Science Advances

Supplementary Materials for

Hypoxia-inducible factor orchestrates adenosine metabolism to promote liver cancer development

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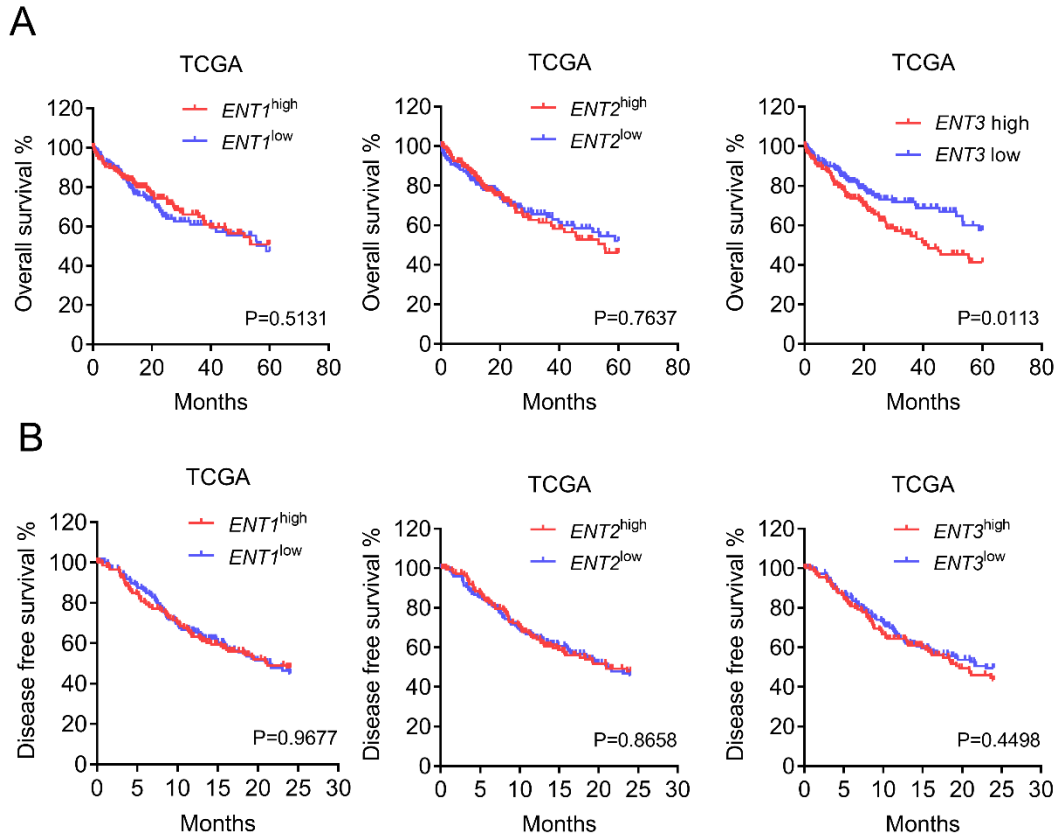Corresponding author: Carmen Chak-Lui Wong, carmencl@pathology.hku.hk

This PDF file includes:

