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Supplementary Information for

Therapeutic Inhibition of USP9x-Mediated Notch signaling in Triple-Negative Breast Cancer

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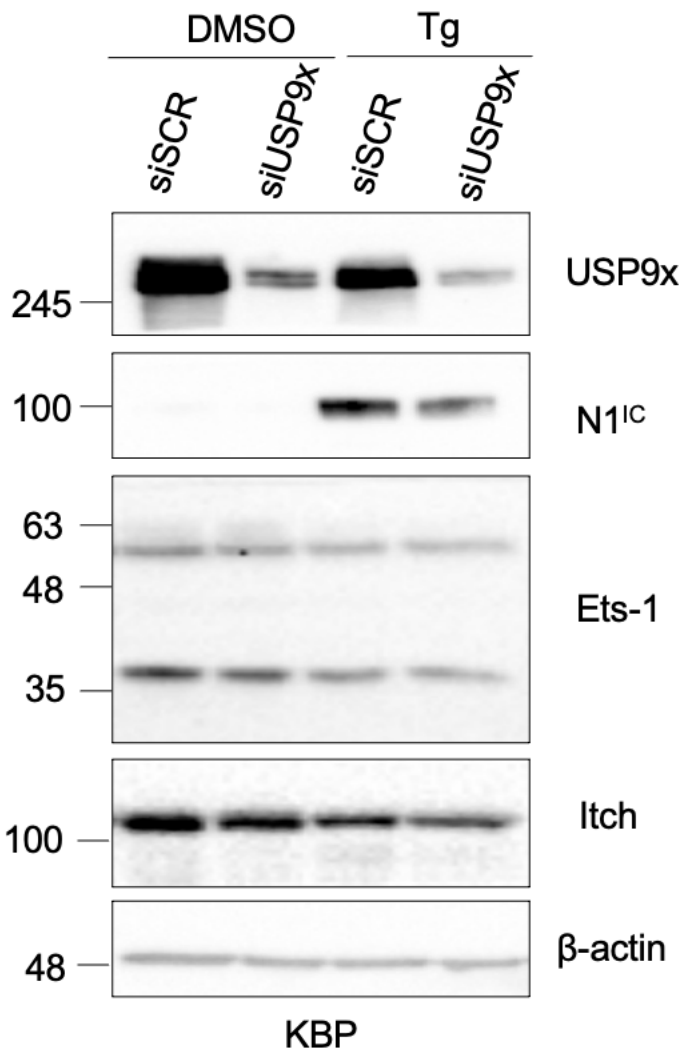
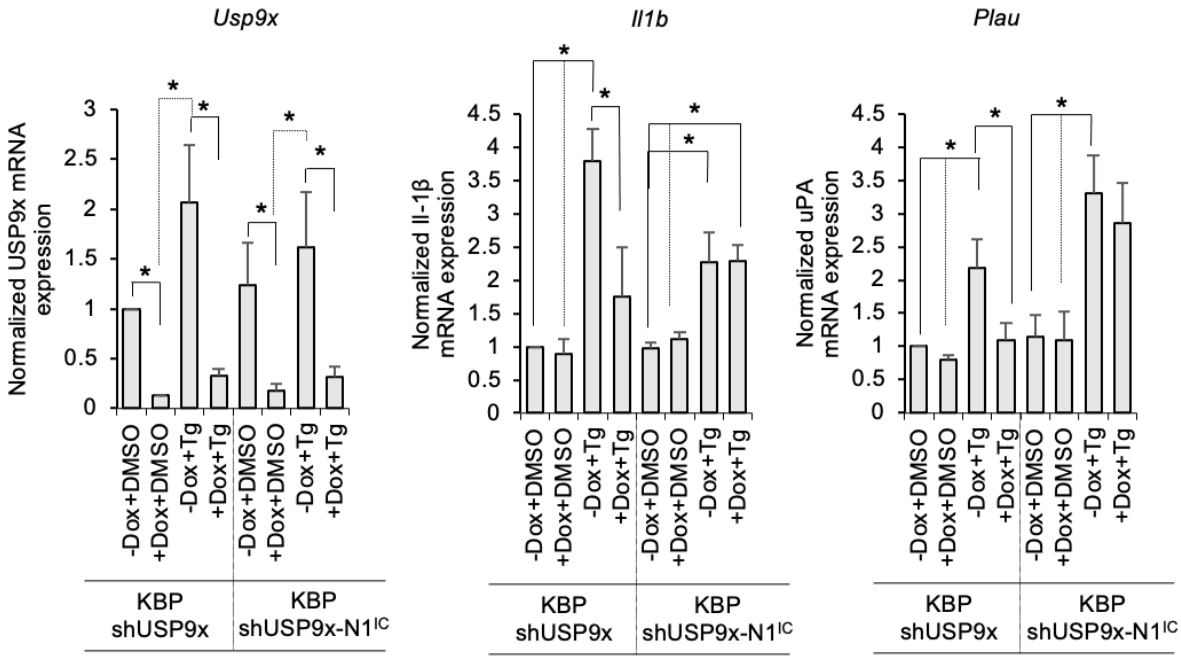
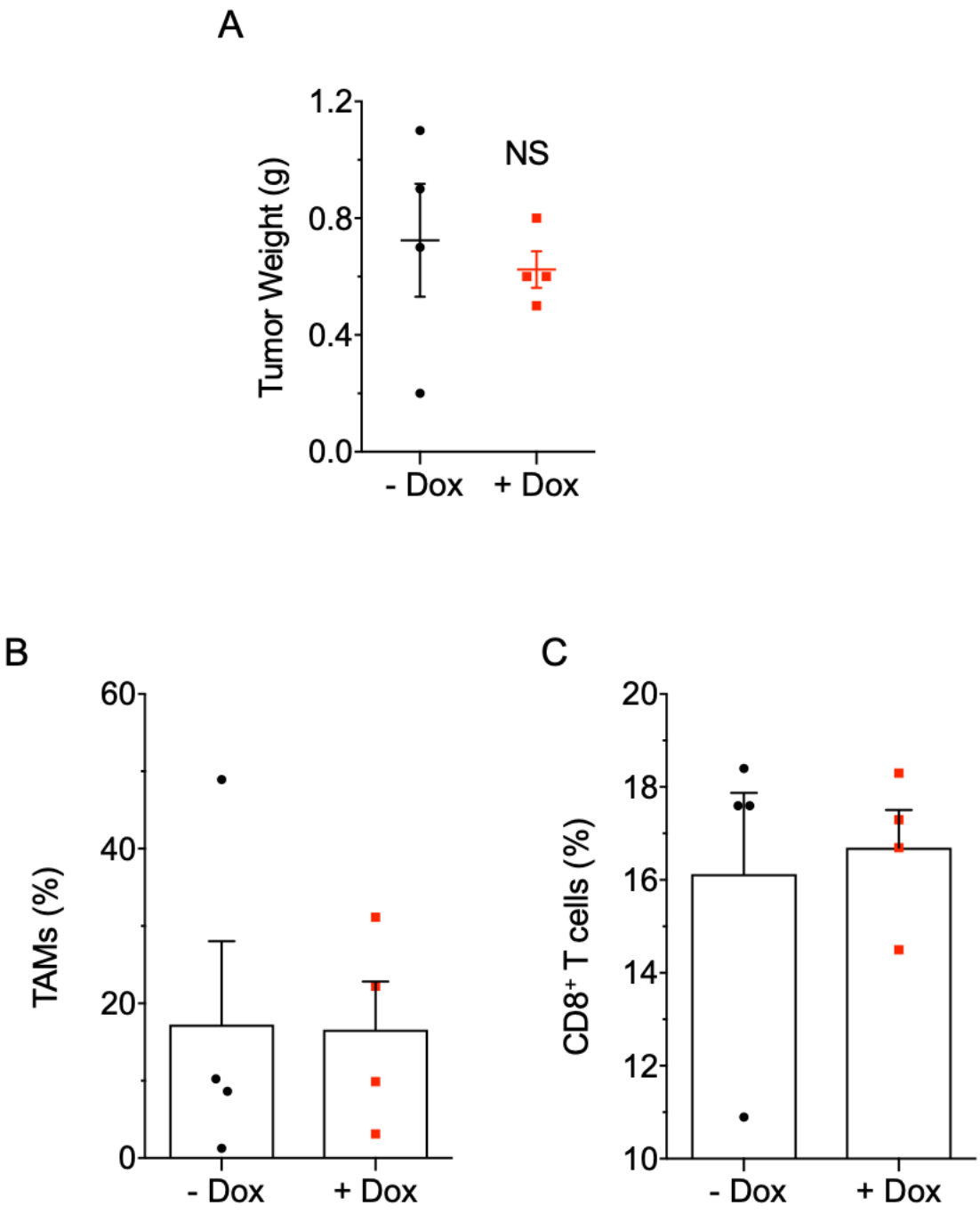


