b matched well to the 3'-UTR of SEMA4C mRNA (**Figure 7B**). Treatment of let-7b had minor effect on colon cancer proliferation (**Figure 7C**). Treatment of neutralizing antibody induced internalization and degradation of SEMA4C protein by 60% in HCT116 cells (**Figure 7D**). Delivery of let-7b also decreased SEMA4C protein level by 60%. Importantly, combinatory treatment of neutralizing antibody and let-7b induced a >95% of inhibition (**Figure 7D**). Moreover, combinatory treatment also elicited a synergistic effect on the inhibition of migration and invasion (**Figure 7E** and **7F**). Bioinformatic analysis showed that let-7b expression is decreased in primary colon cancer when compared with normal tissues (5.02E-07) (Supplementary Figure 3A). In addition, let-7b expression is reduced in all stages of colon tumors (normal vs. s1, 5.35E-07; normal vs. s2, 5.43E-07; normal vs. s3, 5.00E-07; normal vs. s4, 5.15E-07), while SEMA4C is upregulated (normal vs. s1, 2.47E-02; normal vs. s2, 2.61E-02; normal vs. s3, 2.18E-04; normal vs. s4, 3.17E-

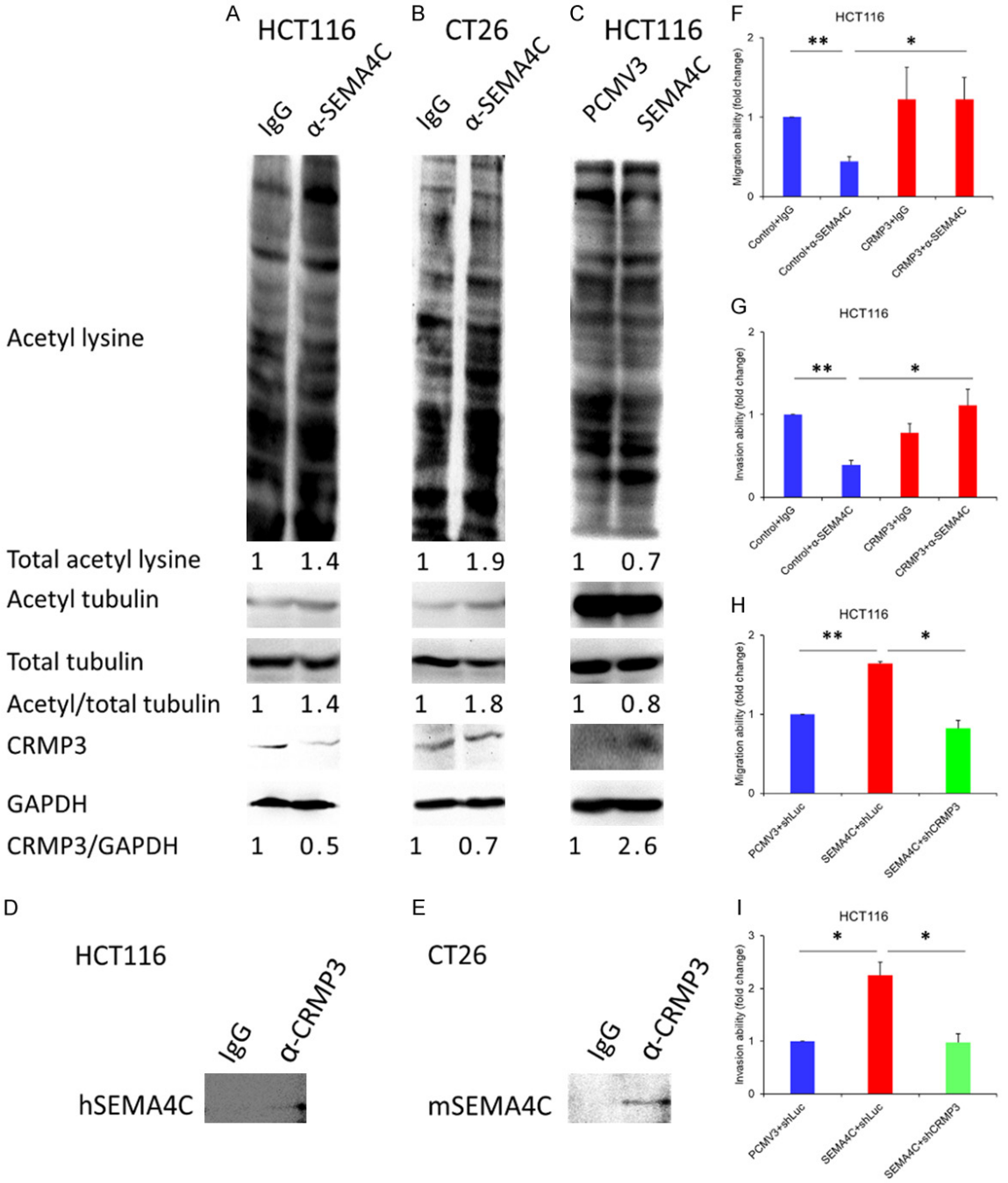

Figure 5. SEMA4C affects tubulin acetylation via CRMP3. HCT116 (A, B, D, F-I) and CT26 (C, E) under the condition of SEMA4C blockage (A, B) or SEMA4C overexpression (C) were analyzed for alterations in total protein acetylation on lysine and tubulin acetylation. In addition, expressions of total tubulin and CRMP3 were also investigated. GAPDH served as internal control. Protein-protein interactions between SEMA4C and CRMP3 in HCT116 (D) and CT26 (E) were determined by immunoprecipitation of CRMP3 and subsequent Western blotting with human- or mouse-specific SEMA4C antibody as described. In functional validation, HCT116 cells were transfected with control vector or CRMP3-overexpressing plasmid and treated with control IgG or SEMA4C antibody (F, G) for cell migration (F) and invasion assays (G). On the other hand, HCT116 cells were transfected with control vector or SEMA4C-overexpressing plasmid, followed by transfection of shRNA against luciferase or against CRMP3 (H, I), with their effects on migration (H) and invasion (I) being analyzed as well.

04; s2 vs. s3, 2.73E-02; s2 vs. s4, 2.01E-02) (Supplementary Figure 3B and 3C). Moreover, increase of SEMA4C is more evident in colon cancer patients with lymph node metastasis

