Targeting the FGF19/FGFR4 Positive Feedback Loop to Disrupt ERK-Mediated Tumor Progression in Head and Neck Cancer

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Conflict-of-interest: The authors declare that they have no conflict of interest.

Supplement information

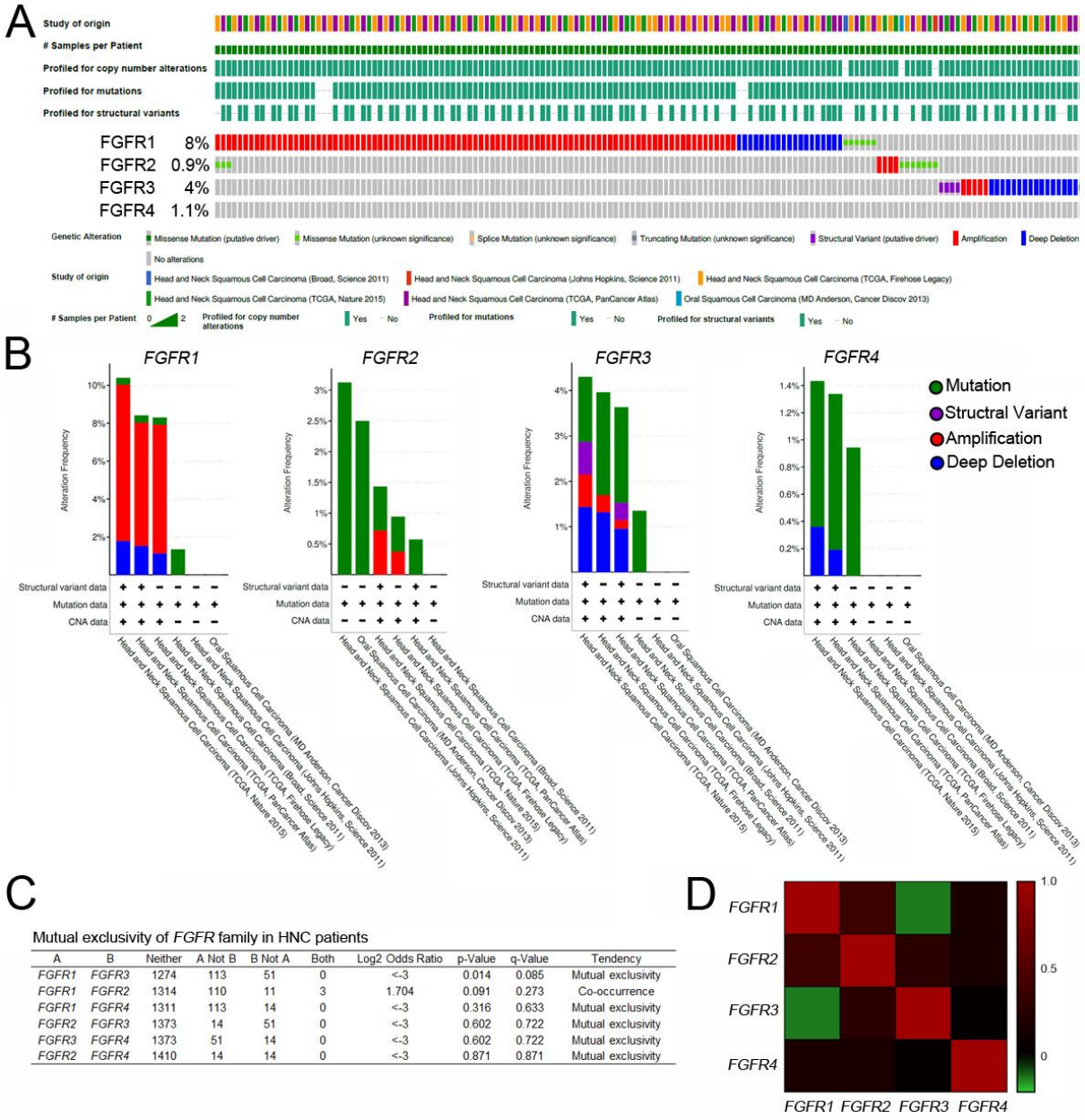
Supplementary data

Supplementary Figure 1	Related to Figure 1
Supplementary Figure 2	Related to Figure 3
Supplementary Figure 3	Related to Figure 5
Supplementary Figure 4	Related to Figure 6
Supplementary Figure 5	Related to Figure 7

Supplementary Tables

Supplementary Table 1	Related to Key Resources
Supplementary Table 2	Related to Figure 1
Supplementary Table 3	Related to Figure 2
Supplementary Table 4	Related to Figure 2
Supplementary Table 5	Related to Figure 4

Figure S1.



Mutual exclusivity of *FGFR* family in HNC patients

A	B	Neither	A Not B	B Not A	Both	Log2 Odds Ratio	p-Value	q-Value	Tendency
FGFR1	*FGFR3*	1274	113	51	0	<-3	0.014	0.085	Mutual exclusivity
FGFR1	*FGFR2*	1314	110	11	3	1.704	0.091	0.273	Co-occurrence
FGFR1	*FGFR4*	1311	113	14	0	<-3	0.316	0.633	Mutual exclusivity
FGFR2	*FGFR3*	1373	14	51	0	<-3	0.602	0.722	Mutual exclusivity
FGFR3	*FGFR4*	1373	51	14	0	<-3	0.602	0.722	Mutual exclusivity
FGFR2	*FGFR4*	1410	14	14	0	<-3	0.871	0.871	Mutual exclusivity

Figure S1. Genetic Alterations and Expression Correlations of FGFR Family Members in Head and Neck Cancer (HNC).

(A) Genetic alteration rates of *FGFR1–FGFR4* in HNC patients from the cBioPortal database. (B) Distribution of alteration types across *FGFR1–FGFR4*. (C) Co-occurrence and mutual exclusivity analysis among *FGFR* family members. (D)

Heatmap showing correlation coefficients among *FGFR1–FGFR4* transcript levels

Figure S2.

