Supplementary Table 1. Features of patients' specimen used in this study.

Characteristics	Descriptions	Number of Patients (%)
Gender	Male: Female	49 (60 %): 32 (40 %)
Age at 1st Diagnosis	Mean±SD (Median)	66.7±12.3 (67) years old
Stage of CRC	1:2: 3: 4	18 (22 %):19 (23 %): 19 (23 %): 25 (31 %)
Survival	≤ 2 years	8 (10 %)
	> 2 years ≤ 5 years	37 (46 %)
	> 5 years ≤ 10 years	20 (25 %)
	> 10 years	15 (19 %)
Chemo/radio therapy before tissue extraction	No chemo/radio therapy	79 (98 %)
	FOLFOX	1 (1 %)
	Xeloda, radiotherapy	1 (1 %)
Family history of cancer	No history of cancer	69 (85 %)
	CRC	6 (7 %)
	Stomach	2 (2 %)
	Oesophageal	1 (1 %)
	Data not available	3 (4 %)

Supplementary Table 2. Antibodies used in this study

Purposes	Antibody targets	Company (clone/catalogue number)
Western blot/ Immunostaining	DUSP6	Abcam (EPR129Y/#ab76310)
Western blot	β-Actin	Cell Signaling (13E5/#4970)
Western blot	pERK1/2	Cell Signaling (197G2/#4377)
Western blot	tERK1/2	Cell Signaling (137F5/#4695)
Western blot	pP38	Cell Signaling (3D7/#9215)
Western blot	tP38	Cell Signaling (#9212)
Western blot	pJNK	Cell Signaling (98F2/#4671)
Western blot	tJNK	Cell Signaling (#9252)
Western blot/IP	NOTCH1	Cell Signaling (D1E11/#3608)
Western blot	c-MYC	Abcam (Y69/#ab32072)

Western blot	COX2	Cell Signaling (D5H5/ # 12282S)
Western blot	NICASTRIN	Cell Signaling (D38F9/#5665)
Western blot	PRESENILIN 1	Cell Signaling (D39D1/#5643)
Western blot	PRESENILIN 2	Cell Signaling (D30G3/#9979)
Western blot	PEN2	Cell Signaling (D6G8/#8598)
Western blot	Anti-rabbit IgG, HRP-linked Antibody	Cell Signaling (#7074)
Western blot	Ubiquitin	Abcam (#ab7780)
Western blot/IP	Flag-tag	Millipore (#F7425)
Western blot/IP	HA-tag	Cell Signaling (C29F4/ # 3724)
Immunostaining	Cleaved NOTCH1	Abcam (#ab52301)
Immunostaining	Ki67	Novus Biologics (#NB500-170)
Immunostaining	Donkey anti-Rabbit IgG Secondary Antibody, Biotin	Invitrogen (#31821)
Immunostaining	High Sensitivity Streptavidin-HRP	Pierce (#21130)

Supplementary Methods

Cell culture and plasmid constructs

Parental Caco2, DLD1 colorectal cancer cell line (ATCC, Manassas, VA) and 293T cells were grown in Dulbecco's Modified Eagle Medium containing 10 % fetal bovine serum and 1 % penicillin/streptomycin (10,000U/ml) in 5 % CO_2 at 37 ^oC. 1 μg/ml puromycin was supplemented in culture media for Caco2 ppy-vector/DUSP6 OE cells.

DUSP6 expression vector was generated by cloning full-length human DUSP6 cDNA into either ppy-vector (for generation of stable overexpression cells) or Flag-tag pXJ40 vector. Notch intracellular domain (NICD) sequence was amplified from pB-TAG-NICD (a gift from James Thomson (Addgene plasmid # 130934 ;http://n2t.net/addgene:130934; RRID:Addgene_130934) (26) using primer set; sense = 5'- ATGGTGCTGCTGTCCCGCAAG-3' and anti-sense = 5'-TTACTTGAAGGCCTCCGGAATGCGG-3' and cloned into HA-tag pXJ40 vector. The resultant NICD protein expressed by this construct includes only the HA-tagged NOTCH1 intracellular domain without the transmembrane domain. CRISPR/Cas9 gene targeting to knockout DUSP6 in DLD1 and Caco2 cells was achieved by transfecting pGL3-U6 sgRNA-Puromycin expression vector containing DUSP6 targeting oligo sequence: GACCGCTTCACCCGGCGCTG and pST1374-N-NLS-

