Supporting information for Karlström *et al.* (2002) *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.252624099

Supporting Text

DNA Constructs and Cloning. The full-length Notch 3 cDNA was obtained by a subcloning and RT-PCR amplification approach and cloned into the pcDNA3.1 plasmid (Invitrogen), generating the pcDNA-N3 vector. The introduction of silent mutations into the mNotch 3 cDNA generated an *Asc*I site immediately 3' of the sequence encoding the site 3 (S3) cleavage site. The Gal4VP16 (GVP)-encoding cDNA was PCR amplified and cloned into the *Asc*I site of pcDNA3-N3, generating pcDNA3-N3GVP (referred to as Notch 3-GVP). To introduce the Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) R142C mutation (CGA→TGT), a 1-kb *Eco*RI/*Cla*I fragment of the 5' end of the pcDNA-N3 construct was subcloned into pBluescript. The codon change was then made on the subcloned fragment by using the QuickChange mutagenesis kit (Stratagene) by using the oligonucleotides: 5'-GGC**T**G**T**TTTGCCTGTGCCTGC-3' and 5'-AAAA**C**A**G**CCATCCGGCCCCAC-3' (mutated nucleotides in bold). The mutated cDNA was subcloned back into the pcDNA3-N3 and pcDNA3-N3GVP backbones, generating the Notch 3^{R142C} and Notch 3^{R142C}-GVP constructs.

N3ICGVP cDNA was made from the Notch 3-GVP construct and subcloned into the pCMX vector. The ΔEN3-GVP construct, UAS-luc, and CMV-βgal plasmids have been reported elsewhere (1, 2).

Western Blot Analysis. Whole-cell lysates of the stable 293 cell lines were lysed in RIPA buffer (20 mM Tris, pH 7.5/2 mM EDTA/150 mM NaCl/1% NP-40/1% deoxycholate/0.1% SDS) containing Complete Protease Inhibitor (Roche Diagnostics). The lysates were sonicated 2× 5 sec and then centrifuged to remove cell debris. Approximately 30 μg of protein was separated on precast 7.5% SDS/polyacrylamide gels (Bio-Rad) and subsequently transferred to nitrocellulose membranes. Filters were blocked in 5% milk in 0.05% Tween-20 in PBS (PBS-T) for 1 h and then incubated with the primary antibody 5E1 (1:500) raised against the extracellular (EC) domain of Notch 3. After several washes, the filters were incubated with a horseradish peroxidase-conjugated goat–anti-mouse antibody (1:2,000, DAKO). Immunoreactive bands were finally visualized by chemiluminescence by using the Super signal kit (Pierce).

Immunocytochemistry. Stable 293 transfectants were plated on glass coverslips at a density of 10,000 cells per well. The cells were fixed in 3% paraformaldehyde solution and incubated with primary antibodies in immunosolution (PBS containing 5% BSA, 10% goat serum, and 0.3% Triton X-100) at room temperature for 1 h. The coverslips were washed three times in PBS and 4',6-diamidino-2-phenylindole (1:1,000), and the appropriate fluorescent secondary antibody was added in immunosolution followed by incubation at room temperature for 45 min. The coverslips were finally analyzed in a Nikon fluorescence microscope.

1. Taniguchi, Y., Karlström, H., Lundkvist, J., Mizutani, T., Otaka, A., Vestling, M., Bernstein, A., Donoviel, D., Lendahl, U. & Honjo, T. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 4014–4019.

2. Beatus, P., Lundkvist, J., Oberg, C. & Lendahl, U. (1999) *Development (Cambridge, U.K.)* **126,** 3925–3935.