SUPPLEMENTARY FIGURES

Supplementary Figure 1

Figure S1. ENT4 is the most clinically relevant in human HCC when compared to other ENT family members. Kaplan-Meier curves showing the association of mRNA expression of *ENT1, ENT2* and *ENT3* with **(A)** overall and **(B)** disease-free survival in HCC patients from TCGA database. Kaplan-Meier followed by log-rank test.
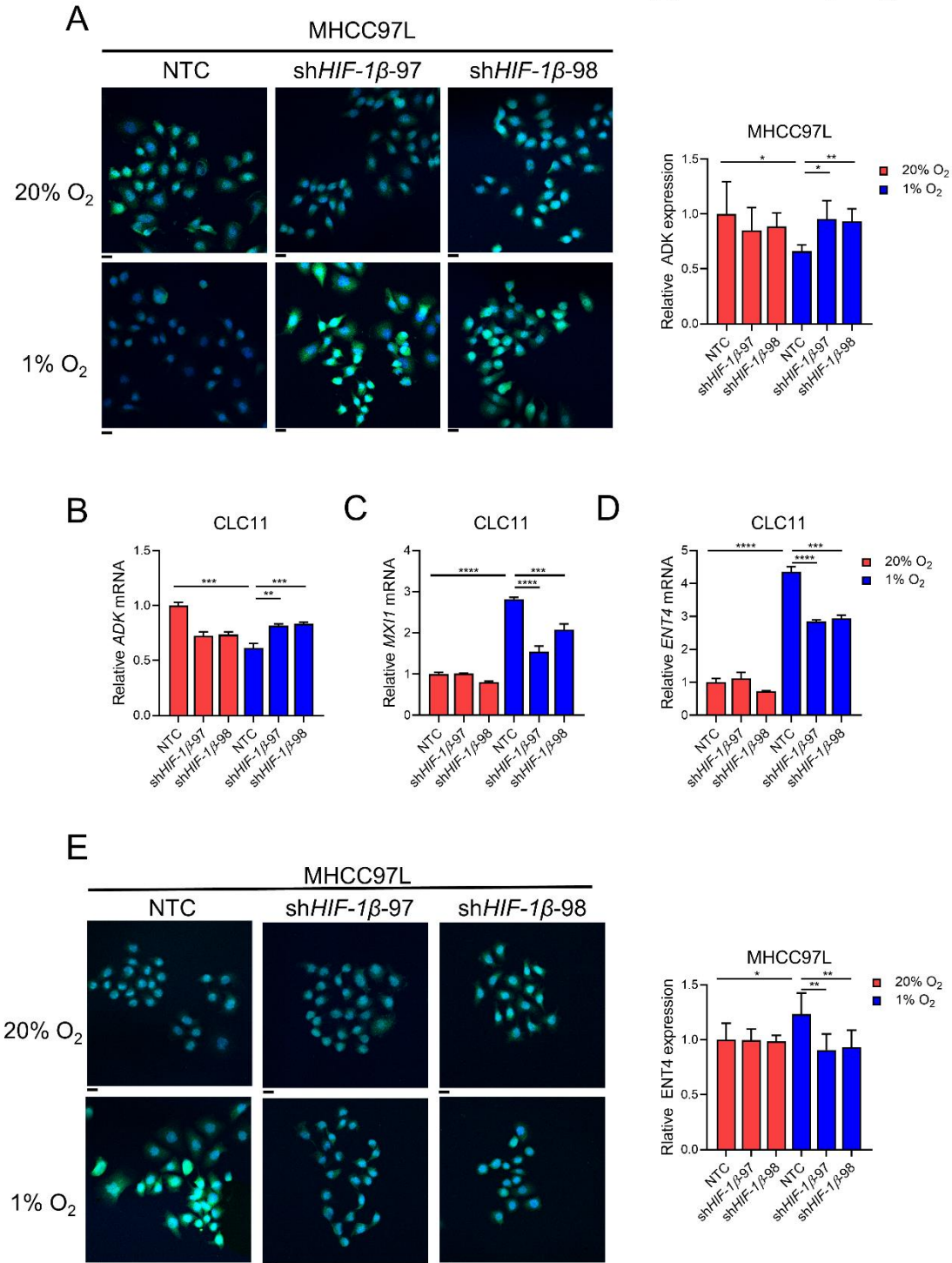
Figure S2. Hypoxia regulates ADK and ENT4 via HIF in HCC cells. (A) ADK protein

expression in MHCC97L-sh*HIF-1β* cells compared to -NTC (non-targeting control)

