SUPPLEMENTAL MATERIAL

table e-1. Clinical information on the investigated family

	Gender F=female M=male	Age at MRI-examination	NOTCH3^{A1604T} mutation	COL4A1^{A1599T} mutation	White matter lesion on MRI	Migraine	Comorbidities
Index patient	F	50	Yes	No	Frontotemporal WML; hypodensities of the white matter, most prominent in the frontal area	Yes, with aura	Hypertension; Mild polyneuropathy; Bilateral cataract; Neuropsychiatric disorder
Sister	F	51	Yes	Unknown	Frontotemporal WML; hypodensities of the white matter, most prominent in the frontal area	Yes, with aura	
Half-sister (same father)	F	58	Yes	Unknown	Single small frontal subcortical WML	Yes	Small infarct in the left SCA; Bipolar disorder
Father	M	-	Yes	Unknown	Not conducted	Unknown	Intracerebral bleeding
Sister	F	58	No	Yes	Confluent WMLs almost equally distributed in the frontal and parietal regions	Yes, with aura	Hypertension; Obstructive sleep apnea syndrome; Sleeping disturbance
Sister	F	54	No	Unknown	No WML revealed on MRI	Yes, with aura	Hypertension
Brother	M	55	No	Unknown	Small, nonconfluent WMLs bifrontally in the subcortical and deep white matter of microangiopathic type	No	Hypertension; Hyperlipedemia; Sleeping disturbance
Half-brother (same mother)	M	29	No	Unknown	Not conducted	Unknown	
Mother	F	77	No	Yes	WML of a microangiopahtic type	Yes, with aura	Hypertension; Sleeping disturbance; Mild polyneuropathy

table e-2. List of all PCR cloning primers used.

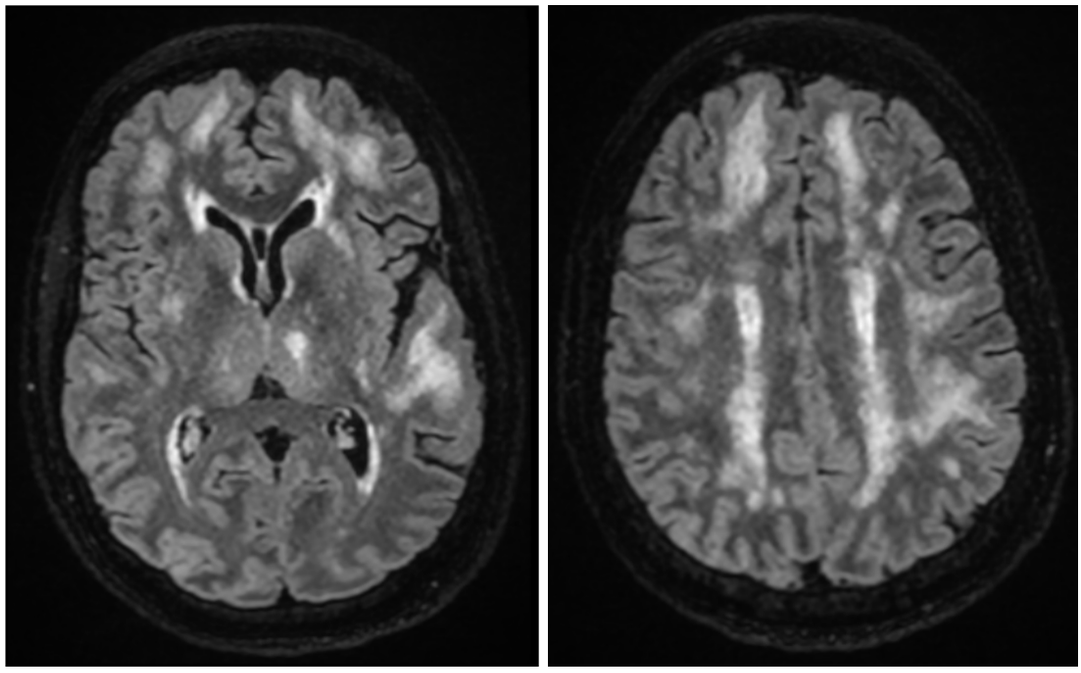
Mutagenesis Primers
5' - ACT GCT TCC CCG ATA CCC AGA GCG CC - 3' (forward)
5' - GGC GCT CTG GGT ATC GGG GAA GCAG T - 3' (reverse)
Ligation primers
5' - AGC GCG GGG ACC AGC GCT G - 3' (forward) for Enzyme AfeI
5' - CTG GTT CCC TGA GGG CTT CTC ACT - 3' (reverse) for Enzyme Bsu36I

table e-3. List of all antibodies used.