Flag-Cas9. Puromycin and blasticidin selection was used to isolate the resultant DLD1 and Caco2 DUSP6 KO cells, which were further validated by Western blot analysis and sequencing to ensure that the top three off-targeting genes were not affected. Notch1 KO Caco2 ppy-vector/DUSP6 OE cells were generated by transfecting pSpCas9(BB)-2A-GFP (PX458) (gift from Feng Zhang (Addgene plasmid # 48138; http://n2t.net/addgene:48138; RRID:Addgene_48138) (27) expression vector containing *Notch1* targeting oligo sequence: GGCACCTGCCACAACGAGGT before selection based on GFP positivity. All transfection reaction to generate stable cell lines were carried our using Lipofectamine LTX + Plus Reagent (Invitrogen, USA).

Immunoprecipitation

For immunoprecipitation assay, Flag-DUSP6 or HA-NICD plasmid were transfected into DLD1 cells using Lipofectamin LTX Reagent (Invitrogen) based on manufacturer's instructions. At 24 h post transfection, cells were lysed in lysis buffer (1 M Tris-Cl, 5 M NaCl, 10 % NP-40) containing Halt Protease and Phosphatase Inhibitor Cocktail and 5mM EDTA (Thermo Scientific, USA) before centrifugation at 16,000 g to remove insoluble fraction. Anti-Flag or anti-HA beads were added to the resultant supernatant for immunoprecipitation overnight at 4 ^{o}C. Beads were washed 3 times with lysis buffer and resuspended in 15 μl protein loading gel and denatured in 95^{o}C for 10 min. Protein were detected using by western blot (see below).

Western blot analysis

Western blot analysis was performed as described previously (25). Briefly, cells were harvested at respective timepoints in lysis buffer (1M Tris-Cl, 5 M NaCl, 10 % NP-40) containing with 1x phosphatase inhibitor (Roche, Switzerland) and 1x protease inhibitor (Roche) before quantification using Bio-Rad Bradford reagent. Protein lysates were resolved in Tris/Glycine gel (10 % or 12 % acrylamide, 1.5 M Tris (pH8.8), 10 % SDS, 10 % APS, TEMED) before transferred to PVDF membrane for detection of proteins (Supplementary Table 2 for information of antibodies used). ImageJ software was used for western blot densitometry analysis where required (28).

***In vitro* cell proliferation and migration assay**

0.0015 x 10^6 Caco2 or DLD1 cell clones were seeded into 96 well plate and allowed to adhere overnight before serum starvation to initiate cell cycle synchronisation between Caco2 DUSP6 OE, DLD1 DUSP6 KO and the respective control cells. Proliferation was carried out using Thiazolyl Blue Tetrazolium Bromide (Sigma) based on standard MTT assay as recommended by the manufacturer. MTT that were converted to formazan were solubilised in DMSO and measured at Abs 570 nm (Abs 690 nm background reference) and read using the BioTek plate reader (BioTeck, USA). For cell migration assay, cells were seeded into ibidi cell culture insert (ibidi, USA) forming a monolayer of cells. On the day of experiment, culture insert was removed before cells were treated with 10 μM of mitomycin-c (Sigma) for 2 hours before imaging to determine the "wound" size at Time 0. Imaging was done after 24 hours to determine percentage "wound" closure for quantification of cell migration.

Immunohistochemistry

Immunohistochemistry (IHC) staining was carried out as described previously (18). Briefly, 10 % neutral buffered formalin fixed, paraffin embedded tissues sections were deparaffinised in Histochoice clearing agent (Sigma, USA) before rehydration in reducing ethanol series. Antigen retrieval was carried out using Tris-EDTA buffer (pH9). Endogenous peroxidase was inhibited using Dako peroxidase blocker (Dako, Glostrup, Denmark) and tissue sections were blocked with 3 % BSA in PBS before addition of primary detection antibody. Target-specific primary and secondary antibodies used are summarised in Supplementary Methods, Table 1. Target peroxidase signal were detected with DAB substrate (Dako) before counter stained with haematoxylin and dehydration for mounting using Histomount mounting reagent (Invitrogen).