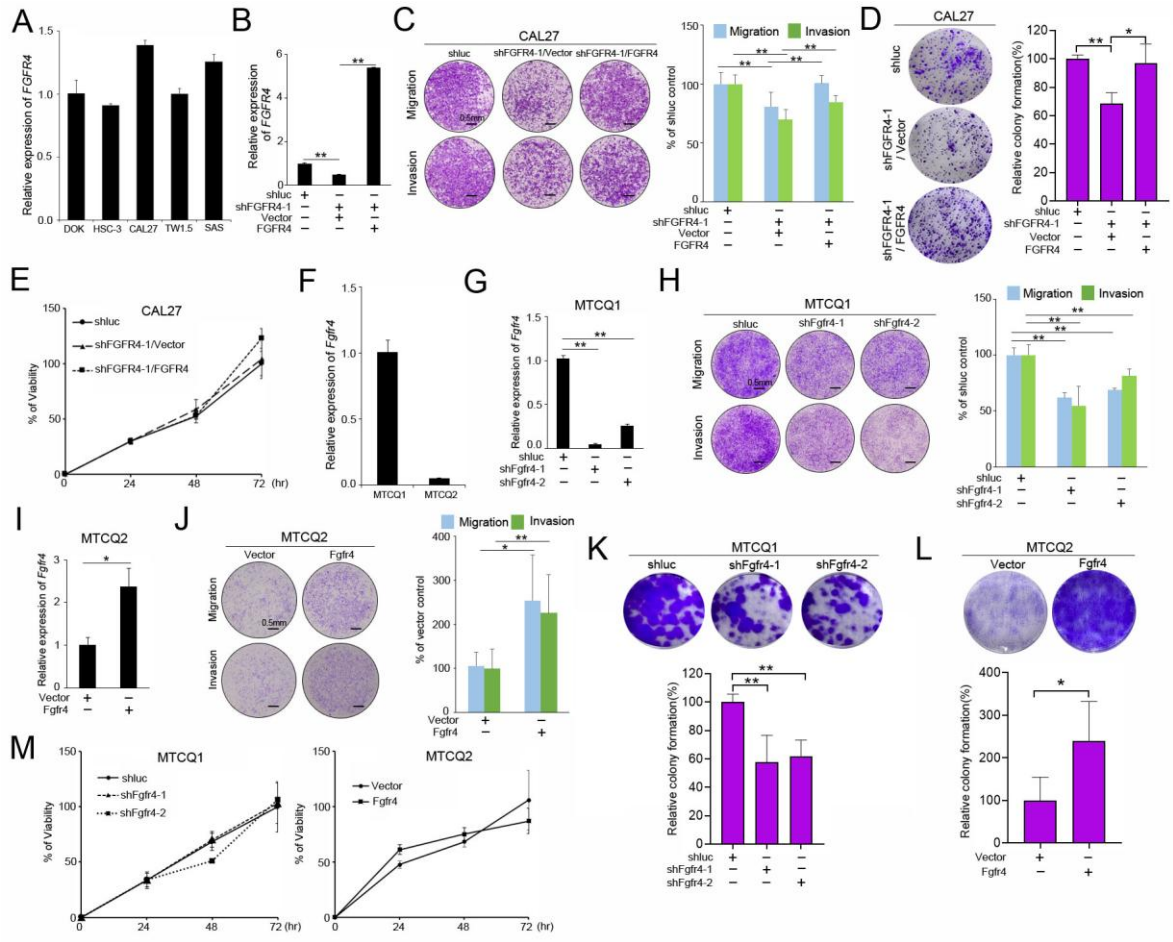


Figure S2. Overexpression of *FGFR4* enhances the migration and invasion abilities of HNC cells.

(A) Endogenous *FGFR4* levels across HNC cell lines. (B) RT-qPCR analysis confirming *FGFR4* knockdown and subsequent overexpression in CAL27 cells (*n* = 3). Migration and invasion assays (*n* = 5) (16×, scale bar: 0.5 mm) (C), colony formation assay (*n* = 3) (1×) (D), and MTT assay (*n* = 8) (E) in CAL27/shFGFR4 cells after *FGFR4* overexpression. (F) RT-qPCR analysis of endogenous *Fgfr4* level in mouse-derived HNC cell lines (*n* = *3*) and (G) validation of *Fgfr4* knockdown in

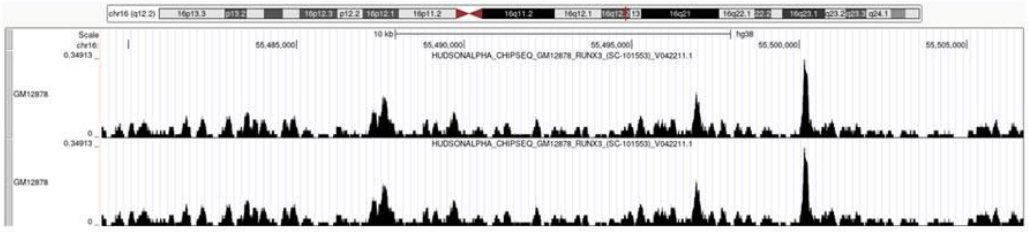
MTCQ1 cells (*n* = 3). (H) Effects of *Fgfr4* knockdown on migration and invasion capacities of MTCQ1 cells (*n* = *5*) (16×, scale bar: 0.5 mm). (I) RT-qPCR analysis confirming *Fgfr4* overexpression in MTCQ2 cells (*n* = *3*). (J) Effects of *Fgfr4* overexpression on migration and invasion abilities of MTCQ2 cells (*n* = *5*) (16×, scale bar: 0.5 mm). Colony formation assay in MTCQ1/shFgfr4 cells (*n* = *3*) (K) and MTCQ2/Fgfr4 cells (*n* = *3*) (1×) (L). (M) MTT assay in MTCQ1/shFgfr4 cells and MTCQ2/Fgfr4 cells (*n* = *8*). **P* < 0.05. ***P* < 0.01.

Figure S3.

