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Supplementary Materials for

Therapeutic targeting of SLC6A8 creatine transporter suppresses colon cancer progression and modulates human creatine levels

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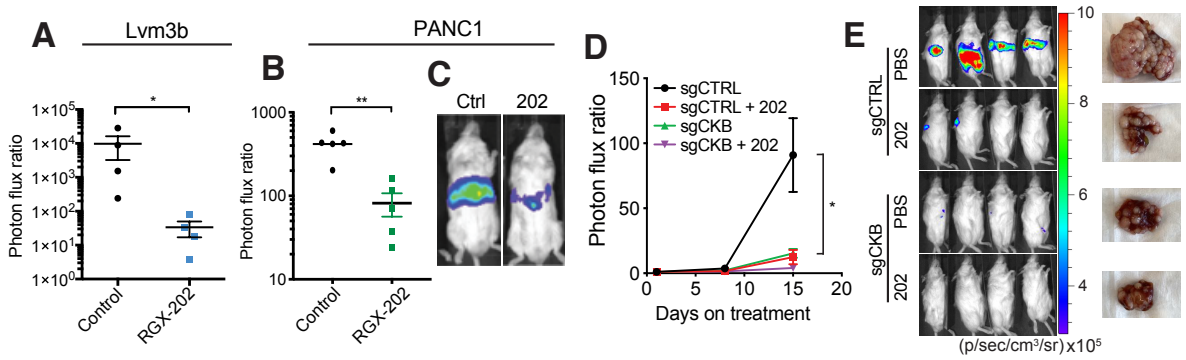

Fig. S1. RGX-202 inhibits liver colonization of metastatic CRC and pancreatic cell lines

(**A**) Bioluminescence plot of liver colonization by $5x10^5$ human Lvm3b metastatic colon cancer cells after intra-splenic injections into NOD-SCID mice. Treatment with control or RGX-202-formulated diet started on day 1, for the duration of the experiment. Mice were imaged on day 22 after injection. Photon flux ratio is the ratio of bioluminescence signal at day 14 normalized to the signal on day 0; n = 4 per group, p = 0.0286. **(B** and **C)** Bioluminescence plot of liver colonization by $5x10^5$ human PANC1 pancreatic cells that were pre-treated with RGX-202 at 10 mM for 48h, after intra-splenic injections into NSG mice. Photon flux ratio (B) is the ratio of bioluminescence signal at day 28 normalized to the signal on day 0; n = 5, p = 0.0079. P values are based on Mann-Whitney test. (**D**) Bioluminescence plot of liver colonization by $5x10^5$ Lvm3b cells expressing either CRISPR control or CRISPR CKB constructs. Mice received daily i.p. injections of PBS control or RGX-202 (650mg/kg). (**E**) Gross liver images derived from (D). Photo Credit: Jia Min Loo, the Rockefeller University (B); Norihiro Yamaguchi, the Rockefeller University (E).