Gamma secretase activity assay

Endogenous gamma secretase activity was measured as previous described (19). Briefly, cells were harvested in hypotonic buffer (10 mM Tris-HCl (pH 7.4), 1mM EDTA, and 1mM EGTA). Before centrifugation at 14,000 g for 20 min. The pellets were

dissolved in 300 µl of a hypotonic buffer containing 0.2 % CHAPS (Sigma) at 4°C for 30 min. Protein concentration was measured using Bradford assay and 100 µg of γ-secretase containing protein supernatant was incubated with 20µM of florescence conjugated peptide substrate (565764-1MGCN, Calbiochem) at 37°C for 2 hours. The amount of substrate cleavage was determined by measuring the emitted fluorescence with an excitation wavelength of 355 nm and emission wavelength of 440 nm.

Detection of phosphorylated sites on Notch1 by mass spectrometry

Caco2 cells with or without DUSP6 overexpression were transfected with HA-NICD DNA plasmid overnight before treatment with 10 uM of proteasome inhibitor MG-132 for 16 hours. The overexpressed NICD were subsequently immunoprecipitated using Anti-HA magnetic beads (Sigma). The affinity purified samples were prepared for mass spectrometry through on-bead digestion. Briefly, beads were washed with PBS before resuspension in 8 M urea, 50 mM Tris, pH 8. Proteins were reduced with 10 mM TCEP for 30min, then alkylated with 55 mM CAA for 20 min in the dark. Proteins were then either digested by addition of 1 µg of LysC for 4 hours followed by 2 µg of trypsin for 16 hours, or by addition of 1 µg of chymotrypsin for 4 hours followed by 4 µg of chymotrypsin for 16 hours. Digested peptides were transferred to a new tube and acidified by addition of TFA, trifluoroacetic acid. Acidified peptides were desalted using an Oasis 10 mg HLB cartridge. HLB cartridge was activated with 100 % acetonitrile, and conditioned twice with 0.1 % formic acid in water. Peptides were then loaded and wash twice with 0.1 % formic acid in water. Elution was performed with 80 % ACN, acetonitrile, and 0.1 % formic acid in water. Eluted peptides were then split with 1 part for un-enriched mass spectrometry analysis and 4 parts for phospho-peptide enrichment before vacuum centrifugation. Phospho-peptide enrichment was performed using C8 stage-tips packed with titanium dioxide beads, TitanSphere TiO (GL Sciences, Japan). Dried peptides were resuspended in 40 % ACN with 6 % TFA. Peptides were loaded onto beads, then washed once with 10 % ACN with 6 % TFA, then 40 % ACN with 6 % TFA, then 65 % ACN with 6 % TFA successively. Phospho-peptides were eluted with 5 % ammonia, the 10 % ammonia with 25 % ACN, 10 % ammonia with 50 % ACN. Eluted phospho-peptides were pooled together and vacuum centrifuged.

Dried peptides and phospho-peptides were resuspended in water with 2 % ACN, 0.5 % acetic acid and 0.06 % TFA for phospho-site identification by mass spectrometry. Peptides were loaded on an Easy-Spray column (75 µm x 50 mm) with an EASY-nLC 1000 system coupled to a Fusion Lumos Mass Spectrometer (Thermo Scientific). Peptides were resolved at a flow rate of 300 nl/min, with mobile phase A (0.1 % formic acid in water) and mobile phase B (95 % acetonitrile in water with 0.1 % formic acid) with a gradient as follows: 2 % B for 2 min, 2-27 % B for 43 min, 27-50 % B for 15 min, 50-90 % B for 5 min, 90 % B for 5 min. Mass spectra were collected in Data-Dependent mode with cycle time of 3 seconds between master scans. MS1 scans were performed in the Orbitrap with 60K resolution, AGC target of 400,000 and maximum injection time of 100 ms. MS2 scans collected with the Ion Trap with normal scan rate, 35 % CID collision energy, AGC target of 15,000, and maximum injection time of 50 ms. MS raw files were searched using Mascot in Proteome Discoverer 2.4 against a human Uniport database (retrieved Jun 2019) with precursor mass tolerance of 10ppm and fragment mass tolerance of 0.06 Da, static modification for carbamidomethyl (C), and dynamic modifications for acetyl (Protein N-terminus), oxidation (M), deamidation (N,Q) and phosphorylation (S,T,Y) and a 1% false discovery rate using Percolator node.

