Ectodomain shedding and intramembrane cleavage of mammalian Notch proteins is not regulated through oligomerization

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Summary

Intramembrane cleaving proteases (I-CLiPs) such as S2P, γ-secretase and SPP hydrolyze peptide bonds within the transmembrane domain (TMD) of signaling molecules such as SREBP, Notch and HLA-E, respectively. All three enzymes require a prior cleavage at the juxtamembrane region by another protease. It has been proposed that removing the extracellular domain allows dissociation of substrate TMD, held together by the extracellular domain or loop. Using γ-secretase as a model I-CLiP and Notch as a model substrate we investigated whether activating and inactivating mutations in Notch modulate γ-secretase cleavage through changes in oligomerization. We find that while the Notch EGF repeats can promote dimer formation, most surface Notch molecules in mammalian cells are monomeric as are constitutively active or inactive Notch1 proteins. Using a bacterial assay for TM dimerization, we find that the isolated TMD of Notch and APP self-associate and that mutations affecting Notch cleavage by γ-secretase cleavage do not alter TMD dimerization. Our results indicate that ligand-induced reversal of controlled TMD dimerization by the Notch extracellular domain is unlikely to underlie the regulatory mechanism of intramembranous cleavage.
Introduction

Notch signaling is a highly conserved signaling pathway that mediates cell fate decisions during multiple stages of development in multicellular eukaryotes; in the adult, Notch signaling is involved in several diseases including cancer, stroke and muscular dystrophy (for a review see (1,2)). Notch receptors are single pass type I transmembrane proteins characterized by a large extracellular ligand binding domain containing 11-36 EGF repeats, three Ca$^{2+}$ binding Lin12-Notch repeats (LNR) that maintain the receptor in an inactive state, and a single predicted TMD (3-5). Mammalian cells have four different Notch receptors and up to six different ligands, five of which are membrane-tethered type I proteins that belong to the DSL family (Delta/Serrate/Jagged)/Lag-2. Notch ligands are also type I proteins containing extracellular EGF repeats and a conserved DSL domain that mediates receptor binding. Binding of DSL ligands to EGF repeats 11-12 leads to Notch activation (6) via a poorly understood mechanism requiring ubiquitination-induced ligand endocytosis (reviewed in (7)).

Ligand binding to Notch receptors triggers juxtamembrane cleavage by ADAM metalloproteases in the extracellular domain of Notch to produce NEXT (Notch Extra Cellular Truncation (8). Shedding of the Notch extracellular domain is followed by intramembranous cleavage of Notch by $\gamma$-secretase, a novel multi-protein complex containing four transmembrane proteins: the aspartyl protease Presenilin (PS; containing the catalytic residues), Pen 2, Aph-1 and Nicastrin (9). Consequently, the Notch Intracellular Domain (NICD) is released from the membrane and translocates to the nucleus where its association with the transcription factor RBPjk leads to activation of target genes (reviewed in (10,11)). Other $\gamma$-secretase substrates include the receptor tyrosine kinase ErbB4, and the cell adhesion molecules CD44, E-Cadherin and Nectin (reviewed in (12,13)). $\gamma$-secretase is best known for its involvement in the intramembranous cleavage of the Amyloid Precursor Protein (APP) that generates the neurotoxic amyloid $\beta$ (A$\beta$) peptides that form aggregates associated with senile plaques in Alzheimer’s Disease. Given that mutations in PS lead to increased production of
amyloidogenic peptides and cause early onset Familial Alzheimer’s Disease, \( \gamma \)-secretase inhibitors could be useful therapeutic tools in the treatment of this disease (reviewed in (14)). Therefore, further insight into the regulation of Notch proteolysis and \( \gamma \)-secretase activity is of great importance in understanding both development and disease.

Like \( \gamma \)-secretase, other intramembranous cleaving proteases (I-CLiPs) are polytopic membrane-spanning proteases that cleave their substrates within the lipid bilayer at one or more sites within their transmembrane domains (TMDs) (15-17). Current models suggest that for iCLiP-mediated proteolysis to occur, a preceding Juxtamembrane Proteolytic step (JP) is necessary. This is thought to permit dissociation of substrate TMD (reviewed (16,18)). The prototype I-CLiP, S2P (site-2 protease) is a metalloprotease involved in the release of sterol regulatory element binding protein (SREBP) from the Golgi as part of a cholesterol/fatty acid sensing system in the cell. The substrate, SREBP, has two TMDs connected by a short loop; after SREBP is transported by the sterol sensing protein SCAP to the Golgi, the loop is cleaved by S1P (site-1 protease) and S2P cleavage of a single SREBP TMD follows. Another I-CLiP, related to the aspartic peptidase PS/\( \gamma \)-secretase is signal peptide peptidase (SPP), which catalyzes cleavage of signal peptides from type II membrane proteins. SPP substrates have two lipid-embedded domains that are separated after cleavage of the precursor protein in the secretory pathway by signal peptidase (SP) (19). The single TMDs of \( \gamma \)-secretase substrates were proposed to exist as dimers prior to ectodomain shedding (see below; (20)) which has been demonstrated for most presenilin substrates including Notch, APP, ErB4, CD44 and E-Cadherin (12). A fourth and distinct class of I-CLiP’s are Rhomboids, a large family of serine proteases (21) that, in contrast to all other known I-CLiP’s, do not require a preceding proteolytic release of an extracellular domain (17); their substrates are thought to be monomeric.