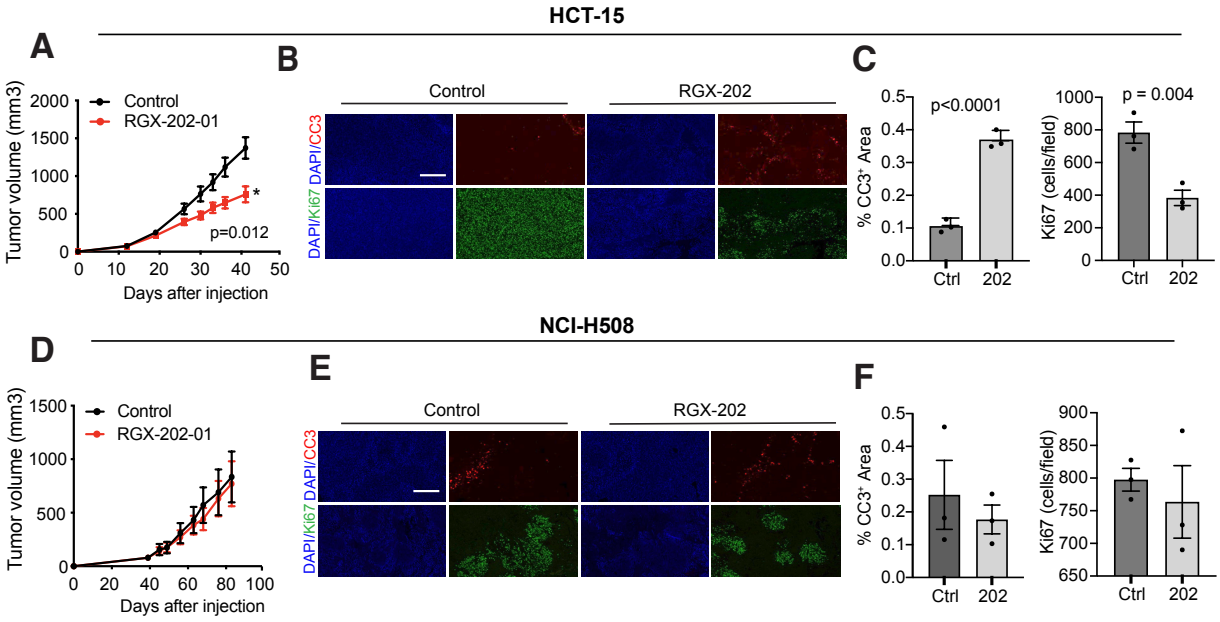

Fig. S2. Anti-tumor efficacy to RGX-202 is associated with induction of tumor apoptosis and inhibition of proliferation

(A to **F)** HCT-15 (A to C) or NCI-H508 (D to F) tumors were extracted from animals that were treated with RGX-202-01 (800 mg/kg) or control diet (A, D, n = 7-11/group) and were immunostained for cleaved-caspase 3 (CC3) or Ki67 and counterstained with DAPI (B and E). Graphs depict quantification of CC3 positive area or the number of Ki67 positive cells in RGX-202 or control diet treated HCT-15 (C) or NCI-H508 (F) tumors. N = 3 tumors per group +/- SEM. Scale bar (B and E) 200µm.

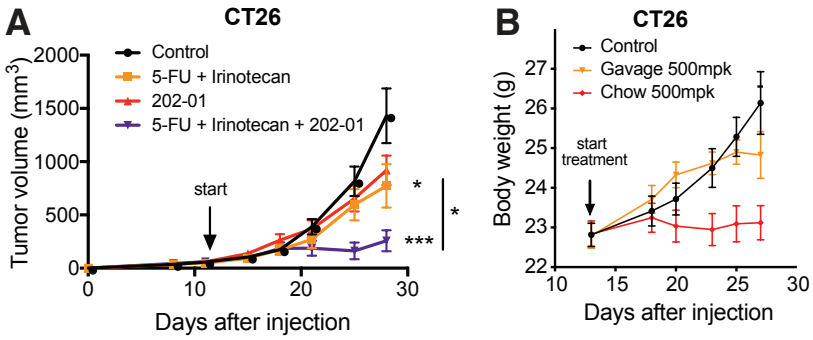

Fig. S3. Anti-tumor efficacy of RGX-202 in combination with 5-FU and irinotecan

(**A**) Tumor growth of $1.5x10^5$ CT26 cells subcutaneously injected into BALB/c mice. As soon as tumors reached an average of 50 mm^3, mice were randomly distributed and received either a control diet, RGX-202-01-supplemented diet (~500 mg/kg, p = 0.092) or a combination of 5-FU (i.p. 30 mg/kg/week) and irinotecan (i.p. 15 mg/kg/week) (p = 0.064) or a combination of RGX-202-01, 5-FU and irinotecan (p = 0.0006); n= 7-8 per group; shown are mean +/-SEM; All p values are based on two-sided t tests; *p = 0.02. (**B**) Body weight curves of BALB/c mice harboring CT26 tumors. Mice were treated with RGX-202 administered by oral gavage (500 mg/kg) or formulated in chow (500 mg/kg) and body weights were measured twice/week; n = 7-11/cohort.