exposed to 20% and 1% O_2 for 48 hours. **(B)** *ADK* mRNA expression in CLC11-sh*HIF1β* cells compared to -NTC exposed to 20% and 1% O_2 for 24 hours. **(C)** *MXI1* mRNA expression in CLC11- sh*HIF-1β* cells compared to -NTC exposed to 20% and 1% O_2 for 24 hours. **(D)** *ENT4* mRNA expression in CLC11-sh*HIF-1β* cells compared to -NTC exposed to 20% and 1% O_2 for 24 hours. **(E)** ENT4 protein expression in MHCC97L-sh*HIF-1β* cells compared to -NTC exposed to 20% and 1% O_2 for 48 hours. **A,E:** Relative protein expression level were normalized to 20% O_2 NTC. **B-D:** mRNA expression was determined by qRT-PCR and values were normalized to 20% O_2 NTC. Error bars indicate mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ vs. 20% O_2 NTC. Student's *t* test. Scale bar = 20 μm.
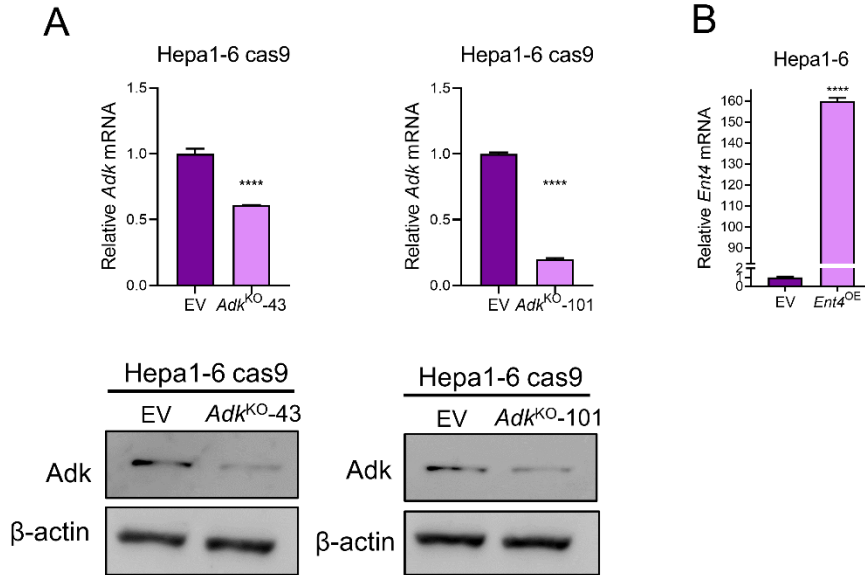
Figure S3. Knockout and overexpression efficiency in mouse HCC cell lines. (A) *Adk* mRNA (top) and protein expression (bottom) in Hepa1-6 cas9-*Adk*^{KO} cells compared to -EV. **(B)** *Ent4* mRNA expression in Hepa1-6-*Ent4*^{OE} cells compared to -EV. Error bars indicate mean ± SD. ****$P < 0.0001$ vs. EV. Student's *t* test.

Supplementary Figure 4
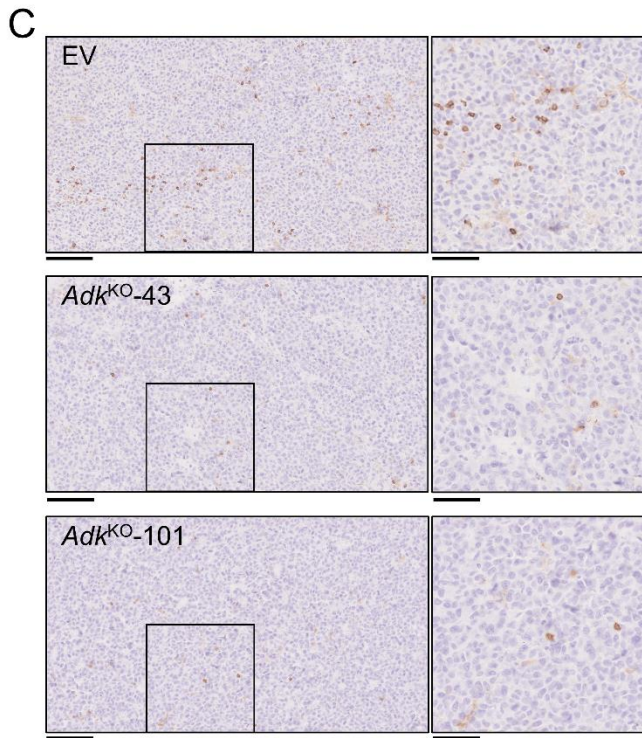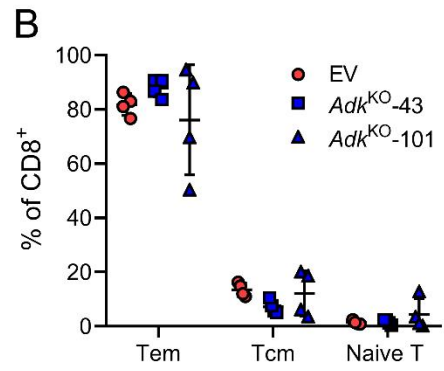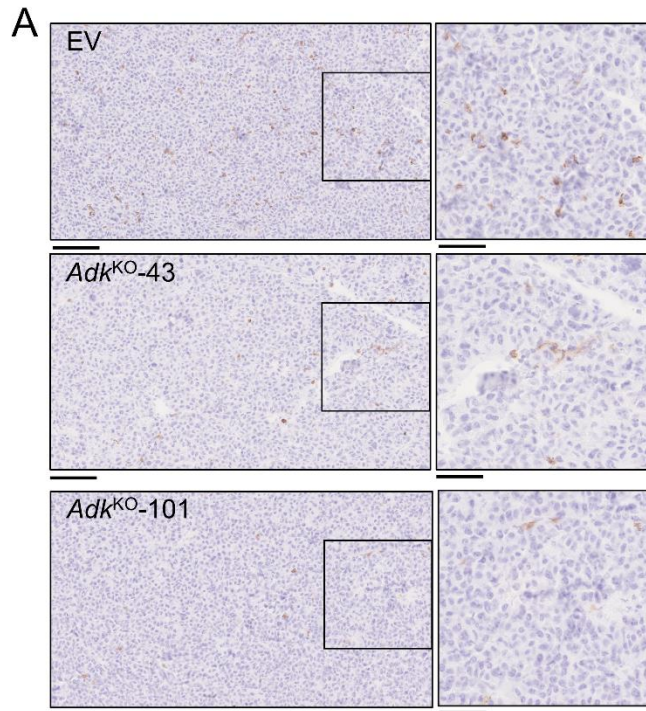
Figure S4. ADK deficiency suppresses T cell tumor infiltration. (A) Representative pictures for the quantification of CD8$^+$ T cells in tumor by IHC staining. **(B)** Percentages of effector memory T cells (Tem: CD44$^+$CD62L$^-$), central memory T cells (Tcm: CD44$^+$CD62L$^+$) and naïve T cells (CD44$^-$CD62L$^+$) in CD8$^+$ T cells. **(C)** Representative pictures for the quantification of CD4$^+$ T cells in tumor by IHC staining. Error bars indicate mean ± SD. Original = 20X magnification, scale bar = 100 µm; Inset = 40X magnification, scale bar = 50 µm.