Data availability

Raw mass spectrometry spectra and search data were uploaded to the jPost repository with the following accession numbers: JPST002116 (jPOST) and PXD041338 (ProteomeXchange).

Supplementary figure legends

Supplementary Figure 1. DUSP6 and cleaved Notch1 (NCID) expression in Caco2 and DLD1 parental cells at basal state. Representative western blot showing lower expression level of DUSP6 and cleaved in Caco2 compared to DLD1 CRC cell line.

Supplementary Figure 2. DUSP6 is a negative regulator of MAPKs. Western blot analysis showing changes in the level of MAPKs (ERK1/2, P38, JNK) upon treatment with 100 ng/ml EGF at early timepoints in (A) Caco2 cells overexpressing DUSP6 (DUSP6 OE) and (B) DLD1 DUSP6 knockout (DUSP6 KO) cells compared to the respective ppy-vector and sg-vector control cells. “OE” and “KO” denote overexpression and knockout respectively. Data shown is representative of three independent experiments.

Supplementary Figure 3: Loss of DUSP6 Caco2 resulted in increased activation of MAPKs, reduced expression of genes associated with cellular proliferation and reduced level of cleaved NOTCH1 after stimulation with EGF. Representative western blot showing changes in MAPKs activation, and level of COX2, c-MYC, cleaved NOTCH1 and key components of **γ**-secretase in Caco2 DUSP6 knockout (KO) cells after stimulation with 100 ng/ml EGF. Data shown is representative of two independent experiments.

Supplementary Figure 4: Loss of DUSP6 resulted in reduced development of Caco2 xenograft tumours *in vivo*.

(Left) Representative photographs of Caco2 xenograft tumours; (Right) Bar chart shows the weight of Caco2 xenograft tumours developed from Caco2 DUSP KO or sg-vector control cells. ***P < 0.001 (Non-parametric Mann-Whitney test). Error bars = standard deviations. Date shown is representative of two independent experiments.

Supplementary Figure 5: DUSP6 dephosphorylate NCID. (A) DUSP6 dephosphorylate endogenous NCID purified from Caco2 cells with or without EGF treatment. (B) Overexpression of DUSP6 resulted in reduced endogenous ubiquitinated NCID.