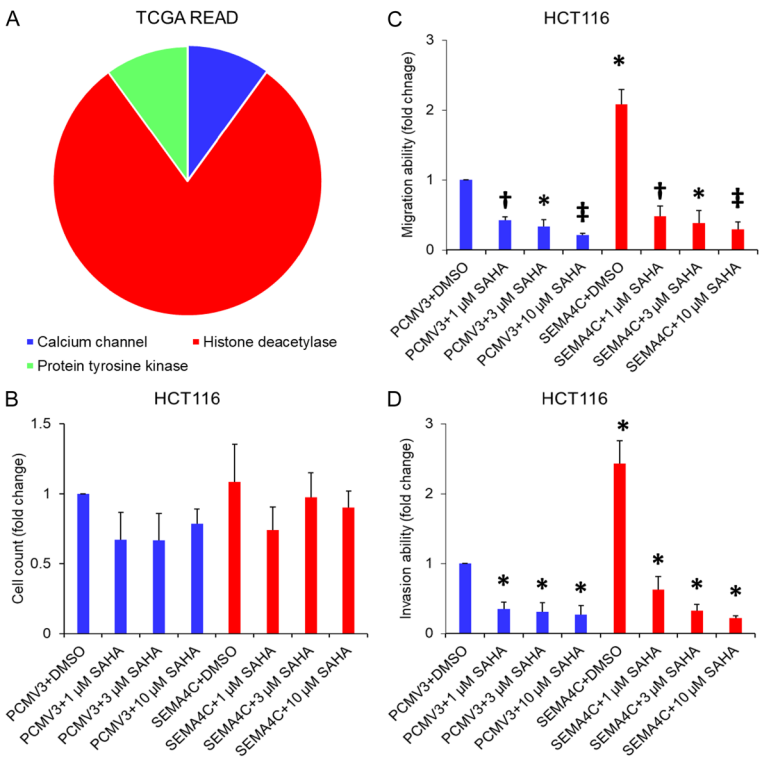

Figure 6. HDAC inhibitor Vorinostat inhibits SEMA4C-increased cell motility. TCGA colon cancer based- and L1000CDS^2-predicted SEMA4C-counteracting therapeutics were shown (A) and HDAC inhibitor was selected for *in vitro* validation on proliferation (B), migration (C), and invasion (D) in HCT116.

(normal vs. N0, 9.28E-03; normal vs. N1, 7.91E-05; normal vs. N2, 9.9E-05; N0 vs. N1, 2.34E-02; N0 vs. N2, 4.36E-02). On the contrary, let-7b was negatively associated with lymph node metastasis (normal vs. N0, 5.24E-07; normal vs. N1, 4.83E-07; normal vs. N2, 5.42E-07) (Supplementary Figure 3D and 3E).

Inhibition of SEMA4C reduces PD-L1 level in colon cancer cells

Study by Hou *et al.* showed that SEMA4C-high colon cancer displayed high microsatellite instability (MSI) [21]. MSI has been suggested to be associated with the response of immunotherapy. We hypothesized that SEMA4C may affect the expression of immune checkpoint molecules. Bioinformatic analysis demonstrated a positive correlation between the expression of SEMA4C and PD-L1, although the *p* value did not reach statistical significance due to the limited patient numbers (**Figure 8A**). Our result showed that SEMA4C blockage decreased PD-L1 expression in HCT116 cells, while SEMA4C overexpression increased it (**Figure 8B** and **8C**). These results suggested SEMA4C promotes the expression of PD-L1 in colon cancer cells.

Discussion

Several important findings in this study should be noted. Firstly, we revealed the mechanism by which SEMA4C controls invasiveness of cancer cells. Although SEMA4C is a well-known neuron guidance molecule which significantly affects neuron migration, how it modulates cancer cell motility is still unclear. Our results demonstrated that SEMA4C interacted with and stabilized CRMP3 to increase α-tubulin deacetylation and cell movement. CRMP3 was originally found as a member of CRMP gene family located at chromosome 7 in developing rodent brain [44]. A previous study pointed out a role of CRMP3 as deacetylase [43]. However, how CRMP3 modulates the organization of tubulin and how CRMP3's function is regulated are not clear. We found that SEMA4C is an interacting protein of CRMP3 and may promote CRMP3 protein stability to modulate tubulin polymerization via deacetylation of tubulin. We also confirmed that another well-known tubulin deacetylase HDAC6 is not involved in the process. Our results provided a molecular basis to explain how SEMA4C enhances migration and invasion of cancer cells. In addition, we also demonstrated a new function of CRMP3 in tubulin assembly in cancer cells.

Secondly, we showed for the first time that SEMA4C is an upstream regulator of PD-L1, a critical immune checkpoint molecule. The immune response is tightly controlled to prevent autoimmune attack and modulate the magnitude of immune reactivity. PD-L1 inhibits the anti-cancer activity of immune cells by interacting with PD-1 receptor [45]. Many cancer cells express high level of PD-L1 to escape the attack of immune cells. The expression of PD-L1 is upregulated by various oncogenic sig-

