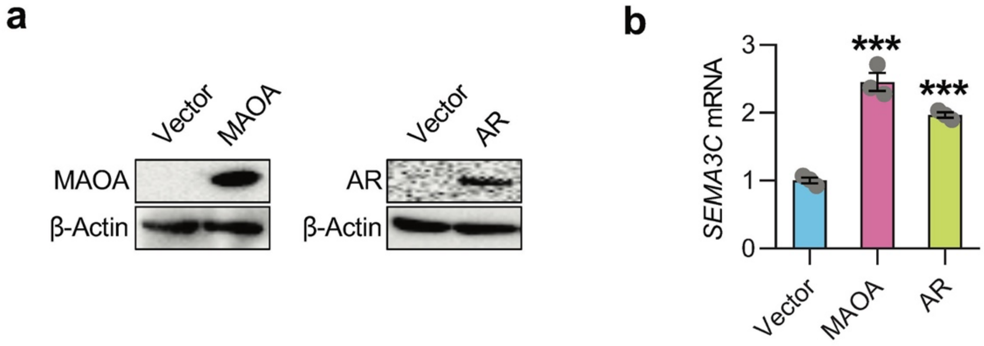
Supplementary Information

MAOA Promotes Prostate Cancer Cell Perineural Invasion through SEMA3C/PlexinA2/NRP1-cMET Signaling

Lijuan Yin, Jingjing Li, Jing Wang, Tianjie Pu, Jing Wei, Qinlong Li, Boyang Jason Wu

Supplementary Fig. 1



Supplementary Fig. 1 MAOA and AR induce SEMA3C in PC cells. **a** Western blot analysis of MAOA and AR in PC-3 cells with enforced expression of MAOA or AR. **b** RT-qPCR analysis of *SEMA3C* mRNA levels in PC-3 cells described in a. Data represent the mean ± SEM. **$p<0.01$.

Supplementary Table 1

Sample Number	MAOA (intensity counts/cell)	SEMA3C (intensity counts/cell)
1	0.058	0.058
2	0.176	0.051
3	0.037	0.029
4	0.059	0.039
5	0.043	0.042
6	0.035	0.027
7	0.029	0.035
8	0.040	0.048
9	0.043	0.032
10	0.083	0.031
11	0.086	0.035
12	0.049	0.029
13	0.117	0.043
14	0.049	0.031
15	0.058	0.031
16	0.020	0.042
17	0.015	0.020
18	0.060	0.027
19	0.027	0.023
20	0.025	0.022
21	0.053	0.025
22	0.036	0.017
23	0.017	0.023
24	0.089	0.057
25	0.042	0.014
26	0.028	0.019
27	0.034	0.024
28	0.020	0.022
29	0.055	0.030
30	0.043	0.017
31	0.046	0.026
32	0.058	0.023
33	0.072	0.024
34	0.014	0.019
35	0.043	0.018
36	0.082	0.024
37	0.061	0.025
38	0.037	0.026
39	0.065	0.023
40	0.043	0.030

Supplementary Table 1 Quantification of IHC staining of MAOA and SEMA3C in serial sections of a PC TMA, related to Figure 3. Average cell-based staining intensity counts for each protein were analyzed by inForm software.