A

Matrix ID	Name	Score	Relative score	Sequence ID	Start	End	Strand	Predicted sequence
MA0684.2	MA0684.2.RUNX3	12.02235	0.9360495	NC_000016.10:55478830-55480829	53	64	+	CTAACCTCAGGA
MA0684.2	MA0684.2.RUNX3	6.724909	0.8180833	NC_000016.10:55478830-55480829	513	524	+	CAAACCCCAGGC
MA0684.2	MA0684.2.RUNX3	6.326364	0.8092083	NC_000016.10:55478830-55480829	105	116	-	ACAGCCTCAGA
MA0605.3	MA0605.3.ATF3	-0.2608683	0.8047564	NC_000016.10:55478830-55480829	61	70	+	AGGACGTCAA
MA0605.3	MA0605.3.ATF3	-0.2851206	0.8045012	NC_000016.10:55478830-55480829	61	70	-	TTGACGTCCT

B



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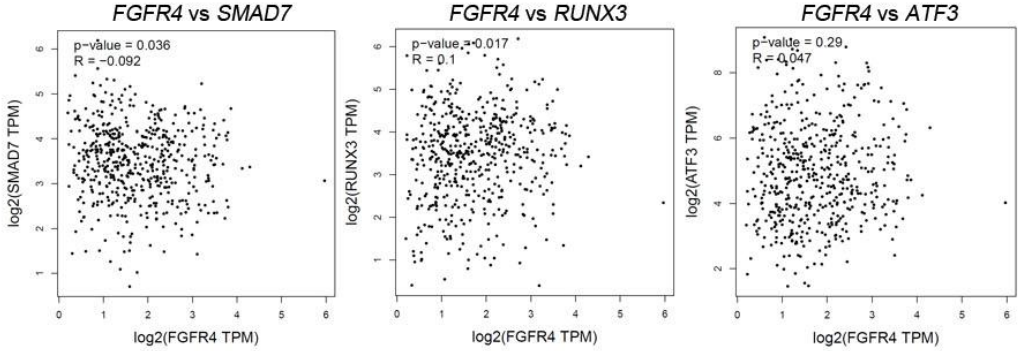

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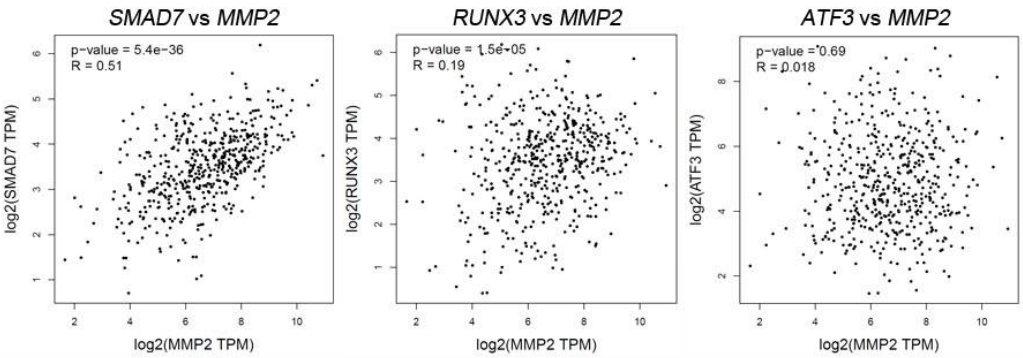

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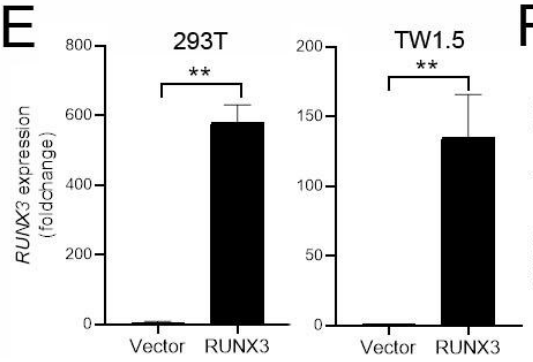

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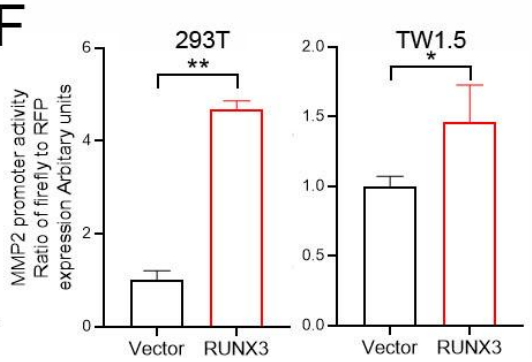


Figure S3. *In silico* prediction of candidate transcription factors for *MMP2* in HNC. (A) Predicted transcription factor binding motifs at the *MMP2* promoter region were identified using the JASPAR database. (B) RUNX3 ChIP-seq data in GM12878 and K562 cell lines were obtained from the ENCODE database and visualized using the UCSC Genome Browser. Correlation analyses of *SMAD7*, *RUNX3*, and *ATF3* with *FGFR4* (C) and *MMP2* (D) levels in the TCGA/HNC cohort were performed using GEPIA2. (E) RT-qPCR analysis of *RUNX3* mRNA in 293T and TW1.5 cells after transfection with *RUNX3* cDNA or vector control ($n = 3$). (F) *MMP2* promoter reporter assay in 293T/RUNX3 and TW1.5/RUNX3 cells. Vector cells were used as controls, and RFP served as an internal control ($n = 4$). *$P < 0.05$; **$P < 0.01$. RFP: red fluorescent protein.

Figure S4.

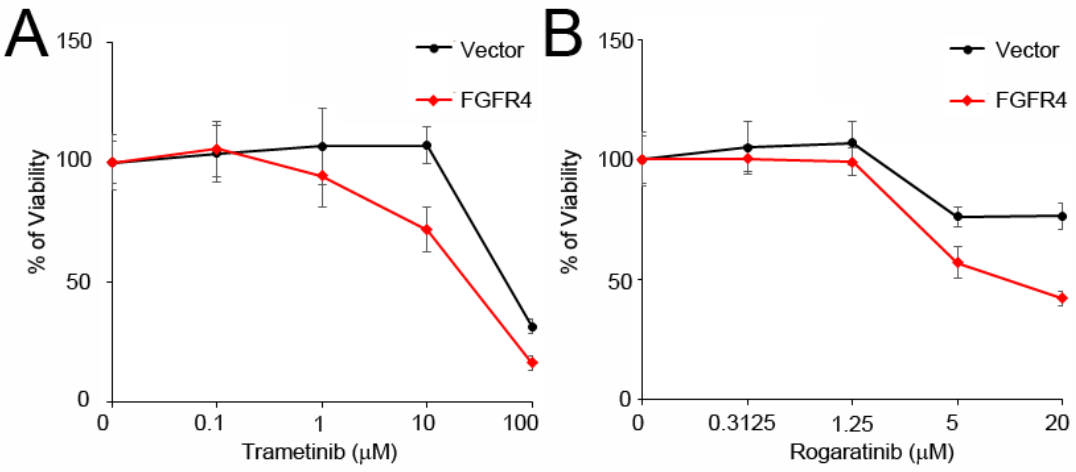


Figure S4. Effects of FGFR4 overexpression on sensitivity to trametinib and rogaratinib in HNC cells.