Figure S5. Effect of Ent4 overexpression on HCC tumor growth. Tumor size of Hepa1-6-$Ent4^{OE}$ orthotopically implanted to livers of C57BL/6N mice compared to -EV. *P < 0.05 vs. EV. Student's t test. Scale bar = 1cm.

Table S1. Clinicopathological significance of ENT4 in HCC

Clinico-pathological features	N (%)	Student's t-test		Fisher's exact test		
		Mean	*p* value	$ENT4^{low}$ (fold<2)	$ENT4^{high}$ (fold≥2)	*p* value
• Tumor microsatellite formation						
• Absent	28 (57%)	1.8962	**0.370**	8 (16%)	20 (41%)	**0.755**
• Present	21 (43%)	2.5043		5 (10%)	16 (33%)	
• Direct liver invasion						
• Absent	29 (64%)	2.0664	**0.497**	6 (13%)	23 (51%)	**0.483**
• Present	16 (36%)	2.5662		5 (11%)	11 (25%)	
• Tumor encapsulation						
• Absent	30 (61%)	2.1360	**0.971**	9 (18%)	21 (43%)	**0.741**
• Present	19 (39%)	2.1613		4 (8%)	15 (31%)	
• Venous invasion						
• Absent	21 (42%)	1.8707	**0.399**	7 (14%)	14 (28%)	**0.346**
• Present	29 (58%)	2.4376		6 (12%)	23 (46%)	
• Tumor stage						
• I – II	21 (42%)	1.8640	**0.437**	8 (16%)	13 (26%)	**0.116**
• III – IV	29 (58%)	2.3948		5 (10%)	24 (48%)	
• Tumor size						
• ≤ 5 cm	21 (42%)	1.3138	**0.020***	10 (20%)	11 (22%)	**0.007***
• > 5 cm	29 (58%)	2.8409		3 (6%)	26 (52%)	
• Cellular differentiation by Edmondson grading						
• I – II	23 (47%)	1.8511	**0.396**	7 (14%)	16 (33%)	**0.747**
• III – IV	26 (53%)	2.4273		6 (12%)	20 (41%)	

Mean: average values of *ENT4* expression in human HCC samples relative to its paired NT tissues determined with the formula: $\Delta Ct^{HCC(ENT4-18S)} - \Delta Ct^{NT(ENT4-18S)}$. Student's *t* test or Fisher's exact test.