Figure S1

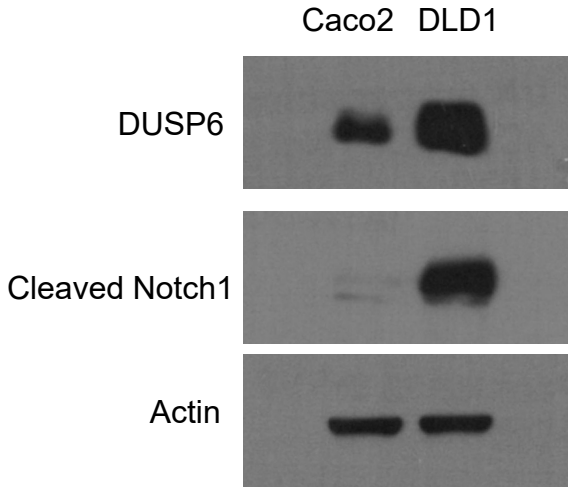
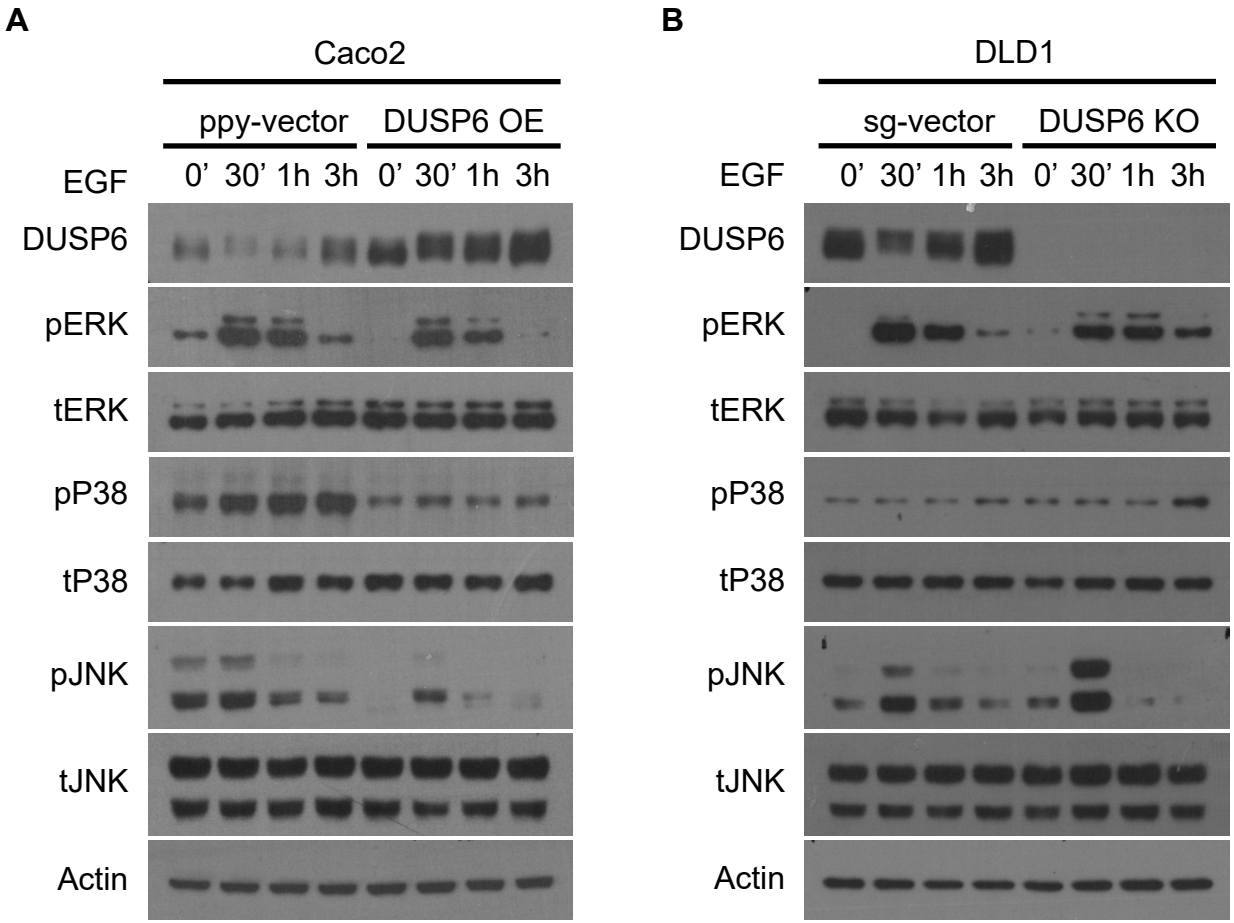