MTT assays in TW1.5/Vector and TW1.5/FGFR4 cells treated with trametinib (A) and rogaratinib (B) (*n* = *8*).

Figure S5.

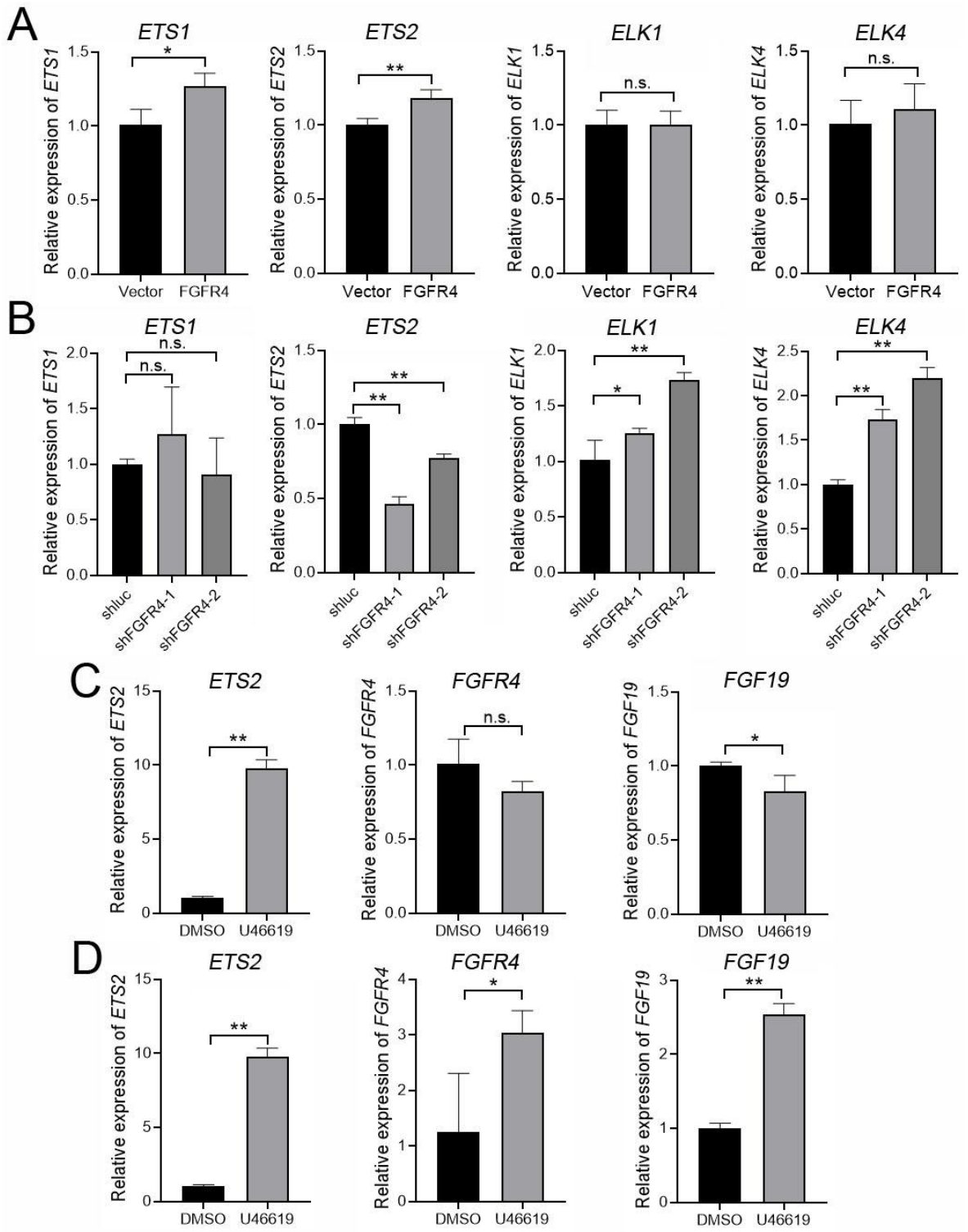


Figure S5. *FGFR4* regulates *ETS2* expression in HNC cells.

RT-qPCR analysis of *ETS1*, *ETS2, ELK1, and ELK4* mRNA in TW1.5/FGFR4 cells (*n* = *3*) (A), and CAL27/shFGFR4 cells (*n* = 3) (B). RT-qPCR analysis of *ETS2*, *FGFR4*,

and *FGF19* expression in TW1.5 cells following treatment with U46619 (10 μM) for 30 min (*n* = *3*) (C) and 60 min (*n* = *3*) (D). *$P < 0.05$; **$P < 0.01$. n.s., not significant.

Table S1. Key resources table

Antibody	Source, catalog number	Application	Dilution
FGFR4	Cell Signaling, #8562	Western blot,	1:1000
FGFR4	abclonal, A9197	IHC	1:200
Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204)	Cell Signaling, #9101	Western blot	1:1000
p44/42 MAPK (ERK1/2)	Cell Signaling, #9102	Western blot	1:1000
β-actin	Sigma-Aldrich, A5441	Western blot	1:5000
RUNX3	Cell Signaling, #9647	Western blot, IHC*	1:1000,1:200*
MMP2	Cell Signaling, #40994	Western blot, IHC*	1:1000,1:200*
FGF19	Cell Signaling, #83348	Western blot,	1:1000