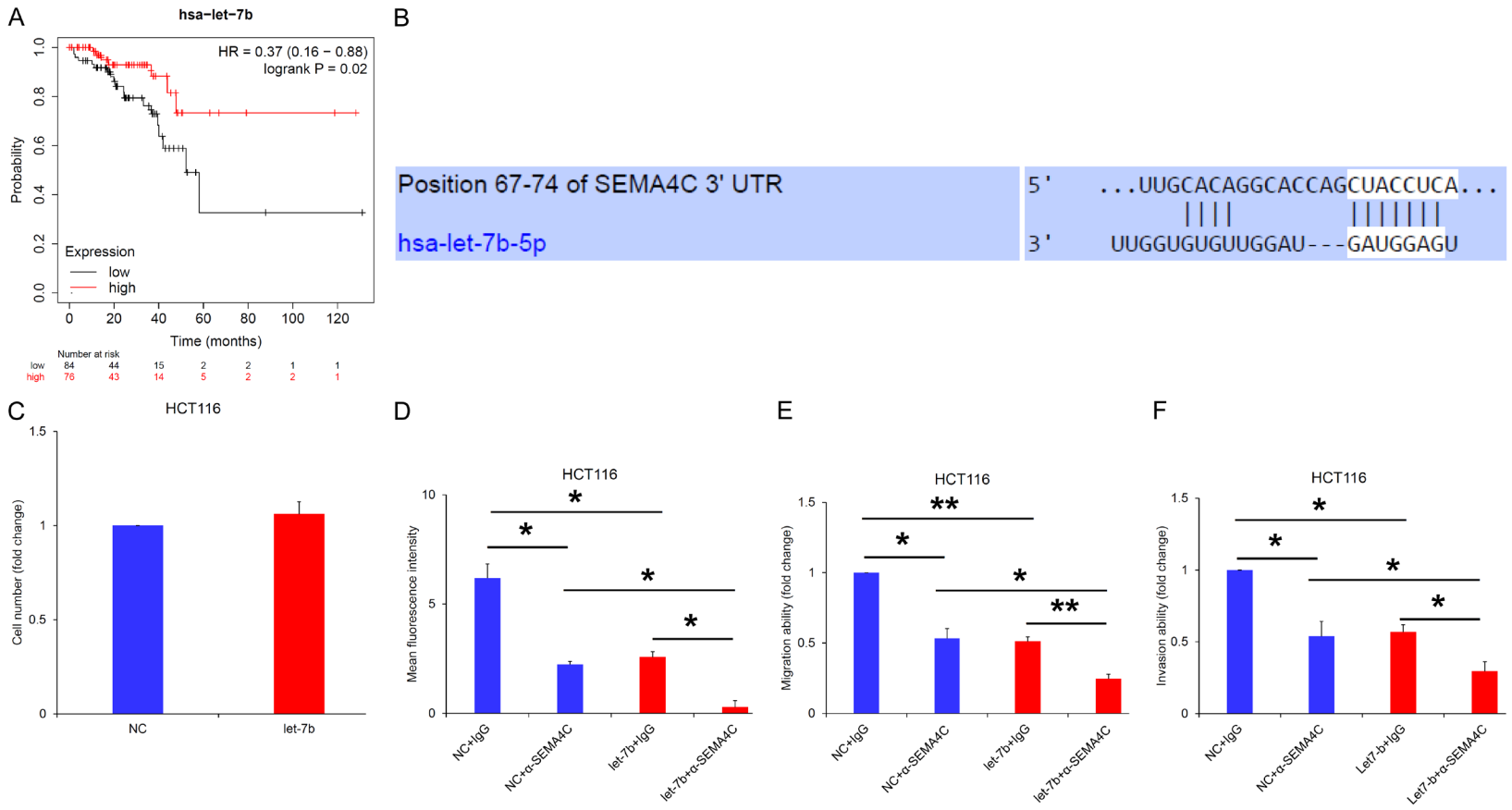

Figure 7. Dual targeting of SEMA4C by neutralizing antibody and miRNA synergistically suppresses cell invasiveness. miRNAs targeting SEMA4C were identified by overlapping predicted miRNA from databases miRDB, TargetScan, and miRWalk, and the prognostic power of predictive miRNA let-7b (A) and its targeting onto SEMA4C 3'-UTR (B) were shown. Effect of let-7b mimic on proliferation in HCT116 was checked (C). Effects of dual targeting by neutralizing antibody and let-7b mimic on SEMA4C expression (D), migration (E), and invasion (F) in HCT116 were analyzed.

