SUPPLEMENTAL FIGURES

Supplemental Figure 1: A) Schematic overview of Notch1 constructs used in this study and B) location of different carboxy-terminal epitope tags.

Supplemental Figure 2: Developmental gain of function Notch1 mutants are cleaved at Val1711. A) Lysates from HEK293 cells transfected with LNR 6Myc constructs with the indicated anti-neurogenic gain of function mutations. B) S3 cleavage assays with Notch1 LNR-GV16 fusions constructs in NIH-3T3 cells. Whereas wild type LNR proteins are only weakly active LNR mutant Notch proteins are highly active and cleaved at Val1744 and Val1711. Note the AV>VH is an activating mutation, but cannot be recognized by the Val1711 antibody.

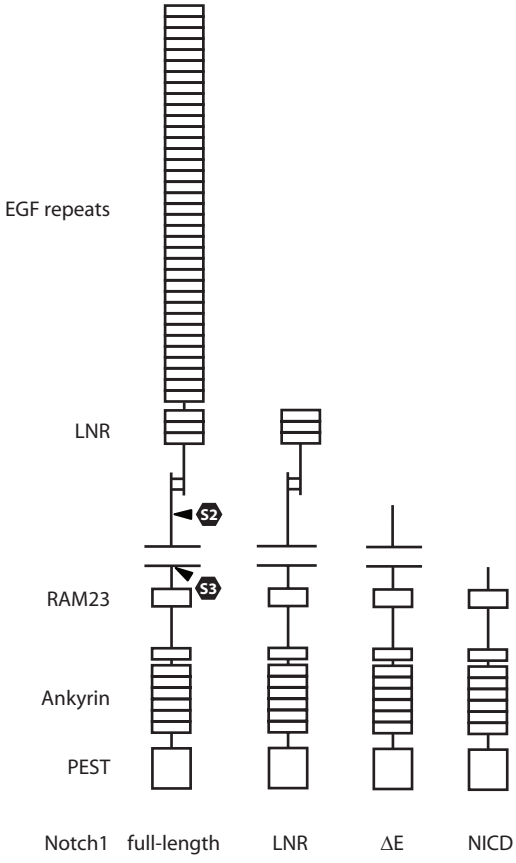
Supplemental Figure 3: NECD or NICD do not influence cleavage at Val1711. A) HEK293 cells transfected with Notch1 ΔE 6Myc molecules. Wild type ΔE is efficiently cleaved at Val1711 and Val1744, the ΔE AV>VH mutant shows comparable cleavage at Val1744. Val1711 cleavage cannot be detected in this mutant. B) Immunoblot showing that HEK293 transfected Notch1 Δice 6Myc molecules, lacking the extracellular as well as the intracellular domain, are very efficiently cleaved at Val1711 and Val1744. C) Notch-CSL transcription reporter assay in transfected HeLa cells showing equal activity of wild type ΔE and ΔE AV>VH mutant, representative of at least two independent experiments in triplicate.

Supplemental Figure 4: S2 cleavage site mutants are cleaved at a different position. Immunoblot showing that a half-site mutant expressed in HEK293 cells AV>EV, still harboring the Val1711 epitope, is not cleaved at Val1711. Both S2 site mutants show Val1744-NICD cleavage and are gain of function mutants. Arrow indicates the S2 cleavage position in the Notch1 extracellular domain.

Supplemental Figure 5: ADAMs 9, 12 and 15 are not involved in Val1711 cleavage. A) *Adam9/12/15* triple knock-out (TKO) cells were transduced with Jagged1 retrovirus and cell lysates were analyzed by immunoblotting. Jagged1 efficiently induces Notch1 receptor cleavage at Val1711 and Val1744 in *Adam9/12/15* TKO cells. B) Lysates from OP9 wild type or ligand expressing cells co-cultured with *Adam9/12/15* TKO cells. Ligand efficiently induces Notch1 receptor cleavage at Val1711 and Val1744 in the combined absence of ADAMs 9, 12 and 15.