Primary antibody	Cat. No.	Company	Dilution	Application
anti-Notch1	3608	Cell Signaling	1:1000	Western blot
anti-Notch2	4530	Cell Signaling	1:1000	Western blot
anti-Notch3	2889	Cell Signaling	1:1000	Western blot
anti-Notch3	AB23426	Abcam	1:200	Immunofluorescence
anti-MYC	MA1-980	Life technologies	1:2000	Western blot
anti-b-Actin	A5441	Sigma-Aldrich	1:10000	Western blot
anti-Phalloidin 633	A22284	Thermo Fisher Scientific	1:100	Immunofluorescence
anti-Paxillin	05-417	Merck-Millipore	1:1000	Immunofluorescence
anti- PDI	AB3672	Abcam	1:100	Immunofluorescence
anti- Giantin	AB24586	Abcam	1:200	Immunofluorescence
anti-EEA1	610457	BDbioscience	1:200	Immunofluorescence
anti- RAB7	9367S	Cell Signaling	1:100	Immunofluorescence
DAPI	10 236 276 001	Roche	1:10000	Immunofluorescence
Secondary antibody	**Cat. No.**	**Company**	**Dilution**	**Application**
anti-mouse HRP	7076	Cell Signaling	1:5000	Western blot
anti- rabbit HRP	7074	Cell Signaling	1:5000	Western blot
IRDye® 680RD Donkey anti-Mouse IgG	926-68072	LICOR	1: 15000	Western blot
IRDye® 800CW Donkey anti-Rabbit IgG	926-32213	LICOR	1: 15000	Western blot
Alexa® Fluor 488	13832	Thermo Fisher Scientific	1:400	Immunofluorescence
Alexa® Fluor 546	13830	Thermo Fisher Scientific	1:400	Immunofluorescence

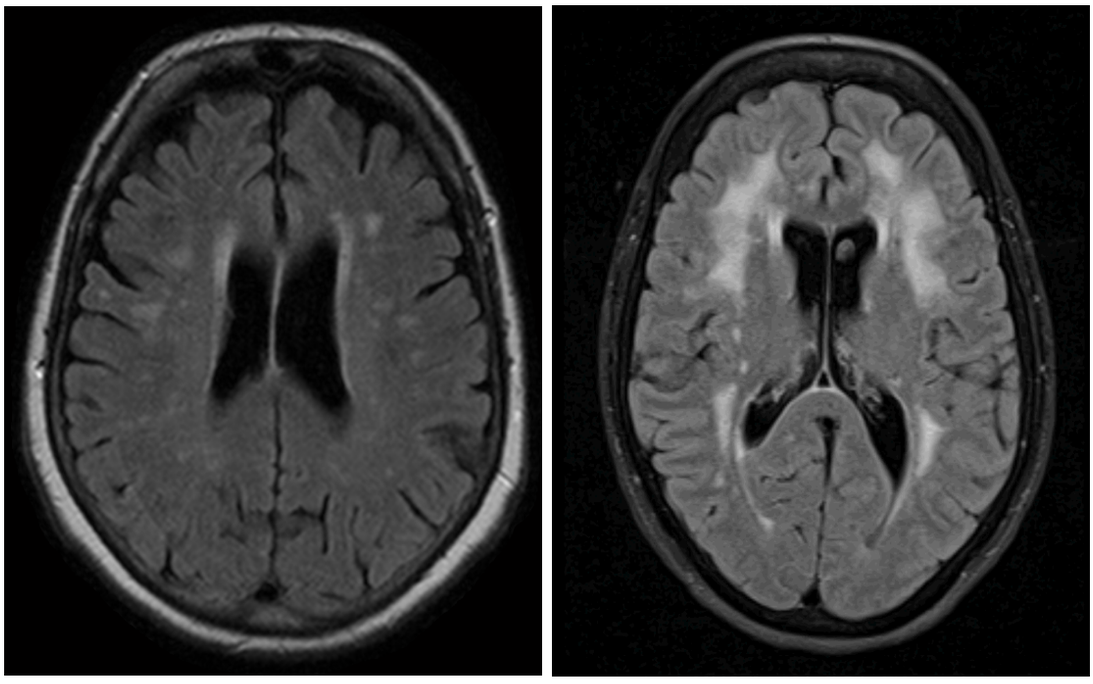
table e-4. List of all RT-qPCR primers used.