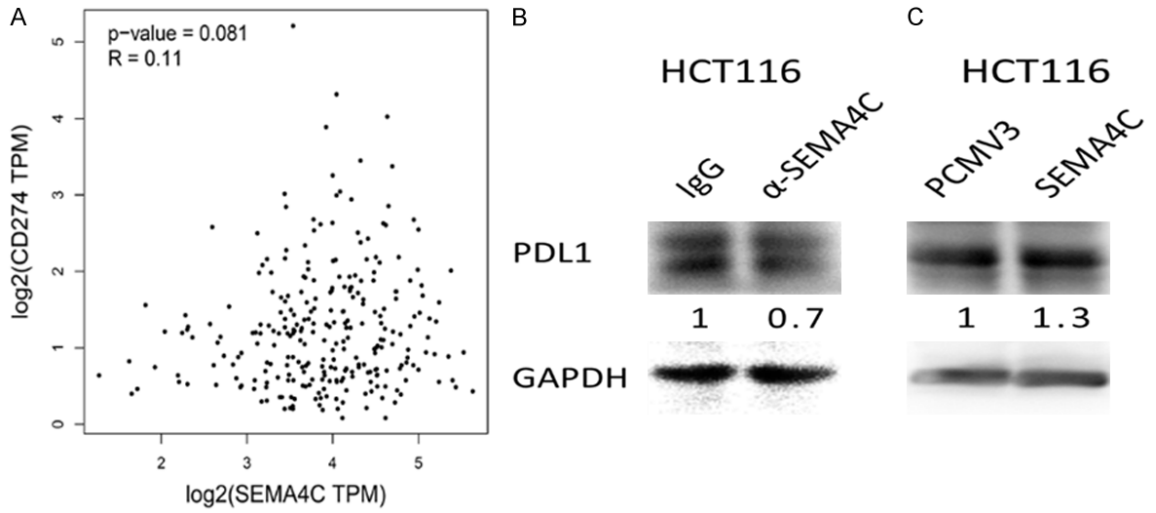

Figure 8. Inhibition of SEMA4C reduces PD-L1 level in colon cancer cells. Correlation of PD-L1 (CD274) and SEMA4C in TCGA COAD dataset was shown (A). Effect of SEMA4C antibody blockage (B) or SEMA4C overexpression (C) on PD-L1 expression in HCT116 was analyzed by Western blotting.

naling pathways and is associated with poor prognosis in a number of tumors [46-48]. Inhibition of PD-L1 by antibody-mediated blockage has been shown to re-activate anti-cancer immunity and prolong cancer patient's survival [46]. In addition, PD-L1 expression is a biomarker to select cancer patients who may be benefited from immunotherapy. Here, we showed a positive link between SEMA4C and PD-L1. More importantly, our data demonstrated that inhibition of SEMA4C reduced PD-L1 level while overexpression of SEMA4C showed an opposite effect in human colon cancer cells. In clinical setting, the detection of PD-L1 by immunohistochemical staining faces some difficulties due to the post-translational modifications of PD-L1. Recently, an elegant study showed that PD-L1 is a glycoprotein and the glycosylated PD-L1 is poorly recognized by anti-PD-L1 antibody, reducing accuracy of immunohistochemical staining and correct selection of patients [49]. This study points out the possibility by using SEMA4C as a biomarker for the selection of colon cancer patients for immunotherapy.