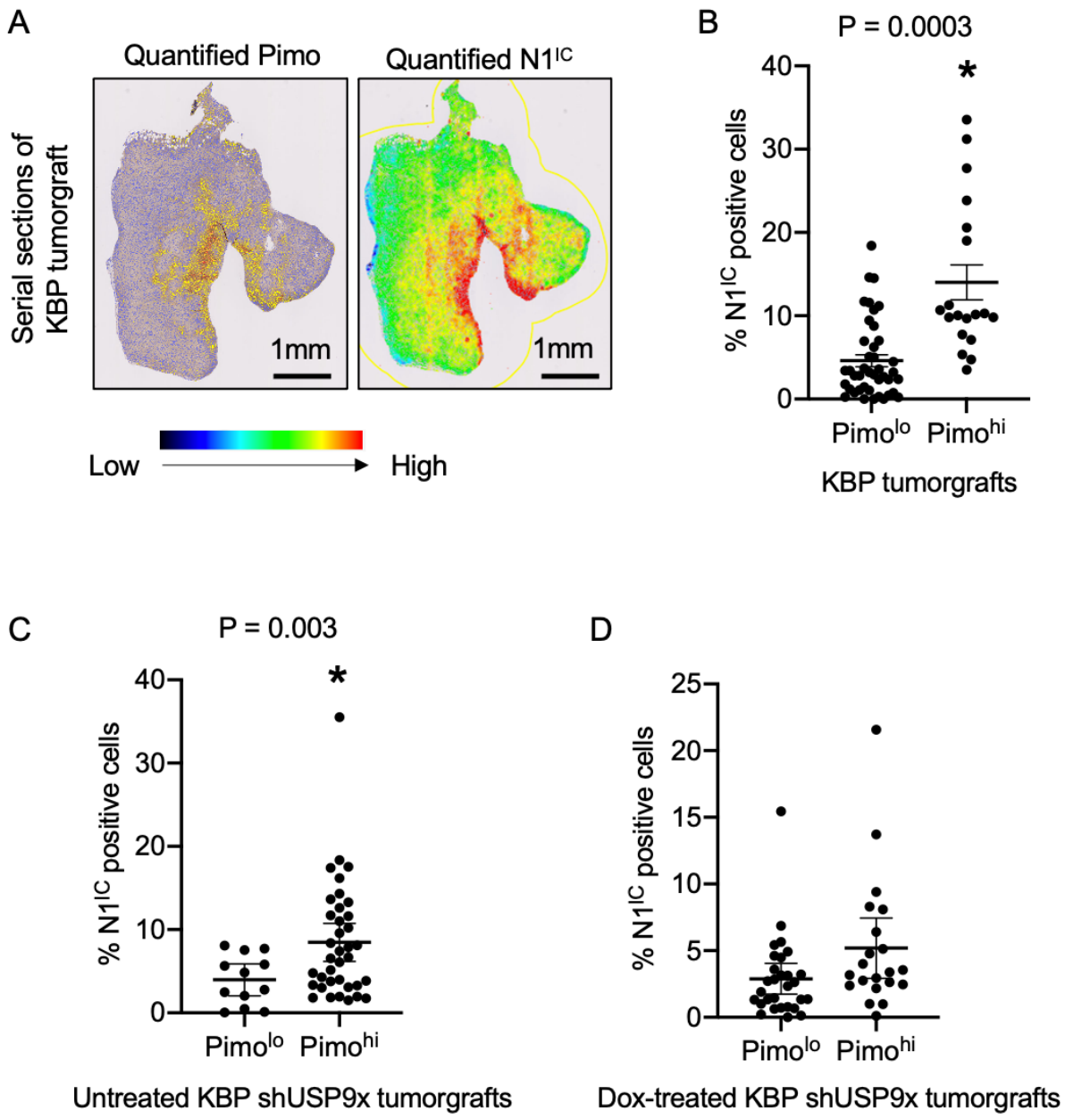
Suppl Fig 1. USP9x knockdown does not affect the expression of Ets-1 or Itch in KBP cells. Immunoblot of USP9x, $N1^{IC}$, Ets-1 and Itch in KBP cells transfected with either siscr or USP9x siRNA and treated with either DMSO or 1 μM Tg. β-actin is included as a loading control. *Mw* markers are shown in kilodaltons.