Primer pairs	Source
hNotch3	Baeten T.J and Lilly B., Journal of Biological Chemistry 2015; 290:16226–16237
5’ - GAG CCA ATG CCA ACT GAA GAG - 3’ (forward)	
5’ - GGC AGA TCA GGT CGG AGA TG - 3’ (reverse)	
GAPDH	Choi S.H. et al., PLoS One 2017; 12: e0185762
5´- CTC TGC TCC TCC TGT TCG AC - 3’ (forward)	
5’- ACG ACC AAA TCC GTT GAC - 3’ (reverse)	
hHES1	Braune E.B. et al., Stem Cell Reports 2016;6:643-651
5' - AGG CGG ACA TTC TGG AAA TG - 3' (forward)	
5' - TCG TTC ATG CAC TCG CTG A - 3' (reverse)	
hNRARP	
5' - TTC TCC CTC TCC CTC AAA TCC - 3' (forward)	
5' - AAC TGC AAA ACA AGC CGG TT - 3' (reverse)	
hHEY1	
5' - AAA AAG CCG AGA TCC TGC AGA - 3' (forward)	
5'- CCG AAA TCC CAA ACT CCG ATA - 3' (reverse)	
hHES5	Xu D. et al., Leukemia 2012; 26:1402–1405
5' - CCG GTG GTG GAG AAG ATG CG - 3' (forward)	
5' - GCG ACG AAG GCT TTG CTG TG - 3' (reverse)	



CADASIL patient
(NOTCH3$^{+/C1015R}$)

figure e-1. MRI scan images of a CADASIL patient (NOTCH3$^{+/C1015R}$). Large, confluent lesions in anterior temporal region, capsula externa as well as periventricular and deep white matter were observed. There were also lesions in basal ganglia and thalami.



The index patient's mother
(COL4A1$^{+/A1599T}$; NOTCH3$^{+/+}$)

The index patient's sister
(COL4A1$^{+/A1599T}$; NOTCH3$^{+/+}$)

figure e-2. MRI scan images of two family members carrying a COL4A1 ^{A1599T} mutation, but not the NOTCH3 ^{A1604T} mutation, show WML of a type distinct from that observed in the index patient. Axial 2D FLAIR of index patient's mother (left). Small WML with occasionally confluent morphology, predominantly in deep white matter and periventricular areas, with an almost equal fronto-parietal distribution, were observed. Axial 2D FLAIR of index patient's non mutation-carrier sister (right). Extensive, confluent WML bilaterally, predominantly in deep white matter in the frontal and to lesser extent parietal and temporal areas were observed. There was also periventricular "capping".