Supplemental Figure 6: Non-metalloprotease inhibitors do not inhibit S2 cleavage at Val1711. Lysates from HEK293 cells transfected with LNR L1594P 6Myc cultured in the presence of broad-spectrum serine, cysteine or aspartyl protease inhibitors. Only the broad spectrum MPi GM6001 can block Val1711 cleavage.

S1A


EGF repeats
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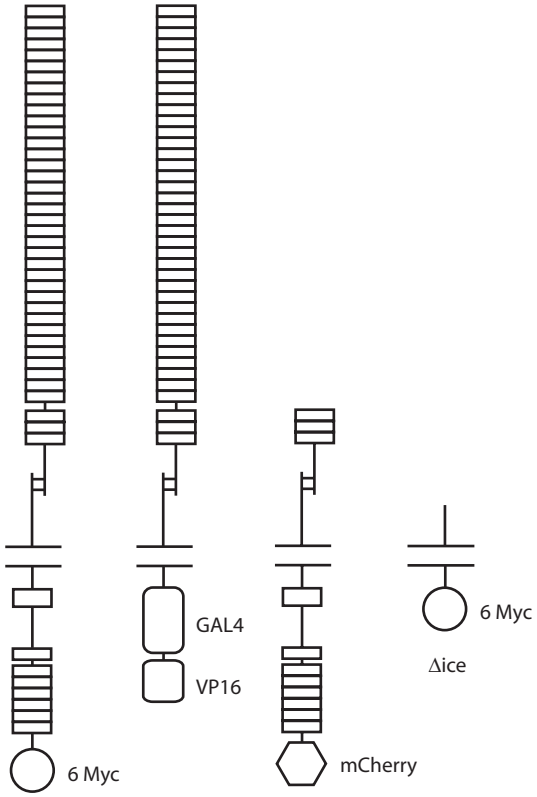
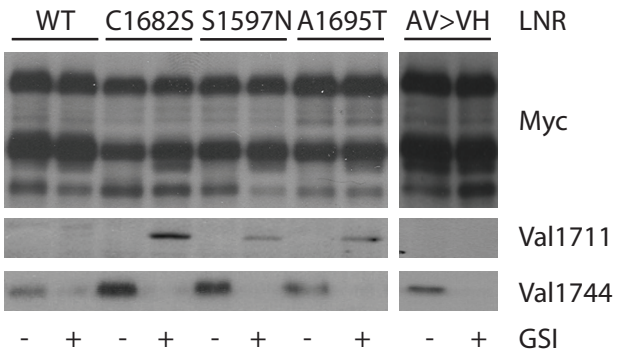
S2
S3
RAM23
Ankyrin
PEST
Notch1
full-length
LNR
ΔE
NICD