Abbreviations: IHC: immunohistochemistry

Reagent	Source, catalog number	Function	Concentration
MMP-2 Inhibitor I	MCE, HY-112546	MMP-2 inhibitor	5-10 μM
5-fluorouracil (5-FU)	Enzo Life Sciences, ALX-480-099	chemotherapy drug	0-50 μM
Cisplatin	MCE, HY-17394	chemotherapy drug	0-25 μM
Docetaxel	MCE, HY-B0011	chemotherapy drug	0-1 μM
Trametinib	MCE, HY-10999	MEK inhibitor	0-100 μM
Rogaratinib	MCE, HY-100019	FGFR inhibitor.	0-20 μM
U46619	Santa Cruz Biotechnology, sc-201242	activator of ERK1 and ERK2	10 μM
PD-98029	MCE, HY-10321	MAPK inhibitor	25 μM

Recombinant Human FGF-19	R&D SYSTEMS,969-FG	100ng/mL

shRNA Target Sequence

Gene symbol	Target Sequence	Region	Score
FGFR4_clone1 (TRCN0000000628)	GCCGACACAAGAACATCATCA	CDS	National RNAicore
FGFR4_clone2 (TRCN0000000629)	CCTATGTGCAAGTCCTAAAGA	CDS	National RNAicore
Fgfr4_clone1 TRCN0000023564	CCTGAAGACAACAGACATCA	CDS	National RNAicore
Fgfr4_clone2 TRCN0000280484	CCTGAAGACAACAGACATCA	CDS	National RNAicore

Primer Sequence

Gene	Sequence
Human	
FGFR4-forward	GAAGTGTATCCACCGGGACC
FGFR4-reverse	TAGTCAATGTGGTGGACGCC
RUNX3-forward	AGGCAATGACGAGAACTACTCC
RUNX3-reverse	CGAAGGTCGTTGAACCTGG
MMP2-forward	CACAGGAGGAGAAGGCTGTG
MMP2-reverse	CATCCACTCGCTGGACATCA
ACTB- forward	AGAAAATCTGGCACCACACC
ACTB- reverse	AGAGGCGTACAGGGATAGCA
FGF19- forward	GGAGATCAAGGCAGTCGCTC
FGF19-reverse	GAGTACTGAAGCAGCCCCTG
ETS1-forward	GATAGTTGTGATCGCCTCACC
ETS1-reverse	GTCCTCTGAGTCGAAGCTGTC

ETS2-forward	CTGGGCATTCCAAAGAACCC
ETS2-reverse	CCAGACTGAACTCATTGGTGG
ELK1-forward	AATCGGAAGAGCTTAATGTGGAG
ELK1-reverse	CTTGGTGGTTTCTGGCACAA
ELK4-forward	CCTGATCTTTGAGTTCGCAAGC
ELK4-reverse	AGTCCCGAGTACAGATGCAGT
Mouse	
Fgfr4-forward	GCTCGGAGGTAGAGGTCTTGT
Fgfr4-reverse	CCACGCTGACTGGTAGGAA
Actb- forward	CAGGTCATCACTATTGGCAA
Actb- reverse	AGGTCTTTACGGATGTCAAC

Table S2. Correlation of *FGFR* family expression in HNC patients

	FGFR1		*FGFR2*		*FGFR3*		*FGFR4*	
Gene	Correlation	*P*-value	Correlation	*P*-value	Correlation	*P*-value	Correlation	*P*-value
FGFR1	1.0	-	0.38	5.5e-19*	-0.12	0.0075*	0.17	0.000*
FGFR2	0.38	5.5e-19*	1.0	-	0.26	1.6e-09*	0.16	0.000*
FGFR3	-0.12	0.0075*	0.26	1.6e-09*	1.0	-	0.023	0.6
FGFR4	0.17	0.000*	0.16	0.000*	0.023	0.6	1.0	-

Table S3. Univariate and multivariate analysis of overall survival in 99 HNC patients

Variables	Item	Univariate			Multivariate		
		HR	95% CI	*P*-value	HR	95% CI	*P*-value
Gender	Male	1.381	(0.175, 10.91)	0.760	-	-	-
	Female	1.00					
Age (y)	≥60	0.963	(0.271, 3.42)	0.954	-	-	-
	<60	1.00					
T classification	3/4	6.345	(1.346, 29.916)	0.02*	7.053	(1.43,34.86)	0.017*
	1/2	1.00					
N classification	N+	3.82	(1.075, 13.58)	0.038*	1.96	(0.49, 7.84)	0.34
	N0	1.00			1.00		
Stage	III/IV	55.068	(0.337, 9315.93)	0.123	-	-	-
	I/II	1.00					
FGFR4	High	4.801	(1.015, 22.72)	0.048*	5.127	(0.97, 27.0)	0.054
	Low	1.00			1.00		

HR = hazard ratio; CI = confidence interval; - = not applicable.

Table S4. Correlation of FGFR4 expression with clinicopathological characteristics in HNC

			FGF4		
			Low	High	
Variables	Item	*N*	*N*	*N*	*P*-value
		99	47	52	
Gender					0.440
	Female	7	2	5	
	Male	92	45	47	
Age (years)					0.220
	＜60	58	31	27	
	≥ 60	41	16	25	
T classification					0.227
	1/2	59	25	34	
	3/4	40	22	18	
N classification					0.234
	N0	76	39	37	
	N+	23	8	15	
Stage					0.423
	I/II	49	21	28	
	III/IV	50	26	24	
Alcohol					0.325
	No	22	9	13	
	Yes	77	38	39	
Betel nut					0.519
	No	31	13	18	
	Yes	68	34	34	
Cigarette					0.027*
	No	20	5	15	
	Yes	79	42	37	
Recurrence					0.561
	No	86	42	44	
	Yes	13	5	8	

Table S5. Upstream regulator prediction of FGFR4-responsive genes based on IPA analysis of the GSE34828 dataset