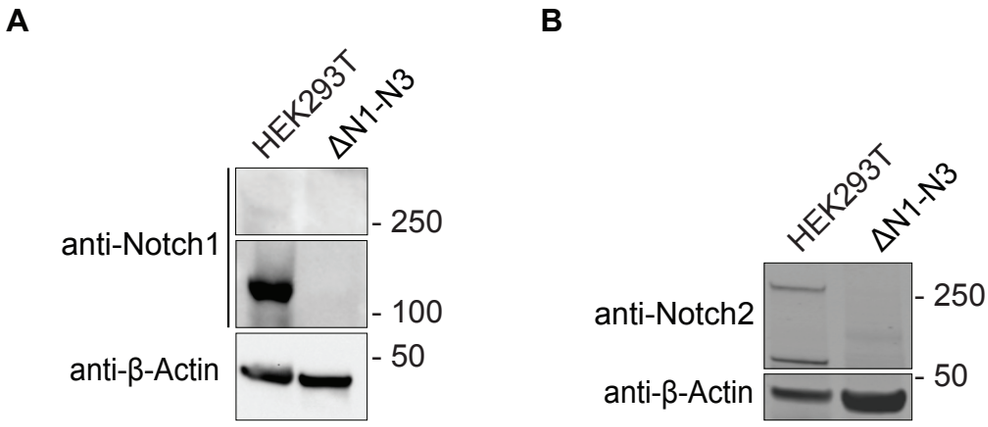


figure e-3. Western blot analysis of HEK293T cells and the 293T(ΔN1-N3) cell line (ΔN1-N3), in which both the A) NOTCH1 and B) NOTCH2 genes have been inactivated by CRISPR/Cas9.

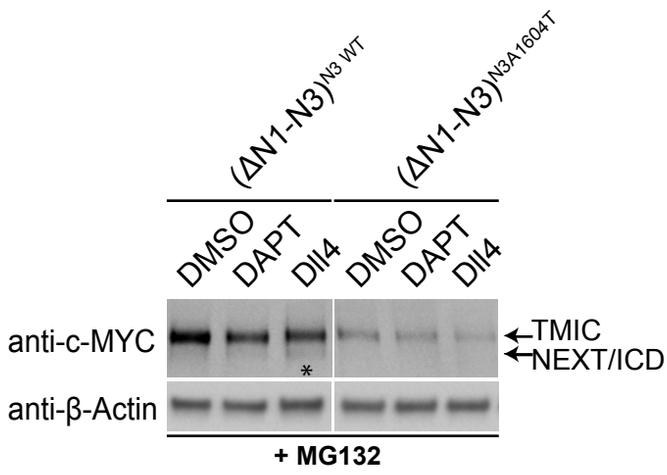


figure e-4. Western blot analysis of TMIC and NEXT/ICD fragments from the 293T(ΔN1-N3)^{N3wt} and 293T(ΔN1-N3)^{N3A1604T} cell lines, following activation by ligand (Delta-like 4) or blockade of S3 cleavage (DAPT), as indicated. MG132 was used as a proteasome inhibitor. β-Actin levels were used as loading controls and the positions of the TMIC and NEXT/ICD forms of the receptor are indicated with arrows.

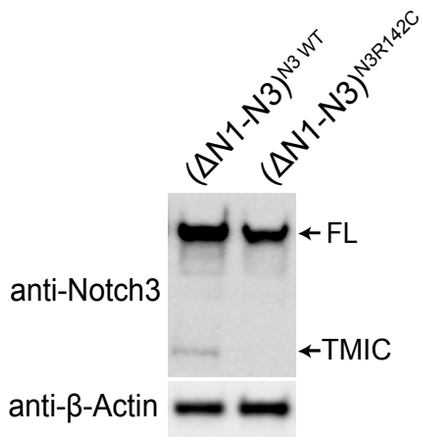


figure e-5 Transient transfection of a known Notch3 mutation (R142C) in 293T(ΔN1-N3) cell line.