S1B


6 Myc
GAL4
VP16
mCherry
6 Myc
Δice

S2A


WT
C1682S
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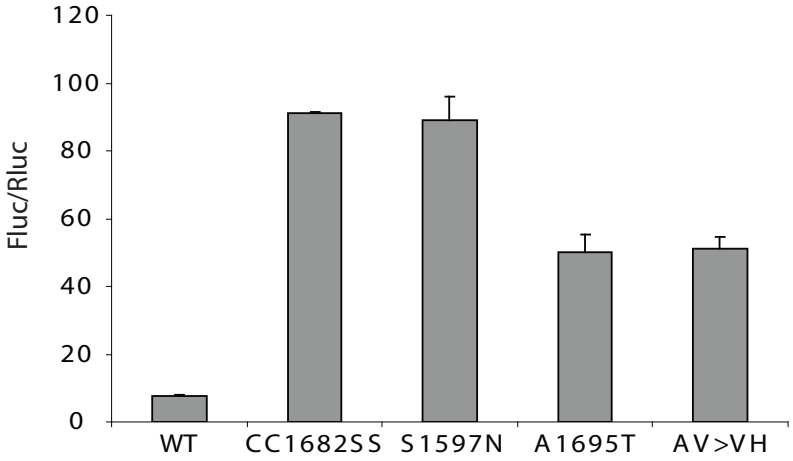
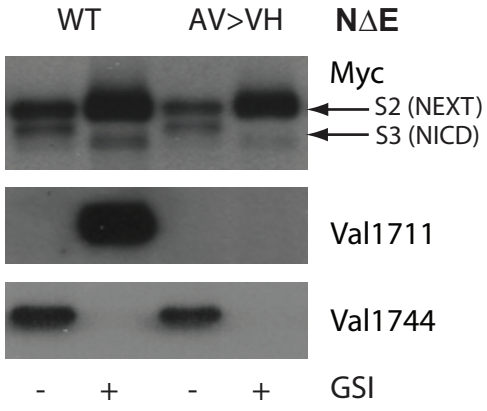
A1695T
AV>VH
LNR
Myc
Val1711
Val1744
- + - + - + - + - +
GSI