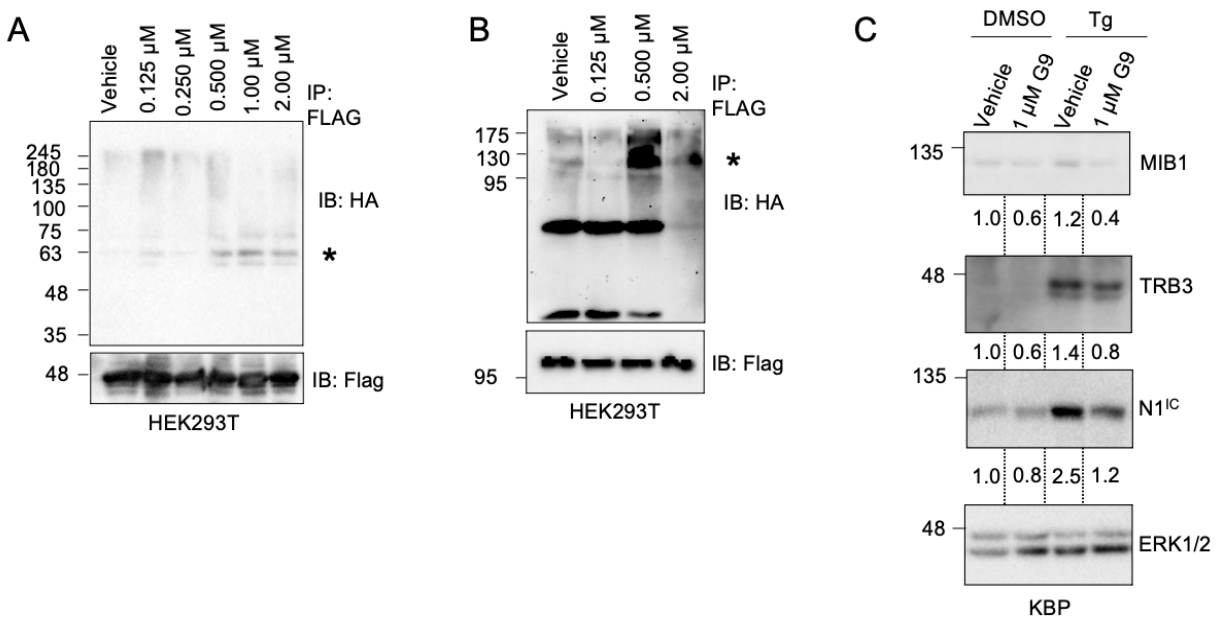
Suppl Fig 2. Ectopic N1^{IC} expression rescues the effect of USP9x knockdown on the expression of Notch target genes. qPCR analysis of *Usp9x* and Notch target genes (*Il1b* and *Plau*) in uninfected or retrovirus infected (expressing N1^{IC}) KBP shUSP9x and treated with either DMSO or 1 μM Tg in the absence or presence of doxycycline. Expression levels were normalized to GAPDH. Experiments were performed in triplicate; error bars represent SEM. The asterisks (*) denotes $P < 0.05$ relative to the respective control. *P* values are determined by one-way ANOVA and corrected for multiple comparisons using Tukey's test.