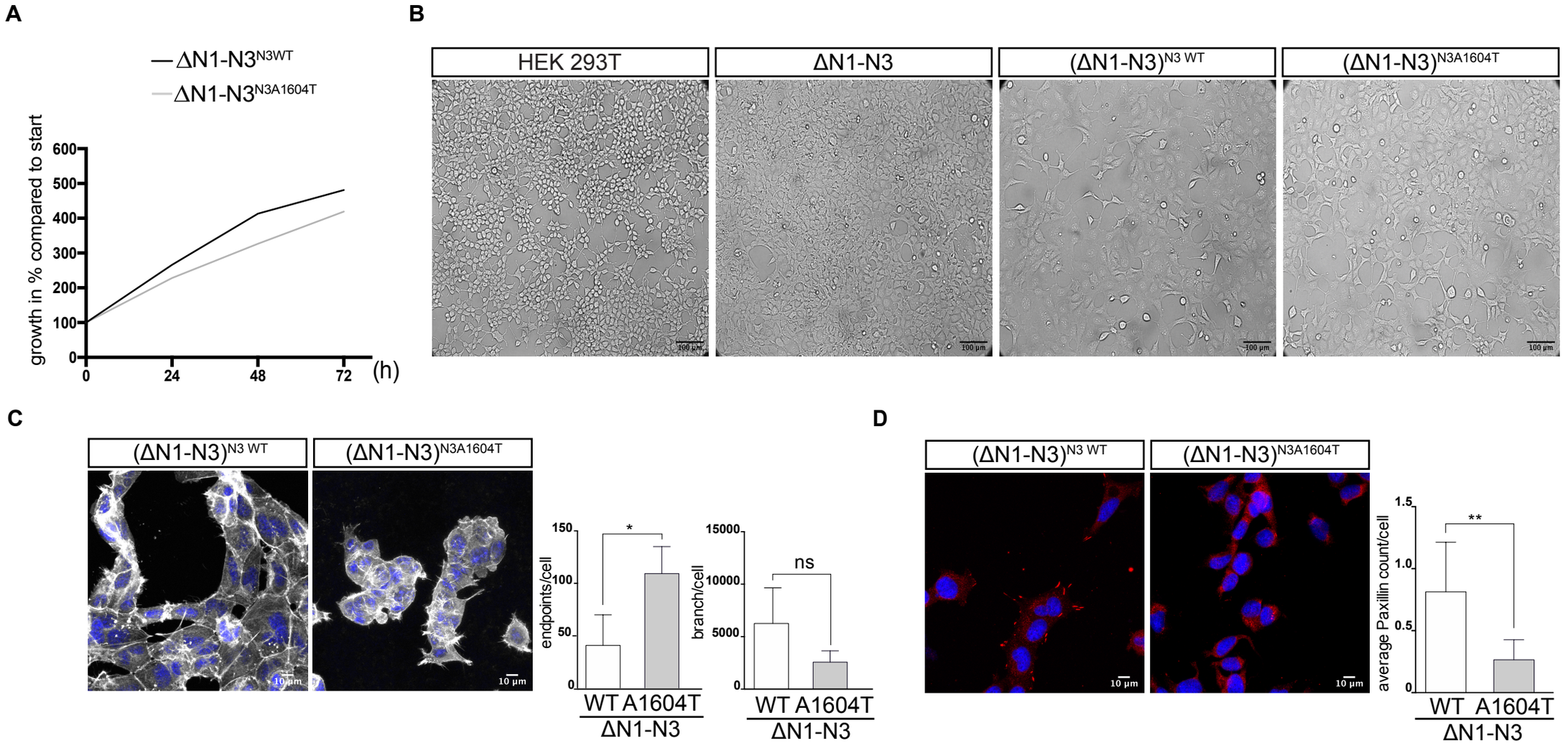


figure e-6. NOTCH3 A1604T-expressing cells show an altered actin cytoskeleton morphology with fewer focal adhesions. (A). Growth curves for the 293T((ΔN1-N3)^{N3wt} and 293T((ΔN1-N3)^{N3A1604T} cell lines. (B) Phase contrast images of cellular morphology. HEK293T cells, 293ΔN1-N3, 293ΔN1-N3^{N3wt} and 293ΔN1-N3^{N3A1604T} cells are indicated. size bar = 100 µm. (C). Staining for phalloidin in the 293T(ΔN1-N3)^{N3wt} and 293T(ΔN1-N3)^{N3A1604T} cell lines (left) and quantification of endpoints/cells and branch/cells from data in B (right). (D). Focal adhesion staining with an antibody for paxillin (red) and nuclear staining with DAPI (blue) to the left and quantification of paxillin counts per cell (right). Values are significant at *** P<0.001, **P<0.01 and *P<0.05. size bar = 10 µm.