Figure S2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tP38
pJNK
tJNK
Actin
B
DLD1
sg-vector
DUSP6 KO
EGF
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pERK
tERK
pP38
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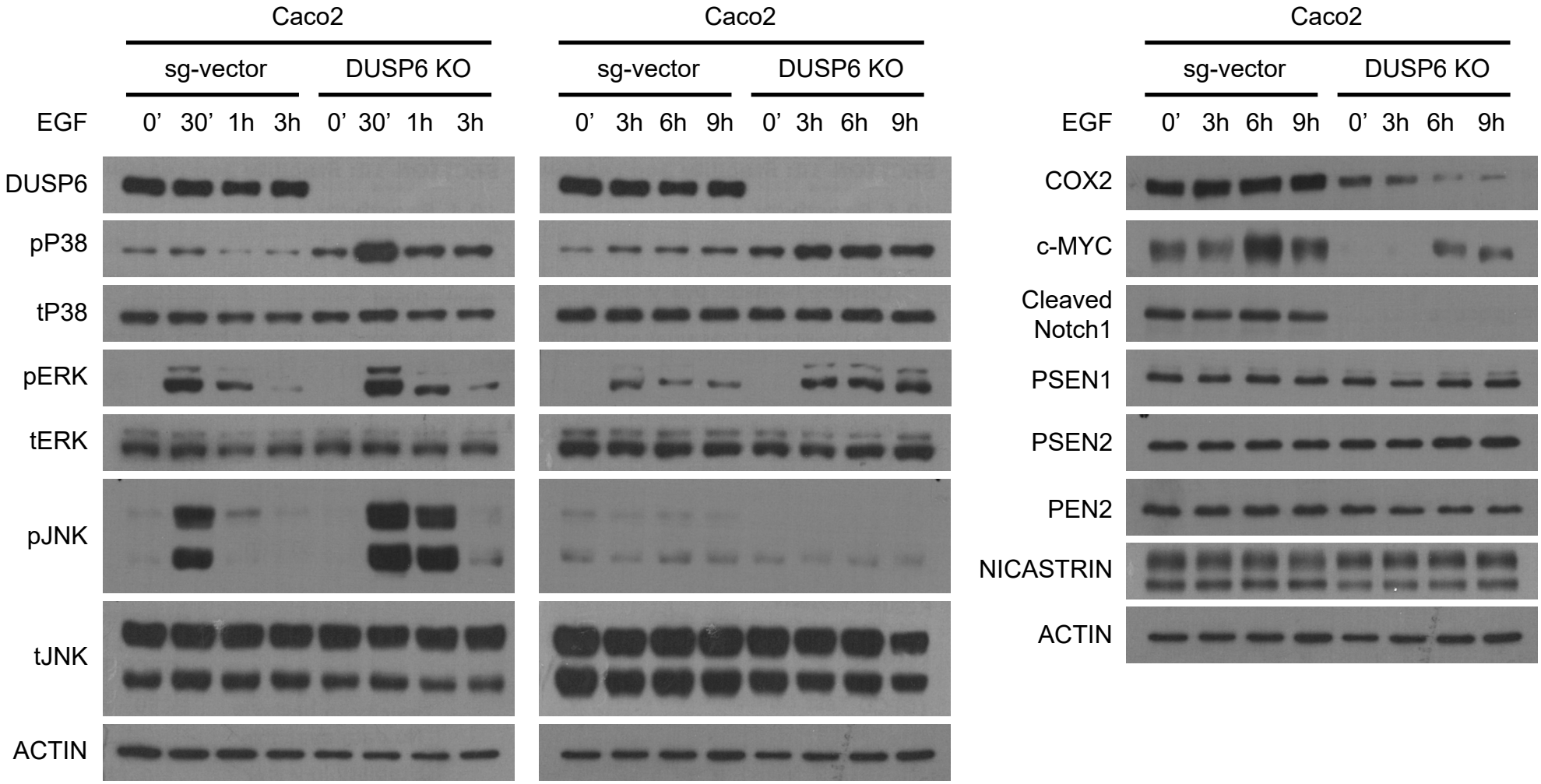
pJNK
tJNK
Actin

Figure S3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sg-vector
DUSP6 KO
EGF
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tJNK
ACTIN
Caco2
sg-vector
DUSP6 KO
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Caco2
sg-vector
DUSP6 KO
EGF
0’ 3h 6h 9h 0’ 3h 6h 9h
COX2
c-MYC
Cleaved Notch1
PSEN1
PSEN2
PEN2
NICASTRIN
ACTIN