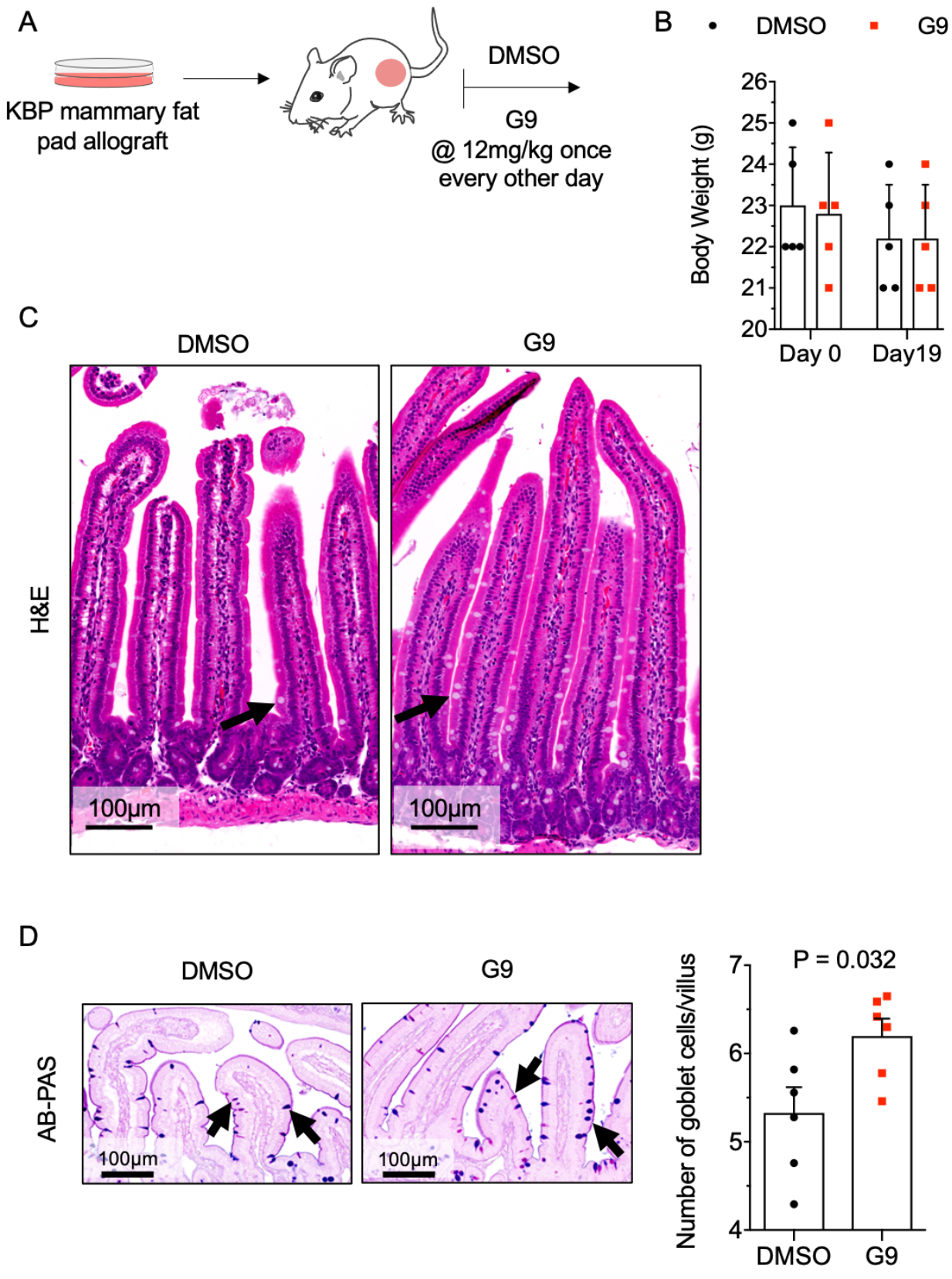
Suppl Fig 3. Doxycycline treatment has no effect on KBP allograft growth. (a) Weight of KBP allografts fed a regular or doxycycline-containing diet (n = 4 per group). **(b and c)** Quantification (by flow cytometry) of TAMs (Gr-1$^-$ F4/80$^+$ CD11b$^+$) **(b)** and CD8$^+$ T **(c)** infiltrates in KBP tumors expressed as % of CD45$^+$ cells. Error bars represent SEM. NS, non-significant. *P*-value is determined by the two-tailed Student's *t*-test.