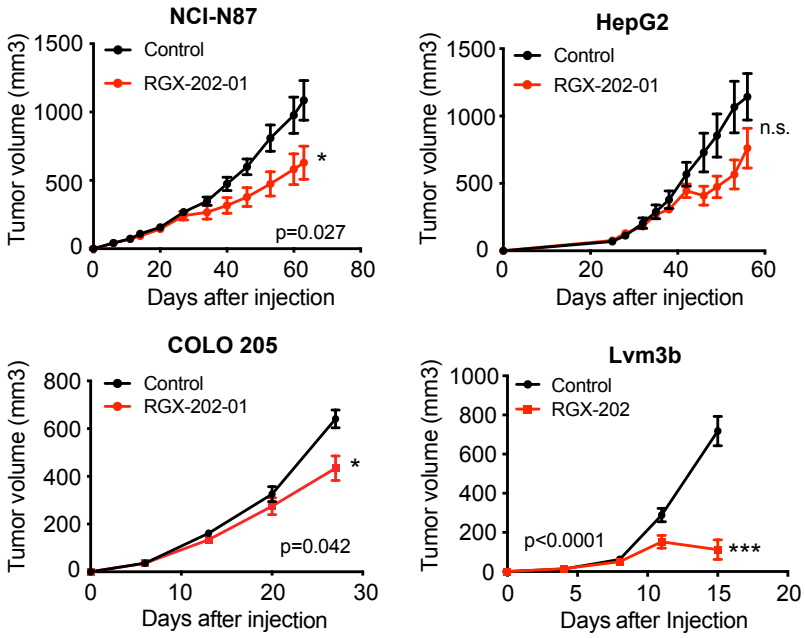

Fig. S4. Xenograft tumor studies

Tumor growth of 4 x 10^6 NCI-N87 ($p = 0.027$), 2 x 10^6 HepG2, 5 x 10^6 Colo205 ($p = 0.042$) and 1 x 10^6 Lvm3b ($p<0.0001$) cells subcutaneously injected into athymic nude mice. Treatment with a control or RGX-202/RGX-202-01-supplemented diet (~800 mg/kg/day) started as soon as tumors reached 10-60 mm^3; n = 8-10 per group. All p values are based on two-sided t tests; n.s. = not significant. Shown are mean +/- SEM.