NOTCH3 NRR A1604T.pdb

figure e-7. PDB file representing the 3D structures of the mutated NOTCH3 NRR domain (the wildtype NOTCH3 NRR domain is already deposited under the name 4ZLP).

appendix e-1

Supplemental Materials and Methods

Cell transfections

After starvation with Opti-MEM medium (Gibco) for two hours, HEK 293T(ΔN1-N3) cells were transfected with human NOTCH3^{WT} or human NOTCH3^{A1604T} constructs by using lipofectamine 3000 (Life Technologies) according to the manufacturer's protocol. DMEM with 10% FBS (Gibco), 1% antibiotic (Pen/-Step, Gibco) and 1% Glutamax (Gibco), was replaced six hours after trasfection. After selection with 600ug/ml Geneticin (Thermo Scientific), single cell clones were isolated and subjected to Western blot analysis. For the transient trasfection experiments, NOTCH3^{WT} and NOTCH3^{R142C} contructs were used. HEK 293T(ΔN1-N3) cells starved for two hours in Opti-MEM, then trasfected with lipofectamine 3000 according to the manifacture's protocol. After six hours, complete DMEM was replaced. After 24 hour of trasfection cells were seeded with or without DAPT on Jagged1 (or Delta like 4) /Fc coated plates and let grow for 24 hours at 37°C.

CRISPR/Cas9 genome editing

To generate the HEK 293T cell line in which NOTCH1, NOTCH2 and NOTCH3 were ablated (HEK 293T ΔN1-3), single guide RNAs (sgRNAs) targeting Notch1 (5'-GGTGAGACCTGCCTGAATGG-3'), Notch2 (5'- GGTGGAGCCTG-GAGTACAGG-3') and Notch3 (5'-GGCTTCCGTCCAGGCAAGGG-3') were cloned into the guide RNA Cas9 vector (Addgene pX459). HEK293T cells were transfected with the gRNA vector, and puromycin dihydrochloride (Sigma-Aldrich) at 1μg/ml was used for selection. Single cell colonies were isolated and subjected to Western blot as previously described [1].

Western blot

HEK 293T(ΔN1-N3)WT and HEK 293T(ΔN1-N3)A1604T cells were cultured in 24 well plates. Western blot was performed as previously described [1], by using Trans-Blot Turbo nitrocellulose membrane transfer pack (Biorad) for protein transfer. For the analysis of the targeting of the NOTCH2 gene, western blot analysis was conducted by using 4-12% Bis-Tris gel (Invitrogen) and wet transfer. The antibody signal was captured by using the LICOR system (LI-COR Biosciences). All antibodies used are listed in table e-3 in the online-only Data Supplement.

Quantification and statistical analysis

All statistical analyses were performed by GraphPad Prism (Ver. 6). Western blot quantification of Figures 3A, 3B, 4B, 4C, 4D was performed with ImageJ software (Ver. 2.0.0). Quantification is based on a minimum of three independent experiments and compared using the unpaired t-test. Where not specified, protein expression levels were normalized with β-Actin. For the RT q-PR (Figure 3C) at least three independent experiments have been performed. The relative transcription levels were calculated using the -ΔΔCT method and then analyzed using the unpaired t-test. For the surface biotinylation (Figure 4D), quantification was performed by taking into account the percentage of loaded protein into the gel: 1/200 for the input fraction and 1/6 for the IP fraction of the whole cell lysate. For the cell growth assay (figure e-6A), three independent experiments were performed. The cell count readout (cells/ml) was compared using the unpaired t-test. For actin cytoskeleton and focal adhesion quantification (figure e-6C, D), signals of at least five randomly chosen images from two biological replicates of each clone were quantified as previously described[2,3] and compared by using the unpaired t-test.

e-references

1. Braune E-B, Tsoi YL, Phoon YP, Landor S, Silva Cascales H, Ramsköld D, et al. Loss of CSL Unlocks a Hypoxic Response and Enhanced Tumor Growth Potential in Breast Cancer Cells. Stem Cell Reports [Internet]. 2016;6:1–9. Available from: http://linkinghub.elsevier.com/retrieve/pii/S2213671116000850

2. Young K, Morrison H. Quantifying microglia morphology from photomicrographs of immunohistochemistry prepared tissue using imagej. J. Vis. Exp. 2018;136:e57648.

3. Horzum U, Ozdil B, Pesen-Okvur D. Step-by-step quantitative analysis of focal adhesions. MethodsX. 2014;1:56–59.