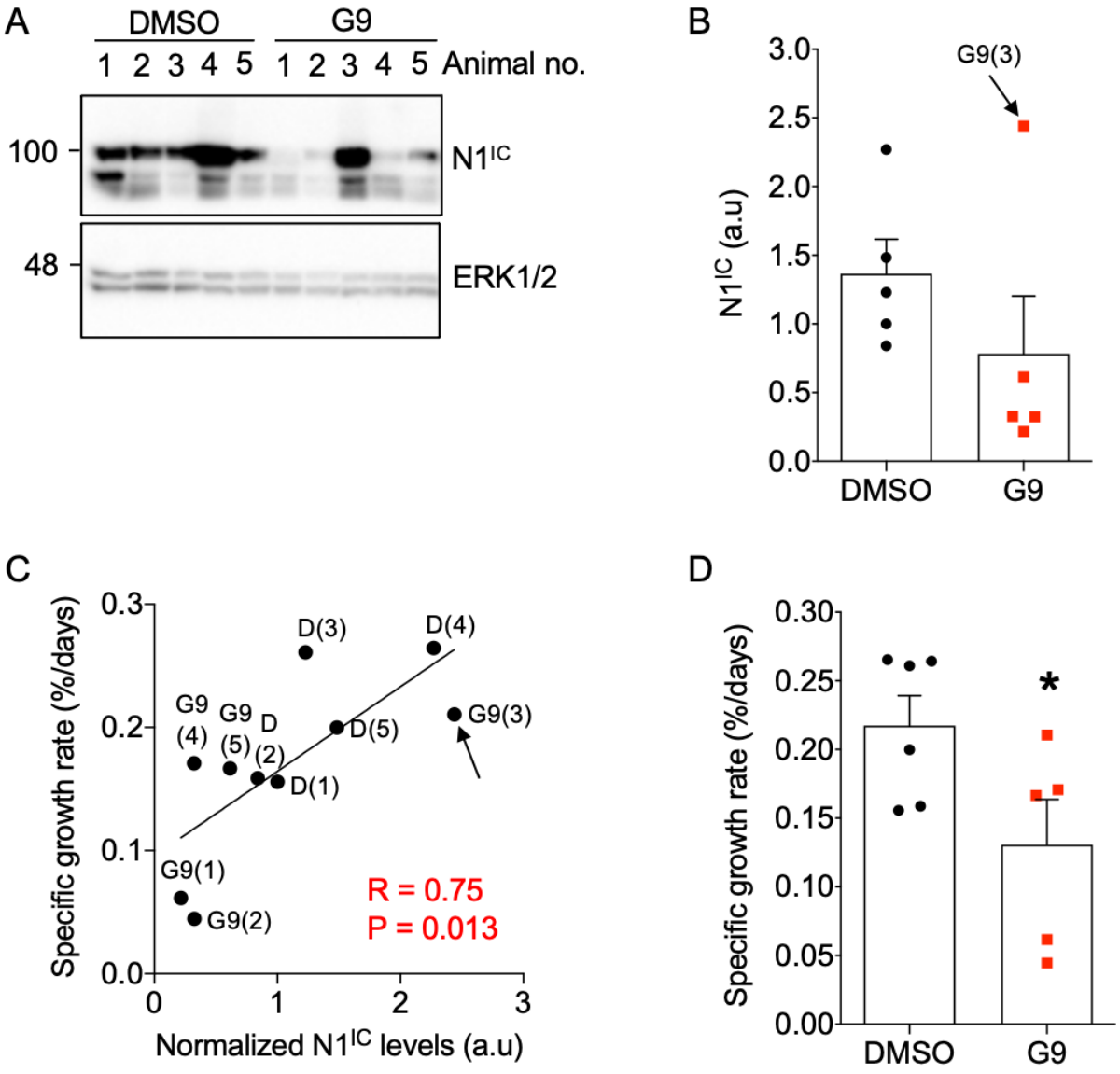
Suppl Fig 4. Co-localization of hypoxia and Notch activation is USP9x-dependent. **(a)** Representative quantified IHC images of the hypoxia marker pimonidazole (Pimo) and $N1^{IC}$ in serial sections of KBP allografts. The color bar indicates the expression level of Pimo and $N1^{IC}$. Scale bars: 1mm. **(b-d)** Percentage of $N1^{IC+}$ cells in $Pimo^{lo}$ (< 20% $pimo^{+}$ cells) *vs* $Pimo^{hi}$ (> 20% cells $Pimo^{+}$) regions of KBP **(b)**, untreated KBP shUSP9x **(c)** and doxycycline-treated KBP shUSP9x **(d)** allografts (14-18 x 20,000 μm^2 regions were surveyed in n = 4 tumors per group). Error bars represent SEM. * = P < 0.05. *P* indicates the significance value determined by the two-tailed Student's *t*-test with Welch's correction for unequal variances.