Upstream Regulator	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap	Target Molecules in Dataset
IKZF1	transcription regulator	Inhibited	-3.693	5.86E-06	*ADAM19, ANTXR1, ARHGEF11,BCL6,CCL4,CCND2,CD3D,CD79B,CDK6,CSF1,CXCL2,CXCR3, CYP7B1, DNM3, DOCK4, EBF1,EMP1,EXOC3L1,FGF13,FGFR4,FLI1,GATA1,HBE1,HBZ, HES1, HHATL,IGF1, IKZF1, IL23A,IL23R, IL27, IL2RA,IL4,IL5,IL6,ITGA2B,KMT2A,LMO2, MAP2,MLXIP, NCAM1,NLRP3, NOTCH3, NR1H4, OPRD1, OTUD1, PAX5,PPP1R2,PRDM1,PTCRA, RAG1,RASSF4, RNF213, RUNX2, SCN9A, SEMA5A, SLAMF7, SLC19A1, SLC27A3, SLC2A1, SMAD3,ST3GAL6, TDO2, TNS3,WNT10B*
SMAD7	transcription regulator	Inhibited	-3.047	1.87E-06	*ADH1C,APOC3,AREG,BGN,BMP5,BMP7,CCL5,CCN2,CFC1/CFC1B,CFLAR,COL1A1, COL1A2, COL3A1, CRP, DCN,DEPTOR,EFEMP1,FAS,FGF5,FOXA2,FST,HDAC9,HMOX1, ICAM1, ID1, ID2, ID3, ITGBL1, MMP2,MUC4, MYL11,NANOG,PLAT,SERPINE1,SFTPC,SMAD3, SP1, TAGLN, TGFB1, TGFBI, TGFBR2, TIMP3*
HEY2	transcription regulator	Inhibited	-2.528	0.0161	*ALPL,ATOH1,CNN1,GJA5,HEY2,IL6,MYH11,NPPA,TAGLN,TBX5,TNFSF8*
RUNX3	transcription regulator	Inhibited	-2.464	0.00509	*CAPN6, CCN2, CD4, CDH11, CDX2, CFLAR, CIITA, CXCL8, FBN1, FOXP3,GRK5,IL17A,IL4,IL5, JAG1, KCTD15, KIR2DL4, KIR3DL1,KIR3DL3,MACF1, MMP2,NEGR1,PAPPA,PTPN21, SCARA3,SGCD, TGFB1, TPSAB1/TPSB2*
EHMT2	transcription regulator	Inhibited	-2.36	0.00275	*CDH1, CDH16, EPAS1, HBE1, IFNB1, IL17A,IL6,MDM2,NDRG1,NFASC,NPPA,PTGS2,SCNN1A, STMN2, SYCE1,TGFB1,VAPB,ZEB2*
VHL	transcription regulator	Inhibited	-2.359	0.00229	*AIF1, ALDH1A1, APCDD1L-DT, BNIP3, CCN2, CDH1, CFL1, CLTC, CNN1, CXCR4, DAB2, EGLN3, EPAS1, EPO, FLCN, GAL3ST1, GNG4, HMOX1, NOX4, NREP,PDGFB,PHF8,SIGLEC8,SKP2, SLC1A3, SLC2A1, TAGLN,TFRC, TH,TMED3,TNFSF10,ZFP36L1*
IKZF3	transcription regulator	Inhibited	-2.309	0.11	*ALOX5,ANTXR1,DNM3,DOCK4,HHATL,MAP2,OTUD1,RASSF4,RNF213,SCN9A,SEMA5A,SMAD3,TNS 3*
ATF3	transcription regulator	Inhibited	-2.218	0.13	*CCL5,CD69,CDK2,CGA,DHODH,EPO,GBP6,GCG,GSN,ICAM1,ID1,IL4,IL6,IMPDH2, ITGA1,LDLR, MDM2, MMP2, MMP3,SERPINB5,SERPINE1,SP1,TSLP,USP22*
HDAC5	transcription regulator	Inhibited	-2.045	0.00121	*CLSPN, DRP2, EGR2, GAP43, HES1, HLA-DRA, IGFBP1, LMOD2, MBP, MEG3, MMD2, MYCN, NPPA, PMP22, PPARA,PPARGC1A,PYGM,RBBP6,RUNX2,SLC8A1,TAGLN,TH*

Original image for checking

Figure 7

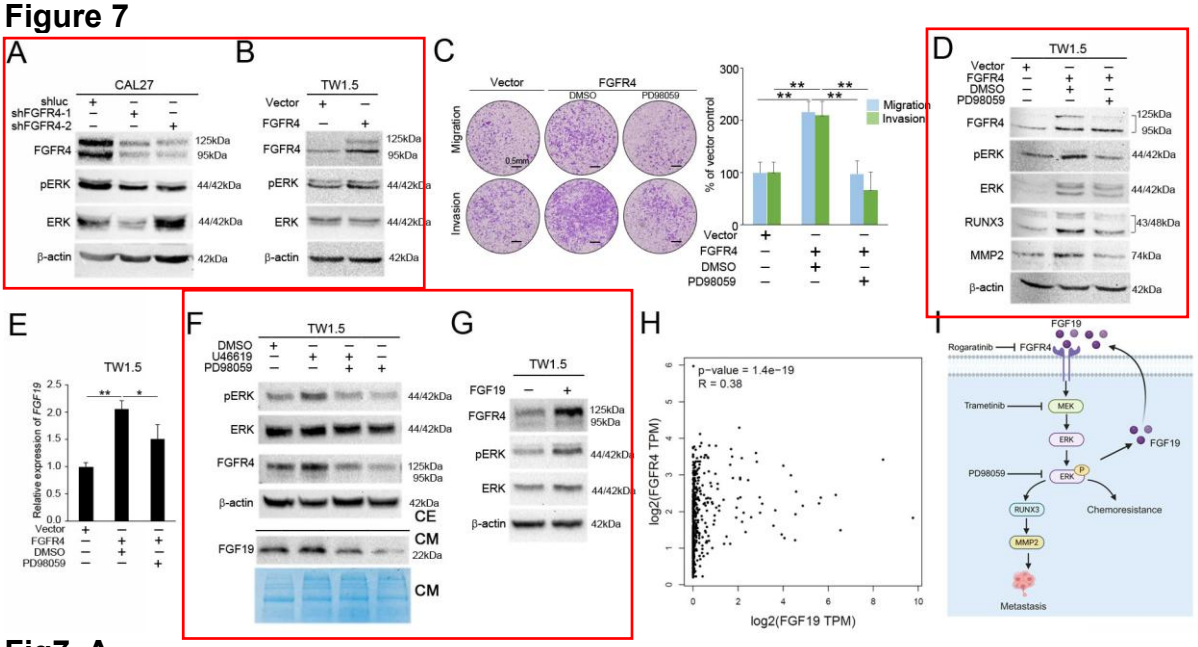
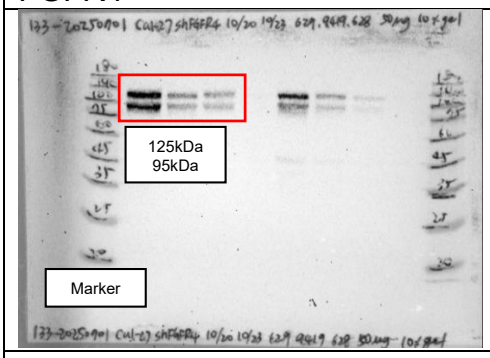
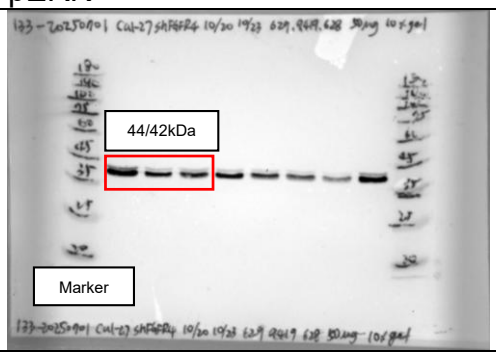