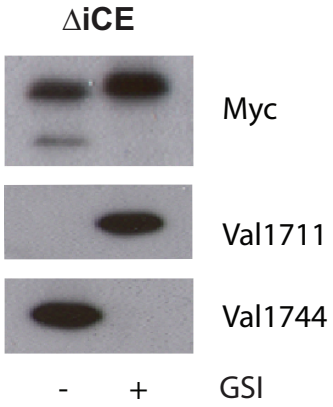
S2B


120
100
80
60
40
20
0
Fluc/Rluc
WT
CC1682SS
S1597N
A1695T
AV>VH

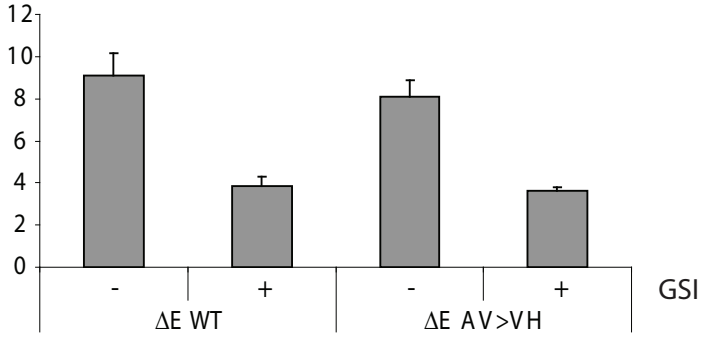
S3A


WT
AV>VH
NΔE
Myc
S2 (NEXT)
S3 (NICD)
Val1711
Val1744
- + - +
GSI

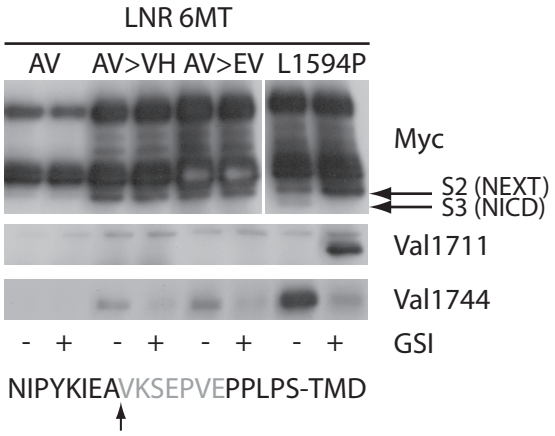
S3B


ΔiCE
Myc
Val1711
Val1744
- +
GSI

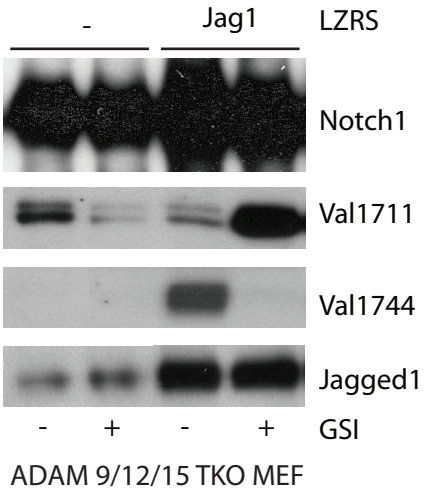
S3C


12
10
8
6
4
2
0
- + - +
GSI
ΔE WT
ΔE AV>VH

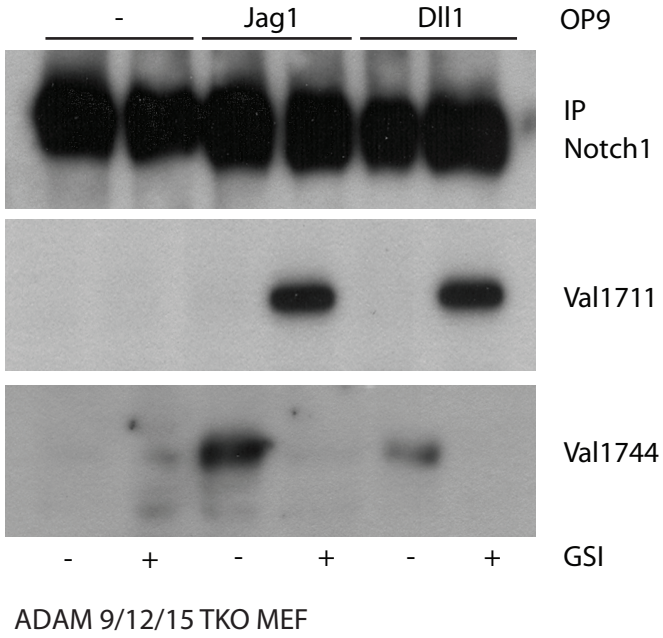
S4

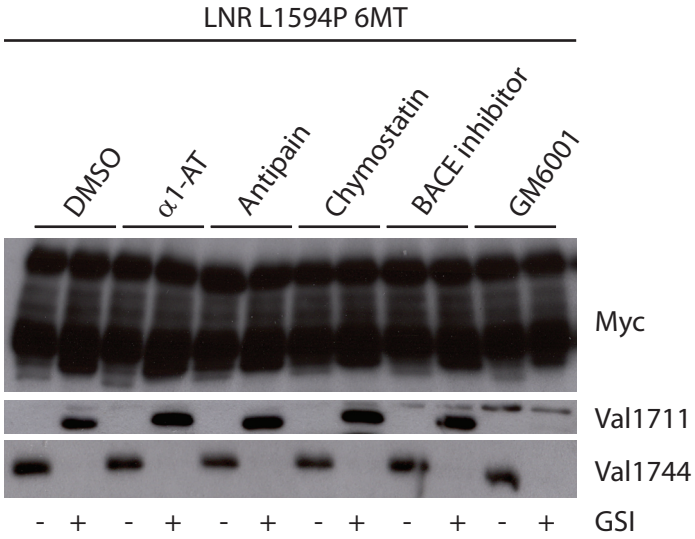

LNR 6MT
AV
AV>VH
AV>EV
L1594P
Myc
S2 (NEXT)
S3 (NICD)
Val1711
Val1744
- + - + - + - +
GSI
NIPYKIEAVKSEPVEPPLPS-TMD

S5A


-
Jag1
LZRS
Notch1
Val1711
Val1744
Jagged1
- + - +
GSI
ADAM 9/12/15 TKO MEF

S5B


-
Jag1
Dll1
OP9
IP
Notch1
Val1711
Val1744
- + - + - +
GSI
ADAM 9/12/15 TKO MEF


LNR L1594P 6MT
DMSO
α1-AT
Antipain
Chymostatin
BACE inhibitor
GM6001
Myc
Val1711
Val1744
- + - + - + - + - + - + GSI