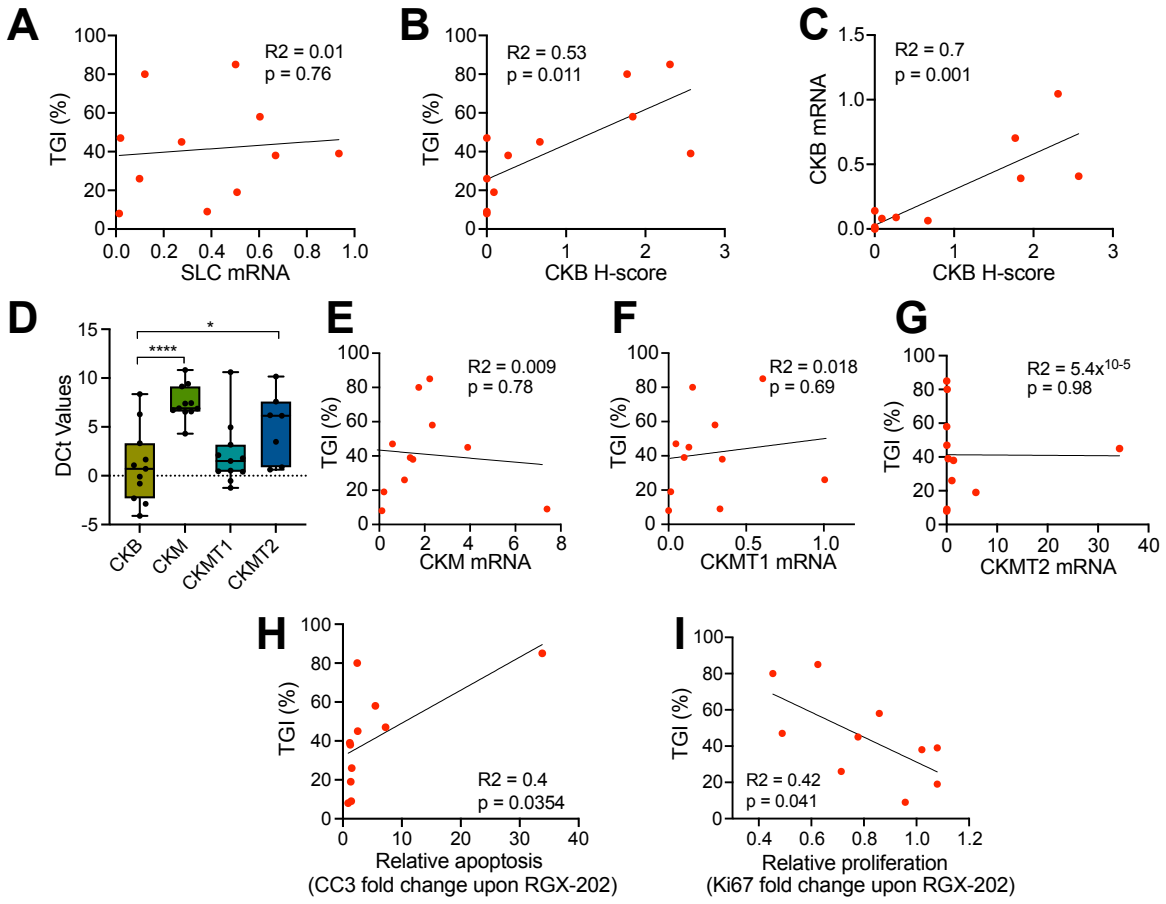

Fig. S5. Linear regression analysis demonstrates anti-tumor efficacy of RGX-202 in CKB high expressing cell lines

(A to **C)** Linear regression analyses of the percentage of tumor growth inhibition (TGI) as a function of SLC6A8 mRNA (A), TGI as a function of CKB H-score (B) and CKB mRNA levels as a function of CKB H-score (C) in the tumors of the xenograft models tested. mRNA levels were measured by qPCR. Each dot represents the mean value of data points from one xenograft model, n=4-6 tumors/xenograft model. (**D**) qPCR analysis of CKB, CKM, CKMT1 and CKMT2 in the xenograft models tested. Relative expression is shown in ΔCt values; n = 7-11 models; 4-5 tumors/models. Shown is the average +/- SEM. (**E** to **G)** Linear regression analysis of TGI as a function of CKM mRNA (E), CKMT1 mRNA (F) and CKMT2 (G) tumoral mRNA levels. mRNA levels were measured by qPCR; Each dot represents the mean value of data points from

one xenograft model, n=4-5 tumors/xenograft model. (**H-I**) Linear regression analyses of the TGI and the fold change of cleaved caspase-3 (H) or Ki67 (I) positive tumor area assessed by IHC in RGX-202-01-treated tumors relative to control tumors; n = 3 tumors/model. Each dot represents the mean value of data points from one xenograft model.