Fig7. A

FGFR4	pERK
	
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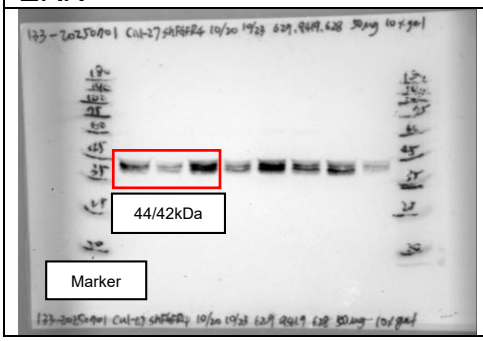
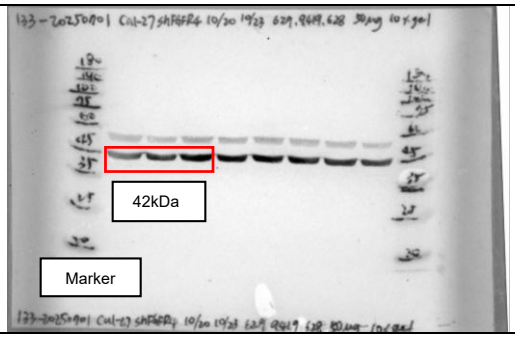
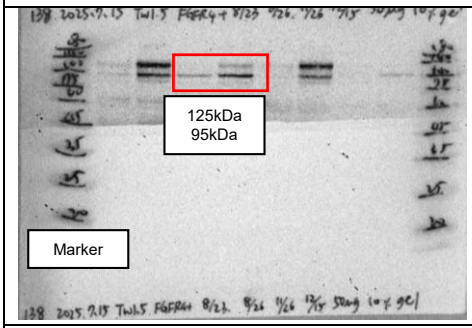
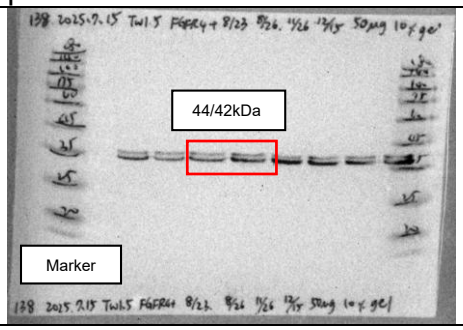
	

Fig7. B

FGFR4



pERK



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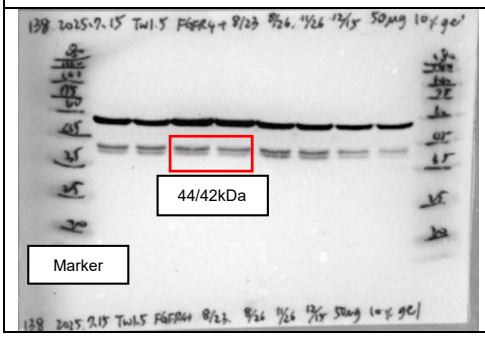


actin

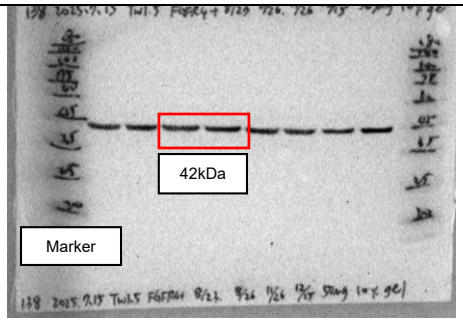
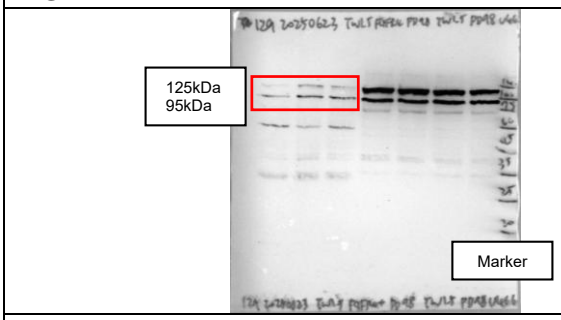
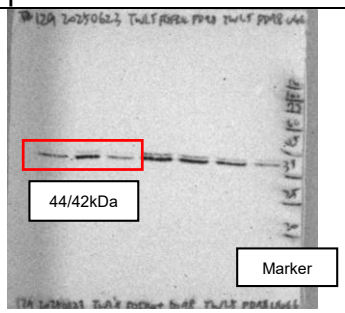