Suppl Fig 5. G9 inhibits stress-induced Notch activation in murine KBP cells. Immunoblot of HA and Flag after anti-Flag immunoprecipitations from HEK293T cells transfected with plasmids expressing HA-Ub and either TRB3-Flag **(a)** or MIB1-Flag **(b)** and treated with either vehicle or varying concentrations of G9 in the presence of proteasome inhibitor, MG132. * marks the predicted size of monoubiquitinated TRB3-Flag-HA-Ub **(a)**, or MIB1-Flag-HA-Ub **(b)**. **(c)** Immunoblot of MIB1, TRB3, and $N1^{IC}$ in KBP cells treated with either vehicle or 1µM G9 and treated with either DMSO or 1µM Tg. Expression of ERK1/2 is included as a loading control. Expression of MIB1, TRB3 and $N1^{IC}$ are determined by densitometry and quantified relative to ERK1/2. *Mw* markers are shown in kilodaltons.


A
KBP mammary fat pad allograft
DMSO
G9
@ 12mg/kg once every other day
B
DMSO
G9
Body Weight (g)
26
25
24
23
22
21
20
Day 0
Day19
C
DMSO
G9
H&E
100μm
100μm
D
DMSO
G9
AB-PAS
100μm
100μm
P = 0.032
Number of goblet cells/villus
7
6
5
4
DMSO
G9

Suppl Fig 6. The effect of G9 on the gastrointestinal epithelium. (a) Schematic diagram of the experimental design. **(b)** Bodyweight of animals treated with DMSO or G9 (n = 5 mice per group). **(c)** Representative images of H&E-stained sections of murine intestinal villi. **(d)** Left panel: representative images of AB-PAS-stained sections of murine intestinal villi. Right panel: quantification of intestinal goblet cells (i.e secretory cells) from AB-PAS-stained sections (n = 6 mice). Arrows in **(c)** and **(d)** indicate goblet cells. Error bars represent SEM. Scale bars: 100μm. *P* values are determined by the two-tailed Student's *t*-test.


A
DMSO
G9
1 2 3 4 5 1 2 3 4 5 Animal no.
100
48
N1IC
ERK1/2
B
N1IC (a.u)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
G9(3)
DMSO
G9
C
Specific growth rate (%/days)
0.3
0.2
0.1
0.0
D(3)
D(4)
G9 (4)
G9 (5)
D (2)
D(5)
G9(3)
D(1)
G9(1)
G9(2)
R = 0.75
P = 0.013
0
1
2
3
Normalized N1IC levels (a.u)
D
Specific growth rate (%/days)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
DMSO
G9