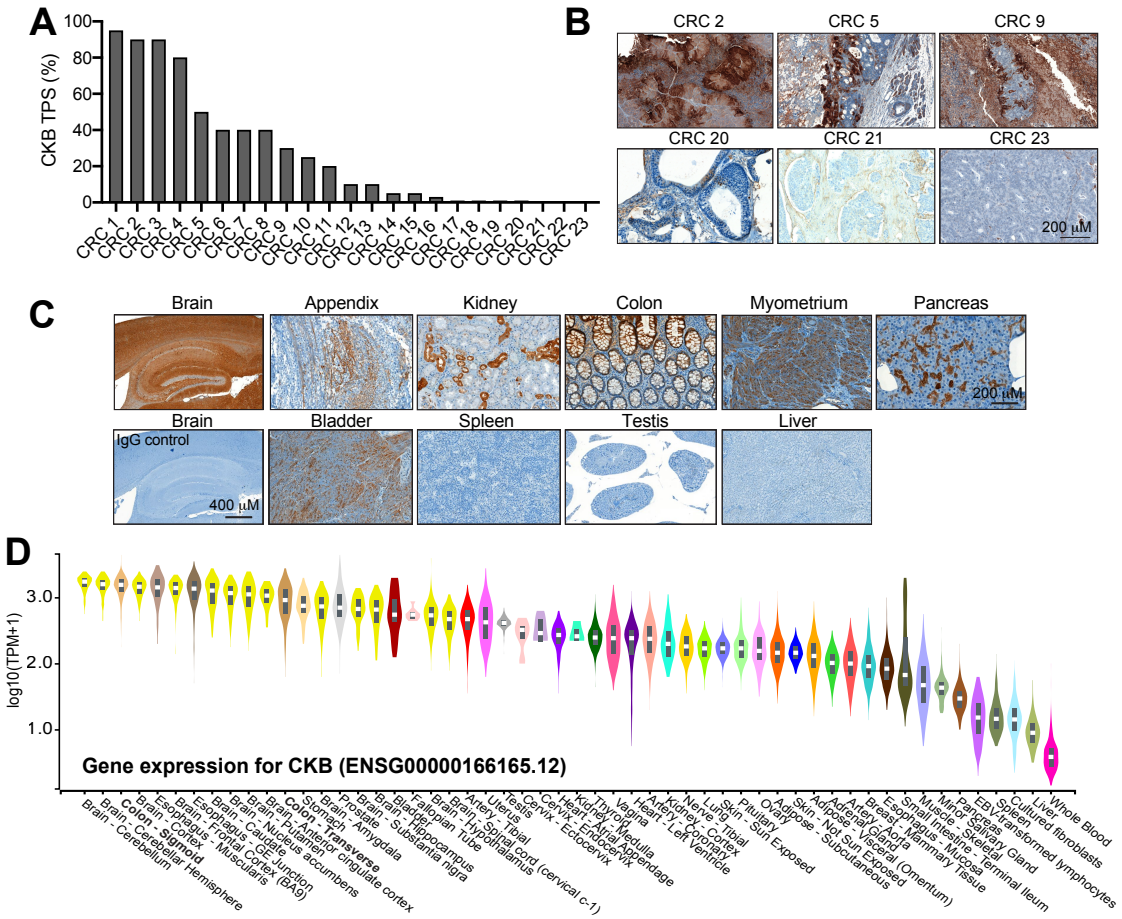

Fig. S6 CKB expression in patient specimen and healthy tissue

(**A** and **B**) CKB expression was assessed by IHC in 23 metastatic CRC specimens. Quantification of TPS is shown in (A) and representative images are shown in (B). (**L**) Human tumor microarray (TMA) comprising 10 healthy tissues were immunostained with CKB and counterstained with hematoxylin. Scale bar (B and C) 200µm; except brain and IgG control, 400µm. (**D**) Organ-based physiological CKB mRNA expression levels from the Genotype-Tissue Expression (GTEx) database in human specimen. Horizontal bars in box plots show 25, 50, and 75 percentiles.

Table S1. PDX models utilized in the PDX trial

Summary of PDX tumor growth data, KRAS and BRAF status. TGI(21) = Tumor growth inhibition on Day 21 = $((C_{21}-C_0)/C_{21}) - ((T_{21}-T_0)/T_0)$. Change in tumor size relative to control.