Supplementary Table 2

Membrane Position	Target Protein	Phosphorylation Site	Intensity (Vector)	Intensity (MAOA)	Ratio (MAOA/Vector)
A3, A4	p38α	T180/Y182	10.991	13.897	1.264
A5, A6	ERK1/2	T202/Y204, T185/Y187	8.178	9.530	1.165
A7, A8	JNK1/2/3	T183/Y185, T221/Y223	64.941	62.816	0.967
A9, A10	GSK3α/β	S21/S9	79.967	80.307	1.004
A13, A14	p53	S392	27.509	28.523	1.037
B3, B4	EGFR	Y1086	15.599	15.288	0.980
B5, B6	MSK1/2	S376/S360	20.844	25.010	1.200
B7, B8	AMPKα1	T183	19.143	15.602	0.815
B9, B10	AKT1/2/3	S473	44.051	46.606	1.058
B11, B12	AKT1/2/3	T308	21.890	24.149	1.103
B13, B14	p53	S46	30.448	30.278	0.994
C1, C2	TOR	S2448	19.643	20.189	1.028
C3, C4	CREB	S133	21.793	27.786	1.275
C5, C6	HSP27	S78/S82	13.886	14.187	1.022
C7, C8	AMPKα2	T172	28.495	30.580	1.073
C9, C10	β-Catenin	—	28.978	29.495	1.018
C11, C12	p70 S6 Kinase	T389	24.934	20.650	0.828
C13, C14	p53	S15	23.474	24.095	1.026
C15, C16	c-Jun	S63	31.772	27.974	0.880
D1, D2	Src	Y419	31.740	32.334	1.019
D3, D4	Lyn	Y397	8.980	9.411	1.048
D5, D6	Lck	Y394	9.289	9.492	1.022
D7, D8	STAT2	Y689	29.221	27.840	0.953
D9, D10	STAT5α	Y694	15.083	16.659	1.104
D11, D12	P70 S6 Kinase	T421/S424	24.854	19.769	0.795
D13, D14	RSK1/2/3	S380/S386/S377	16.067	18.392	1.145
D15, D16	eNOS	S1177	15.630	19.687	1.260
E1, E2	Fyn	Y420	10.835	11.090	1.024
E3, E4	Yes	Y426	18.856	15.980	0.847
E5, E6	Fgr	Y412	5.448	6.618	1.215
E7, E8	STAT6	Y641	29.472	29.185	0.990
E9, E10	STAT5b	Y699	11.207	11.468	1.023
E11, E12	STAT3	Y705	27.158	24.347	0.896
E13, E14	p27	T198	17.143	13.779	0.804
E15, E16	PLC-γ1	Y783	16.675	18.833	1.129
F1, F2	Hck	Y411	16.833	19.516	1.159
F3, F4	Chk-2	T68	28.846	18.087	0.627
F5, F6	FAK	Y397	24.606	22.536	0.916
F7, F8	PDGFRβ	Y751	9.758	10.306	1.056
F9, F10	STAT5a/b	Y694/Y699	32.397	34.191	1.055
F11, F12	STAT3	S727	12.842	11.939	0.930
F13, F14	WNK1	T60	29.387	27.439	0.934
F15, F16	PYK2	Y402	17.935	18.937	1.056

G3, G4	PRAS40	T246	76.004	70.074	0.922
G11, G12	HSP60	—	47.729	59.254	1.241

Supplementary Table 2. Quantification of phosphoprotein expression levels in a phospho-kinase antibody array from control and MAOA-OE PC-3 cells, related to Figure 4. Average chemiluminescent signal intensity of a pair of duplicate spots representing each phosphorylated kinase protein is shown.

Supplementary Materials and Methods

Plasmids and reagents

A human *MAOA* lentiviral expression construct was generated by insertion of the human *MAOA* coding region at *EcoR*I/*Xba*I sites in pLVX-AcGFP1-N1 vector (Clontech) containing a puromycin-resistant gene. The pCMV-MAOA expression construct was generated as described previously [1]. The pcDNA-AR expression construct was kindly provided by Gerhard Coetzee (University of Southern California). The human 1.5-kb *SEMA3C* promoter *Gaussia* luciferase reporter construct that simultaneously expresses secreted alkaline phosphatase (SEAP) as an internal control for signal normalization was purchased from GeneCopoeia. Human *MAOA* and non-target control shRNA lentiviral particles were purchased from Sigma-Aldrich. The lentiviral expression construct expressing the *Firefly* luciferase gene for labeling PC cells were purchased from GenTarget. *Twist1* and *cMET* siRNAs were purchased from Santa Cruz. *SEMA3C*, *PlexinA2* and *NRP1* siRNAs were purchased from Origene. Clorygyline and forskolin were purchased from Sigma-Aldrich. Recombinant human NGF protein was purchased from Thermo Fisher Scientific. NGF protein levels in culture media were quantified by ELISA (PeproTech).