Thirdly, we provided evidence that HDAC inhibitors could be useful for the prevention of SEMA4C-promoted migration and invasion. HDAC inhibitors have been shown to exhibit anti-cancer activity *in vitro* and *in vivo* and one HDAC inhibitor Vorinostat has been approved to treat cutaneous T cell lymphoma. Although the application of HDAC inhibitors in the treatment of solid tumors is still under intensive studies, the combinatory use of HDAC inhibitors with other chemotherapeutic or targeted therapeutic drugs could be a novel strategy to improve the treatment response in cancer patients. Results of this study suggest that HDAC inhibitors may benefit colon cancer patients with SEMA4C overexpression.

Fourthly, we also showed a novel combination of antibody and miRNA to inhibit SEMA4C-overexpressing tumors. By using bioinformatic approach, we identified several miRNAs that may target the 3'-UTR of SEMA4C mRNA. A potential miRNA is let-7b. Because let-7b has been found to be a tumor suppressor in different cancers, we focused our attention on this miRNA and demonstrated for the first time that let-7b suppresses SEMA4C in colon cancer cells. Although therapeutic antibodies have been used successfully in cancer treatment, their efficacy is target- and cancer type-dependent. In our study, we showed that SEMA4C neutralizing antibody only induces SEMA4C internalization and degradation by 50-60%. We tested the combination of SEMA4C antibody and let-7b on the migration and invasion of colon cancer cells and found a synergistic effect on the inhibition. Collectively, this study unveiled the underlying mechanism of SEMA4C-promoted colon tumorigenesis and provided new strategies for the treatment of SEMA4C-overexpressing colon cancer.

Acknowledgements

This study was supported by grants CA-107-PP-19, CA-108-PP-22, CA-109-PP-22, CA-110-PP-20 from National Health Research Institutes; 110-2321-B-037-002 from Ministry of Science and Technology; KMU-KI110001 from Kaohsiung Medical University, Taiwan to Li-Tzong Chen.

Disclosure of conflict of interest

None.