Figure S4


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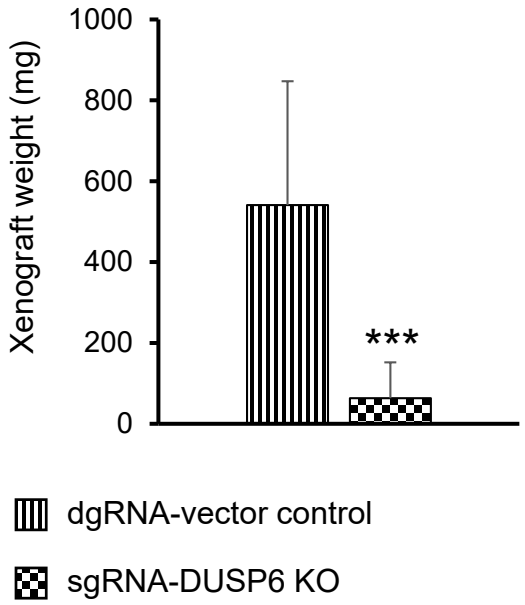
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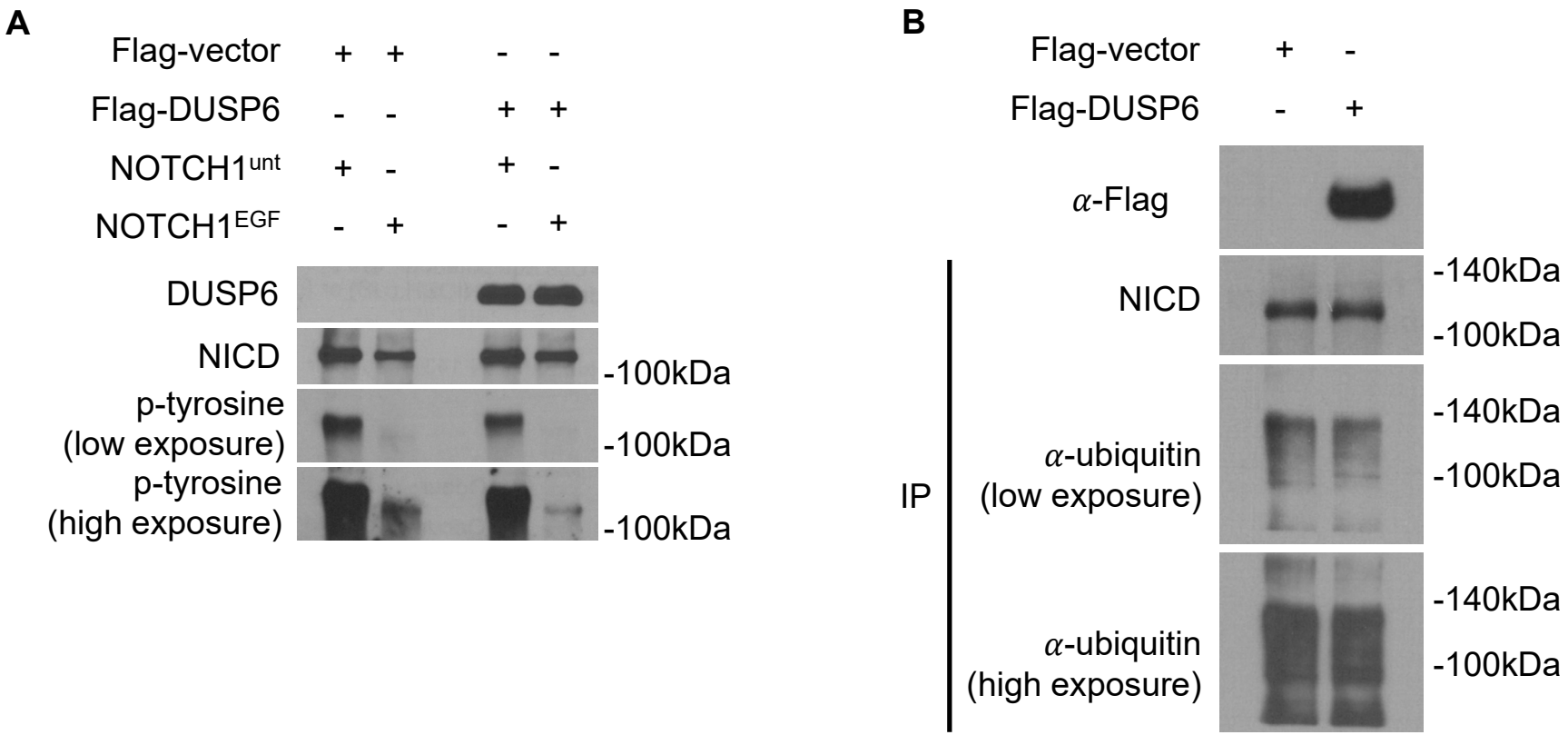
Figure S5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NOTCH1unt + - + -
NOTCH1EGF - + - +
DUSP6
NICD
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(low exposure)
-100kDa
p-tyrosine
(high exposure)
-100kDa
B
Flag-vector + -
Flag-DUSP6 - +
α-Flag
IP
NICD
-140kDa
-100kDa
α-ubiquitin
(low exposure)
-140kDa
-100kDa
α-ubiquitin
(high exposure)
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