Generation of stable knockdown and overexpression cells

Stable shRNA-mediated MAOA knockdown was achieved by infecting cells with lentiviral particles expressing *MAOA* shRNA TRCN0000046009 (shMAOA#1, mainly used in this study and usually dubbed “shMAOA”) or TRCN0000046011 (shMAOA#2), followed by 2-week puromycin selection (2 µg/ml) for establishing stable cell lines. A non-target control shRNA (shCon) was used as control in stable knockdown cells. Lentivirus production was performed for stable OE of MAOA in LAPC4 cells. Briefly, 293T cells were co-transfected with a *MAOA*-expressing lentiviral construct, pCMV delta R8.2 (Addgene) and pVSVG (Addgene) in a 4:2:1 ratio using Lipofectamine 2000 reagent (Thermo Fisher Scientific) following the manufacturer’s instructions. The medium was changed 6 hrs after transfection. The medium containing lentivirus was harvested 48 hrs after transfection. Viral particles were concentrated and purified using a Lenti-X concentrator (Takara Bio). LAPC4 cells were infected with lentivirus in the presence of 8 µg/ml polybrene followed by 2-week puromycin selection (2 µg/ml). An empty lentiviral construct was used as a control for stable OE cells. Stable OE of MAOA in PC-3 cells were generated as described previously [1].

Biochemical analyses

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and reverse-transcribed to cDNA by M-MLV reverse transcriptase (Promega) following the manufacturers’ instructions followed by qPCR. Details on primers and methods used for qPCR are provided below. For immunoblots, cells were extracted with RIPA buffer in the presence of a protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific), and blots were performed as described previously [2] using primary antibodies against MAOA (H-70 or G-10, Santa Cruz), SEMA3C (PA5-75455, Thermo Fisher Scientific), PlexinA2 (D44D4, Cell Signaling), NRP1 (C-19, Santa Cruz), p-cMET (D26, Cell Siganling), cMET (C-12, Santa Cruz), Twist1 (10E4E6, Novus Biologicals), AR (441. Santa Cruz), GAPDH (14C10, Cell Signaling) or β-Actin (AC-15, Santa Cruz).

Luciferase reporter assay

To determine the effects of MAOA and Twist1 on *SEMA3C* promoter, PC-3 cells subjected to manipulation of MAOA and Twist1 levels were transfected with *SEMA3C Gaussia* luciferase promoter that simultaneously expresses SEAP to normalize for transfection efficiency. After 24-48 hrs following transfection, relative light units were calculated as the ratio of *Gaussia* luciferase to SEAP activity by a Secret-Pair Dual Luminescence kit (GeneCopoeia).

Quantitative real-time PCR

qPCR was conducted using SYBR Green PCR Master Mix and run with the Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). PCR conditions included an initial denaturation step of 3 min at 95°C, followed by 40 cycles of PCR consisting of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. The PCR data were analyzed by $2\text{-}^{\Delta\Delta CT}$ method [3].

Gene	**Forward**	**Reverse**
SEMA3A	GGTTAACTAGGATTGTCTGTC	GTGATCACATTGTTGGATTC
SEMA3B	ATGCCTACAACCGCACCCA	GGTGGCCCACTTCCACAAAG
SEMA3C	ATCGAGTGAACGCTGCTGATG	GCTCGCCACTGACAGAGTTGTT
SEMA3D	TGGAATTGTCTCTGAAGCAGCA	TGCGCAAGCTTTCCCATAAG
SEMA3E	CAACAGGCACACATGCAA	GTCTTATCCAAAGCATCCC
SEMA3F	ACCAGTGGATGCCCTTCT	GCGCATGAAGTTGATCAC
SEMA3G	GCTCAAAGTCATCGCTCTCCAG	CATTTCGGTGATAGGTGTTGGC
PLXNA1	TCCTGGTGGACCTCTCAAAC	ACTGCACACAGCTCTCCACA
PLXNA2	CATCTCGTACTGGACCCCAC	TTTACAACGGCTACAGCGTG
PLXNA3	ACCACGAAGGCACGGAAG	AGCCAGCGGAGGGACAG
PLXNA4	TCTCAGTACAACGTGCTG	TAGCACTGGATCTGATTGC
NRP1	AAGGTTTCTCAGCAAACTACAGTG	GGGAAGAAGCTGTGATCTGGTC
NRP2	GGATGGCATTCCACATGTTG	ACCAGGTAGTAACGCGCAGAG