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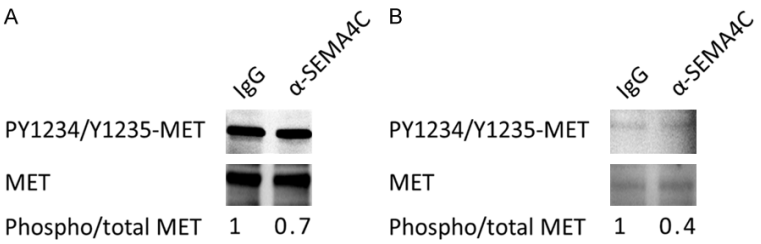
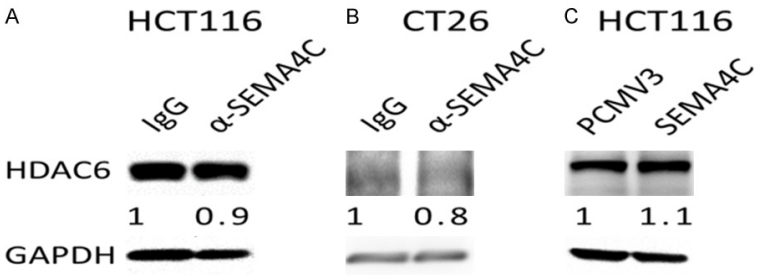
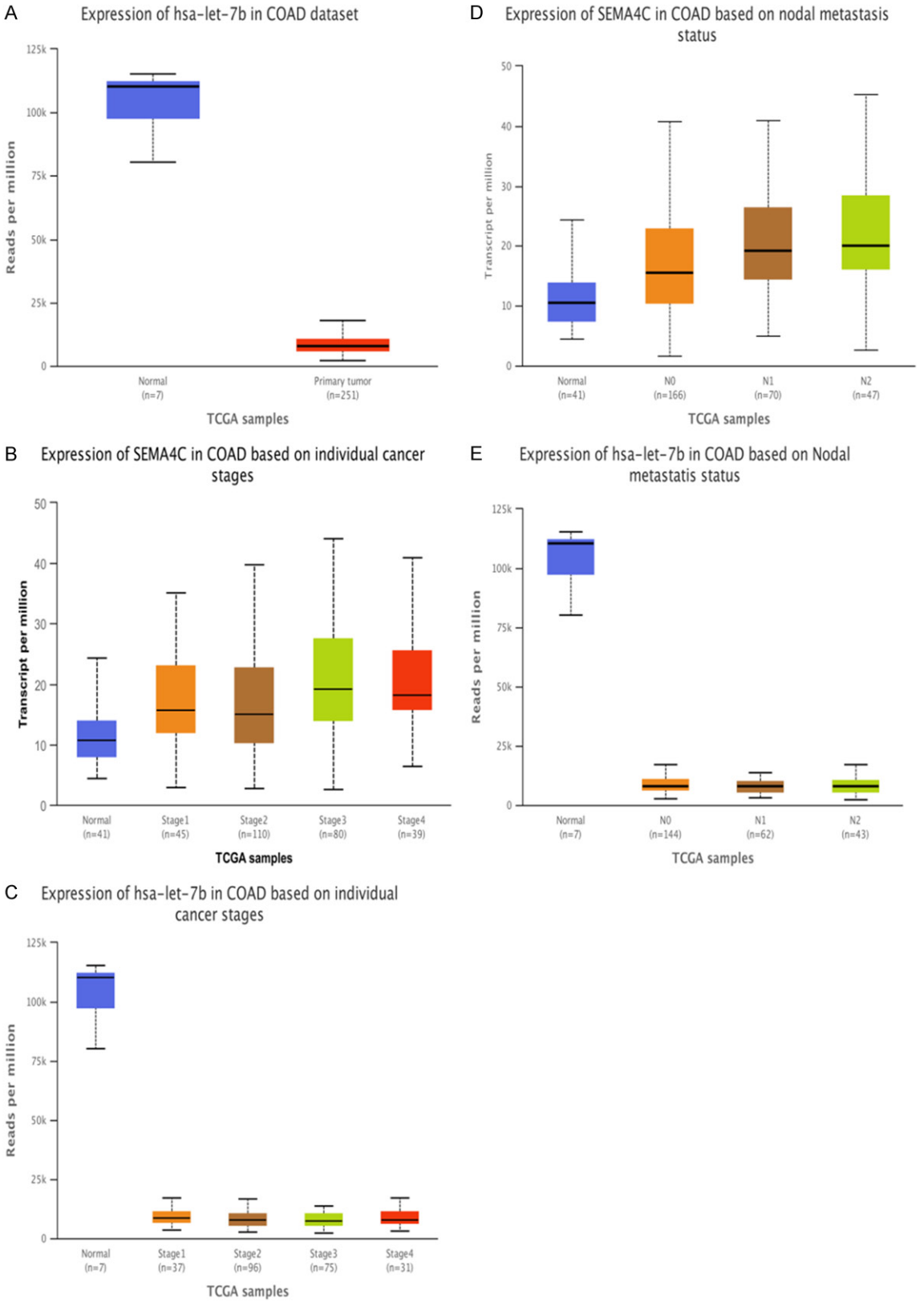


Supplementary Figure 1. Effect of SEMA4C antibody on MET phosphorylation. HCT116 (A) and CT26 (B) cells were treated with control IgG or SEMA4C antibody and the inhibition of downstream signaling (determined by phosphorylation of MET) was investigated.



Supplementary Figure 2. No effect of SEMA4C on HDAC6 expression. HCT116 (A, C) and CT26 (B) cells were subjected to SEMA4C antibody blockage (A, B) or SEMA4C overexpression (C). The expression of HDAC6 was investigated.



Supplementary Figure 3. Let-7b inversely correlates with SEMA4C during colon tumorigenesis. Expression of let-7b in normal vs. tumor (A), at different status of cancer stage (C) or nodal metastasis (E) was shown. Accordingly, expression of SEMA4C at different status of cancer stage (B) or nodal metastasis (D) was shown as well.