Cancer Type	ID	TGI(21)	Change in tumor size	KRAS	BRAF
CRC	**CR5039**	-3.67	147%	WT	WT
CRC	**CR3280**	-3.74	141%	p.G12R	D22N
CRC	**CR5053**	-2.78	71%	p.A146V	WT
CRC	**CR2554**	-1.03	44%	p.G12V	WT
CRC	**CR5032**	-1.91	41%	p.G13D	WT
CRC	**CR6251**	-2.79	29%	WT	WT
CRC	**CR5066**	-0.61	24%	p.G12C	WT
CRC	**CR5075**	-1.23	17%	WT	V600E
CRC	**CR5033**	-0.47	14%	WT	WT
CRC	**CR5080**	0.50	6%	p.G12D	WT
CRC	**CR5081**	-0.15	1%	p.G12D	WT
CRC	**CR1287**	0.04	0%	p.G12D	WT
CRC	**CR5059**	-0.18	-2%	WT	V600E
CRC	**CR3151**	0.23	-2%	p.G12A	WT
CRC	**CR2491**	0.26	-4%	p.G12V	WT
CRC	**CR5052**	0.11	-5%	WT	V600E
CRC	**CR5046**	0.43	-8%	p.G12R	WT
CRC	**CR6460**	2.26	-14%	p.Q61L	WT
CRC	**CR5082**	0.35	-16%	WT	V600E
CRC	**CR6863**	0.55	-19%	WT	V600E
CRC	**CR5043**	0.57	-26%	p.G13D	WT
CRC	**CR3085**	3.28	-27%	WT	WT
CRC	**CR5058**	1.81	-32%	WT	WT
CRC	**CR5044**	1.69	-32%	WT	WT
CRC	**CR5048**	1.00	-33%	p.G12C	WT
CRC	**CR5038**	-0.07	-34%	p.Q61H	WT
CRC	**CR5101**	1.10	-36%	WT	WT
CRC	**CR2526**	1.01	-42%	WT	WT
CRC	**CR5040**	6.75	-42%	p.G12V	WT
CRC	**CR2179**	3.34	-45%	WT	V600E
CRC	**CR6249**	3.08	-45%	WT	WT
CRC	**CR11372**	1.79	-47%	WT	WT
CRC	**CR6256**	3.82	-48%	p.G12C	WT
CRC	**CR1795**	9.37	-48%	WT	WT
CRC	**CR5026**	2.94	-49%	WT	V600E
CRC	**CR3079**	3.01	-49%	p.G12D	WT
CRC	**CR3099**	4.05	-49%	p.G12D	WT
CRC	**CR2533**	4.18	-51%	WT	WT
CRC	**CR5030**	4.43	-51%	WT	WT
CRC	**CR3448**	5.94	-56%	p.G12D	WT
CRC	**CR2110**	5.72	-63%	p.G12S	WT
CRC	**CR5062**	6.37	-66%	p.G12D	WT
CRC	**CR1451**	14.05	-83%	p.G12C	WT

Table S2. Information on cell lines

Anti-tumor efficacy and mutational status of the cell lines used in the xenograft studies. TGI = Tumor growth inhibition.

Cancer Type	Cell line	TGI	CKB TPS	KRAS	BRAF	p53	Mismatch repair mutations
CRC	**HCT-8**	80%	75	G13D			MSH6
CRC	**Lvm3b**	85%	87	G12D	V600E		MSH3
CRC	**HCT116**	58%	100	G13D			MLH1, MSH2, MSH3, MSH6
CRC	**HCT15**	47%	0	G13D		S241F	MSH2, MSH6
CRC	**SW480**	45%	65	G12V		R273H	wild type
CRC	**HT29**	39%	100	WT	V600E	R273H	MLH1, MLH2
CRC	**Colo205**	26%	0	WT	V600E		
CRC	**NCI-H508**	9%	2	WT		R273H	
Gastric	**NCI-N87**	38%	14	WT		R248Q	
Gastric	**Hs746T**	8%	0	WT		K319	
Hepatic	**HepG2**	19%	3	WT			