Table S2. Antibodies used

Antibody	Application	Clone	Dilution / Amount used	Cat no.	Company
Mouse CD8a	IHC	D4W2Z	1:200	98941	Cell Signaling Technology
Mouse CD4	IHC	EPR19514	1:1000	ab183685	Abcam
ENT4	IF	/	1:100	PA5-65742	Thermo Fisher Scientific
ADK	IF	/	1:100	PA5-83416	Thermo Fisher Scientific
ADK	WB	/	1:1000	PA5-27399	Thermo Fisher Scientific
β-actin	WB	AC-74	1:2500	A5316	Sigma-Aldrich
Human MYC	ChIP	/	5µg	sc-40 X	Santa Cruz
Human MXI1	ChIP	/	5µg	sc-130627	Santa Cruz
Human HIF-1β	ChIP	/	5µg	ab2	Abcam
IgG control	ChIP	/	5µg	sc-2027	Santa Cruz
IgG control	ChIP	/	5µg	sc-2762	Santa Cruz
Mouse CD16/32	FC	93	1:100	101301	Biolegend
Mouse CD45	FC	30-F11	1:100	103127	Biolegend
Mouse CD8b	FC	YTS156.7.7	1:100	126619	Biolegend
Mouse CD8b	FC	YTS156.7.7	1:100	126609	Biolegend
Mouse CD44	FC	IM7	1:100	103005	Biolegend
Mouse CD62L	FC	MEL-14	1:100	104431	Biolegend
Mouse PD-1	FC	29F.1A12	1:50	135219	Biolegend
Mouse TIGIT	FC	1G9	1:100	142103	Biolegend
Mouse LAG-3	FC	C9B7W	1:100	125225	Biolegend
Mouse TIM-3	FC	RMT3-23	1:100	119705	Biolegend
Mouse CD45	FC	30-F11	1:100	103115	Biolegend
Mouse CD3	FC	17A2	1:100	100205	Biolegend

Mouse CD3	FC	17A2	1:100	100215	Biolegend
Mouse CD4	FC	GK1.5	1:100	100429	Biolegend
Mouse CD4	FC	GK1.5	1:100	100405	Biolegend
Mouse FOXP3	FC	FJK-16s	1:100	45-5773-82	eBioscience
Mouse IFNγ	FC	XMG1.2	1:50	505839	Biolegend
Mouse Granzyme B	FC	QA16A02	1:50	372225	Biolegend
Mouse CD11b	FC	M1/70	1:100	101205	Biolegend
Mouse Gr-1	FC	RB6-8C5	1:100	108415	Biolegend
Mouse CD11c	FC	N418	1:100	117337	Biolegend
Mouse F4/80	FC	BM8	1:100	123115	Biolegend
Mouse I-A/I-E	FC	M5/114.15.2	1:100	107625	Biolegend
Mouse CD206	FC	C068C2	1:50	141717	Biolegend

IHC: immunohistochemistry; IF: immunofluorescence; WB: western blotting; ChIP: chromatin immunoprecipitation; FC: flow cytometry

Table S3. Primer sequences

Primer	Sequence
Human *ADK* (ChIP)	Forward: AATCCCACTCCCGAAGACCT
	Reverse: CCTAGTAACAGCGTCCCGTC
Human *ENT4* (ChIP)	Forward: AGAGGAGCGAGAGGGAAGA
	Reverse: CAGACAAAGGCTCCGACAG
Human *ADK*	Forward: AGAGAGCAAGGCTTTGAGACTA
	Reverse: CCCTTGGGTGAAGATCACGA
Human *ENT4*	Forward: AGGGACCTCCATCGTGTTTG
	Reverse: TAAGAGGTAGCCTGCGGTGA
Human *MXI1*	Forward: GAGCGAGAGGAGATTGAAGTGG
	Reverse: ACTGGAGTAACCCTCGTCAC
Human *18S*	Forward: GAGGATGAGGTGGAACGTGT
	Reverse: AGAAGTGACGCAGCCCTCTA
Mouse *Adk*	Forward: TGGGGAGATCCTGAAGCGTA
	Reverse: GTTAGCAACGAGGGACCTGT
Mouse *Ent4*	Forward: AGACATCCACTTTGAGCACCA
	Reverse: CCAGCTTCGTTTGACTCGTG
Mouse *18s*	Forward: ACATCGACCTCACCAAGAGG
	Reverse: TCCCATCCTTCACATCCTTC

Table S4. shRNA / sgRNA target sequences and insert sequences for luciferase reporter assay

shRNA/sgRNA	Target Sequence
sh*HIF-1β*-97	GGCTCAAGGAGATCGTTTATT
sh*HIF-1β*-98	ACTAGGTCCCACAGCTAATTT
Adk^{KO}-43	TTCAAAGTTGAATATCATGC
Adk^{KO}-101	TAAGGCTGCTGACGCACATG
Insert	**Sequence**
Wild-type (WT)	TCGAGCGGGGCCGGGCGCGGGGGGCGGGCAGAGGCGGG<u>GCGTG</u>GTG
Mutant (MUT)	TCGAGCGGGGCCGGGCGCGGGGGGCGGGCAGAGGCGGG<u>AAAAA</u>GTG