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assay was used to determine the association of endogenous Twist1 protein with a Twist1-binding sequence of *SEMA3C* promoter identified from analysis of a published ChIP-seq dataset in control and MAOA-OE PC-3 cells by a SimpleChIP Enzymatic Chromatin IP kit (Cell Signaling) following the manufacturer's instructions. Briefly, the chromatin was crosslinked with nuclear proteins, enzymatically digested with micrococcal nuclease followed by sonication, and immunoprecipitated with anti-Twist1 (Twist2C1a, Abcam) antibody. Normal IgG included in the kit was used as a negative control for IP. After being pelleted with agarose beads and purified, the immunoprecipitates were subjected to qPCR with a pair of primers specifically targeting the *SEMA3C* proximal promoter region encompassing the Twist1-binding sequence. Primer sequences for *SEMA3C* promoter region are forward 5'-GGGCGAGCGCTCTTGGTGTC-3' and reverse 5'-TGCGAAAGGAGCAGGGTTGCG-3'. Primers sequences for *SEMA3C* exon 2 serving as negative control are forward 5'-ACTTCAGCCTTTCCCACCATCC-3' and reverse 5'-TCAGGGAAAGAATGTGATCTTTGCT-3'.

Proximity ligation assay

Cells were seeded on chamber slides. The cells were fixed with 4% formaldehyde for 10 min at room temperature, washed twice with PBS containing 0.02% Tween 20, and permeabilized with 0.5% Triton X-100/PBS solution (blocking solution) for 30 min at room temperature. Primary antibodies against PlexinA2 (A-2, mouse IgG, Santa Cruz), NRP1 (A-12, mouse IgG, Santa Cruz) or cMET (C-12, rabbit IgG, Santa Cruz) were incubated in blocking solution at 4°C overnight. The proximity ligation assay was then performed with the Duolink *In Situ* Red Starter Kit Mouse/Rabbit (Duolink, Sigma-Aldrich) according to the manufacturer's instructions. Anti-mouse MINUS and anti-rabbit PLUS PLA probes (Duolink) were used. Images were acquired by a Nikon Ti-E inverted microscope using a x40 objective and analyzed for cytoplasmic fluorescence signals per cell with inForm software (PerkinElmer).

Immunohistochemistry and multiple quantum dot labeling analyses

IHC analysis of clinical and xenograft tumor samples was performed using antibodies against MAOA (H-70, Santa Cruz), cleaved caspase 3 (Asp175, Cell Signaling), NGF (2046, Cell

Signaling), SEMA3C (PA5-75455, Thermo Fisher Scientific) or p-cMET (44-888G, Thermo Fisher Scientific). The IHC staining protocol was modified for multiple QD labeling as described previously [4]. Xenograft tumor samples were stained with antibodies against Ki-67 (SP6, Abcam), NF-L (C28E10, Cell Signaling) and NF-H (RMdO 20, Cell Signaling) sequentially by single QD labeling. Cell-based averages of MAOA and SEMA3C staining in TMAs and QD signal intensity counts for Ki-67, NF-L and NF-H in xenograft tumor samples were analyzed by inForm or HALO (Indica Labs) software, both enabling automated per-cell analysis of stained samples, after areas of interest were defined using manual tissue segmentation by a pathologist.

Bioinformatics analysis

The ChIP-seq dataset GSE80151 available in Gene Expression Omnibus was analyzed for Twist1-binding region in *SEMA3C* genomic sequences by TACGenomics. Bowtie was used to map the human hg19 genome and unique mapped reads were used for peak calling. MACS2 was used to perform the peak calling and ChIPseeker was used for peak annotation.

Supplementary References

1 Wu JB, Shao C, Li X, Li Q, Hu P, Shi C *et al*. Monoamine oxidase A mediates prostate tumorigenesis and cancer metastasis. *J Clin Invest* 2014; 124: 2891-2908.

2 Wu JB, Chen K, Ou XM, Shih JC. Retinoic acid activates monoamine oxidase B promoter in human neuronal cells. *The Journal of biological chemistry* 2009; 284: 16723-16735.

3 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; 25: 402-408.

4 Hu P, Chu GC, Zhu G, Yang H, Luthringer D, Prins G *et al*. Multiplexed quantum dot labeling of activated c-Met signaling in castration-resistant human prostate cancer. *PloS one* (Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't) 2011; 6: e28670.