Suppl Fig 7. G9 suppresses Notch-dependent KBP tumor growth. (a) Immunoblot of $N1^{IC}$ in lysates of spontaneous KBP tumors treated with DMSO or G9. The expression of ERK1/2 is included as a loading control. *Mw* markers are shown in kilodaltons. **(b)** Expression of $N1^{IC}$ determined by densitometry relative to ERK1/2. The arrow indicates $N1^{IC}$ expression in G9-treated tumor #3. **(c)** Correlation analysis of specific growth rate (SGR; calculated using the formula $\ln(V_2/V_1)/\Delta t$, where V_1 and V_2 are the measured tumor volumes at the beginning and end of treatment respectively, and Δt is the treatment duration) with tumoral $N1^{IC}$ levels. Each data point is labelled with the sample ID (treatment type followed by sample number). R indicates the degree of correlation and P indicates the significance value determined through the Pearson correlation test. **(d)** SGR of KBP tumors treated with DMSO or G9 (DMSO, n = 6 tumors; G9, n = 5 tumors). Error bars represent SEM. * = $P < 0.05$. *P* values are determined by the two-tailed Student's *t*-test for (b) and (d).

Suppl Table 1. Sequences and Primers

siRNA	Sequence
siscr	UGGUUUACAUGUCGACUAA
	UGGUUUACAUGUUGUGUGA
	UGGUUUACAUGUUUUCUGA
	UGGUUUACAUGUUUUCCUA
siTRB3	GCAGAUACCCAUUCCACGA
	GAGAGAGGAUCACGGCCGA
	CUGGAAGAUGCCUGCGUGA
	AGGUGGGGCUGUACGGCUA
siUSP9x	CAAAGGAGAUUUACUAGAA
	AGAAAUCGCUGGUAUAAAU
	ACACGAUGCUUUAGAAUUU
	GUACGACGAUGUAUUCUCA

shRNA	Sense	Anti-sense
shUSP9x (5'-3')	GATGAGGAACCTGCATTTC	GAAATGCAGGTTCCTCATC

qRT-PCR	Forward Primer	Reverse Primer
Mouse *Usp9x*	AAGGAGCTACTGGCTTTTCAGA	GATGCGGGAAAGATGAAGTC
Mouse *Plau*	GACAGCCTGGCCTACCATAA	AGTCTGAACCAAACGGAGCA
Mouse *Il1b*	CTCTTGTTGATGTGCTGCTG	GACCTGTTCTTTGAAGTTGACG

Suppl Table 2. Antibodies

Immunoblot and IHC	Source	Catalog no.
USP9x	Bethyl Laboratories	A301-351A
TRB3	Santa Cruz Biotechnology	sc-365842
MIB1	Abcam	ab245766
JAG1	CST	2620
Cleaved Notch1 [Val1744]	CST	4147
Notch1 (to detect ectopic $N1^{IC}$)	Santa Cruz Biotechnology	sc-6014
p44/42 MAPK (ERK1/2)	CST	9102
HA	Santa Cruz Biotechnology	sc-805
Flag	Santa Cruz Biotechnology	sc-807
β-actin	Santa Cruz Biotechnology	sc-1615
Ets-1	CST	14069
AIP4 / Itch	Santa Cruz Biotechnology	sc-11890
CCL2/MCP-1	Santa Cruz Biotechnology	sc-1785
IL-1β	CST	12242
anti-Mouse IgG, HRP-linked	CST	7076
anti-Rabbit IgG, HRP-linked	CST	7074
anti-goat IgG, HRP-linked	Santa Cruz Biotechnology	sc-2020
Pimonidazole	Hypoxyprobe	PAb2627
SMA	Abcam	ab5964
ELISA	**Source**	**Catalog no.**
CCL2	R&D	DY479-05
IL-1β	R&D	DY401-05
Flow cytometry	**Source**	**Catalog no.**
CD45	eBioscience	63-0451-82
CD3	Biolegend	100234
F4/80	eBioscience	17-4801-82
CD11b	BD biosciences	553310
CD206	Biolegend	141719
CD8	eBioscience	45-0081-82
GrmB	Invitrogen	48-8898-82
FoxP3	eBioscience	12-5773-80
Gr-1	eBioscience	45-5931-80
Viability	eBioscience	65-0865-14