Fig7.D

FGFR4



pERK



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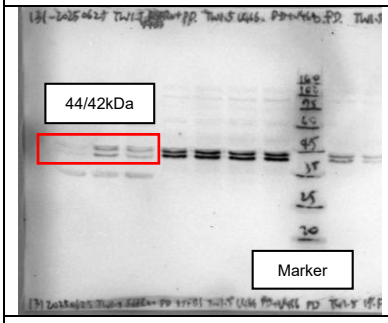
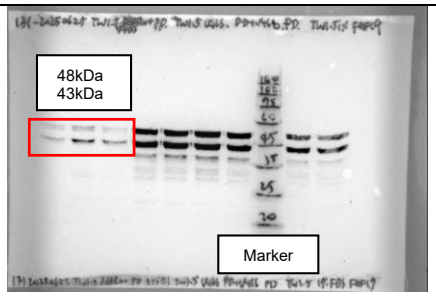
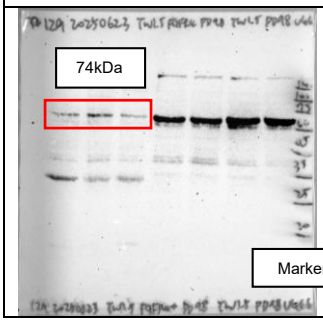


RUNX3

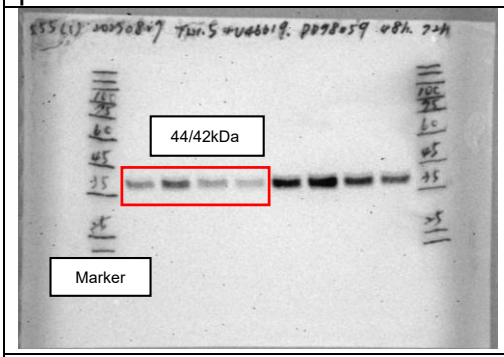
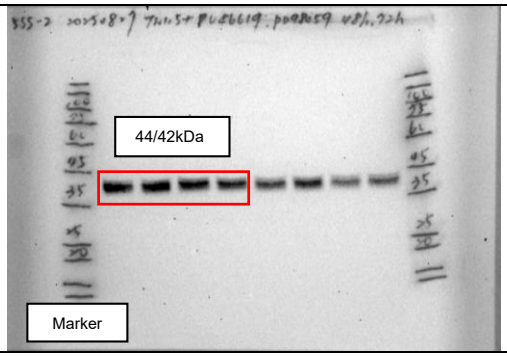
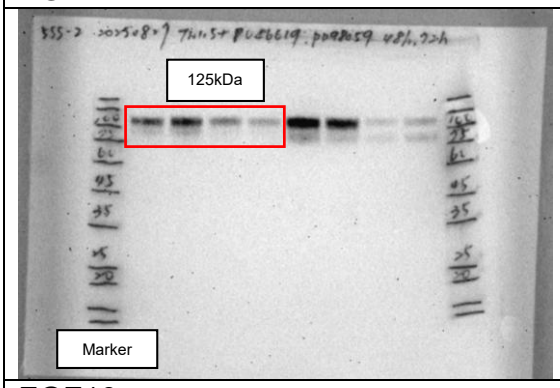
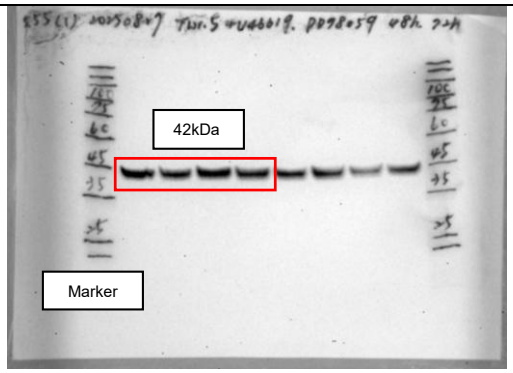


MMP2



actin

Fig7.F

pERK	ERK
	
FGFR4	**actin**
	
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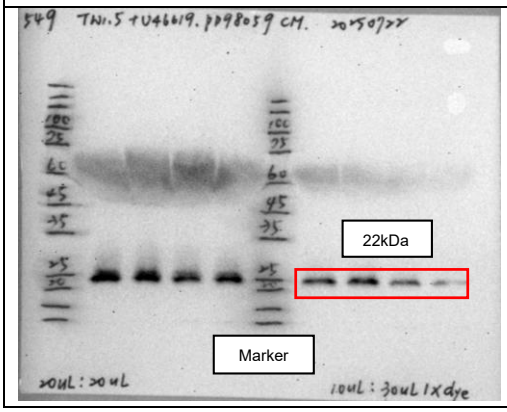
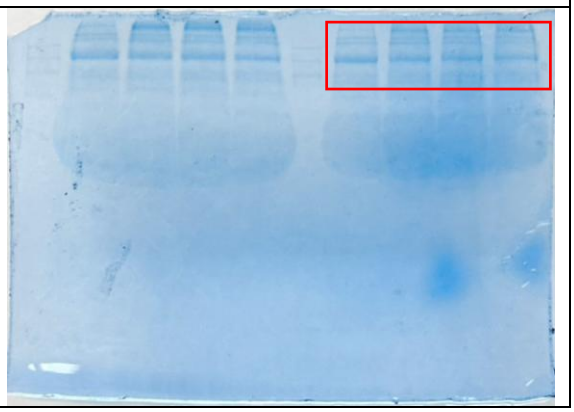
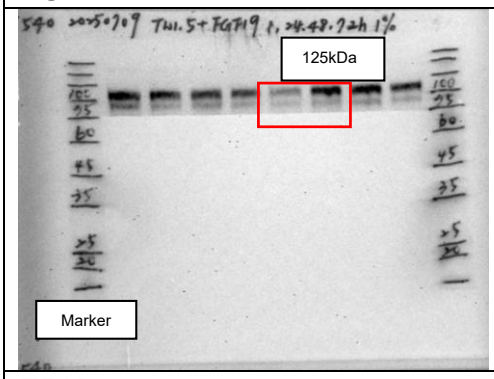
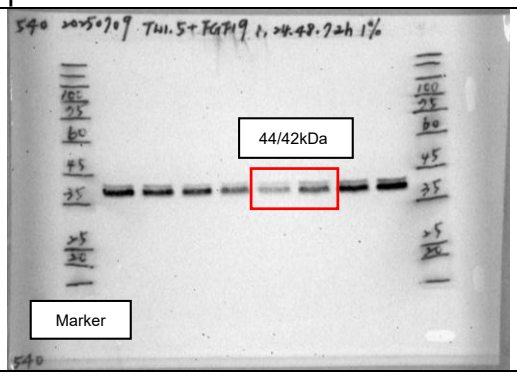
	

Fig7.G

FGFR4	pERK
	
ERK	**actin**