At present, the oligomerization status of surface Notch molecules is unknown. The role of oligomerization on Notch function has been addressed by indirect genetic and biochemical studies but no clear conclusions have emerged. Genetic complementation of abruptex alleles (mutations in EGF repeats 24-29) in Drosophila lead to the hypothesis that Notch was a dimer (22,23) and genetic evidence in
Caenorhabditis elegans raised the possibility that ligand drives Notch dimerization (24). The Notch intracellular domain contains seven ankyrin repeats, of which repeat 2-7 assume an ankyrin fold (11) and was shown to dimerize in yeast two-hybrid assays (25). However, structural and biochemical analyses using the purified ANK domain have shown unequivocally that the Notch intracellular domain is monomeric (11,26).

Why juxtamembrane cleavage is a prerequisite for intramembrane cleavage is unclear, as is the mechanism by which these proteases recognize and cleave scissile bonds within the lipid bilayer. Activating mutations in Notch (Drosophila), Glp-1 and Lin-12 (C. elegans) indicate that the extracellular juxtamembrane region of Notch functions to inhibit receptor activation (4,27-30). It has been proposed that juxtamembrane cleavage of Notch, and by inference of other I-CLiP substrates, is needed because in the absence of ligand the extracellular domain imposes a conformational state protecting scissile bonds from hydrolysis (3,20,29,31). This model of I-CLiP regulation emerged from elegant in vivo reporter assays in Drosophila utilizing a transcription factor (TMD-gal4VP16) that depended on γ-secretase mediated cleavage of its TMD to activate an UAS-LacZ reporter (20). These studies demonstrated that LacZ activation was attenuated in transgenic flies expressing gal4VP16 proteins containing the Glycophorin-A (GpA) TMD; this domain forms a stable dimer within the lipid bilayer (32). Furthermore, engineered dimerization of the Notch TMD in TMD-gal4VP16 using a heterologous, extracellular leucine zipper also impaired reporter gene activation (20). In contrast, engineering mutations in the GpA TMD or in the extracellular leucine zipper forcing them to adopt a monomeric conformation resulted in LacZ activation that was comparable to that seen with TMD-gal4VP16 containing a wild type Notch TMD. These findings supported a model in which in the absence of ligand, oligomerization, mediated by the extracellular domain, prevents ectodomain shedding and maintains the TMD domain in a dimeric ‘inaccessible’ conformation. This model predicts that constitutively active Notch proteins are monomers whereas intact or inactive proteins are dimers (20); it also predicts that TMD lacking the extracellular domain will not self associate. While the behavior of chimeric Notch proteins supported this interpretation (29), no direct measurement of oligomerization state or its effect on proteolysis have been performed.
To test the hypothesis that oligomerization status functionally influences Notch processing, we investigated the oligomerization state of the Notch proteins that are direct substrates for γ-secretase and compared them to others that require ligand binding in order to become substrates. We find that in contrast to the predictions of the current model, all Notch1 proteins exist predominantly as monomers regardless of their ability to act as efficient γ-secretase substrates. We identified the Notch 1 TMD as the minimal substrate sufficient for PS dependent cleavage, indicating that the intracellular domain does not contribute to the interaction with γ-secretase in the cell. In order to evaluate the association tendencies of the Notch TMD and to determine if cleavage-impairing TMD mutations altered the oligomerization state of Notch TMD, we used the established bacterial TOX-CAT system (33). We discovered that the Notch and APP TMDs are dimers irrespective of their ability to act as an effective substrate in mammalian cells. Together, our results indicate that controlled dimerization of TMD by the Notch extracellular domain and its reversal by ligand binding is unlikely to underlie the regulatory mechanism of ectodomain shedding and hence intramembranous cleavage. Instead, we favor induced conformational change as the regulatory mechanism.

Experimental procedures

Plasmids and vectors

All Notch plasmids were initially cloned into pCS2+6MT as described (34). Notch1∆E contained a TMD substitution (M1726V) facilitating biochemical analysis (34). Site-directed mutagenesis was performed using the Quickchange kit according to manufacturer’s instructions (Stratagene). A construct containing 4xCSL synthetic binding sites in tandem was used for Notch transcription assays (35). Notch Renilla fusions were made by PCR-directed cloning and removal of the 6MT (6Myc tag) for replacement with the Renilla Luciformis gene from pCMV-RL (Promega). mNotch2 Full length-flag (36) and RBPjk-flag (37) have been described elsewhere. mPtc1-RL has been described(38).
The bacterial expression vectors pccKAN and its derivatives containing GpA wild type and G83I mutant TMDs have been described (33). TMD domain sequences of murine N1 (Q1723- S1747), mN2 (L1679- A1700), mN3 (L1645-A1666), mN4 (L1446-I1467), hAPP (G342-L365) and mFGFR1(I378-K398) sequences were synthesized with Nhel and Sau3A1 compatible ends annealed and cloned into Nhel/BamH1 digested pccKAN (33).

Cell culture and transfection
HEK293 and PS1/2dKO cells (39) were grown and maintained in DMEM with 10 % FBS. NIH 3T3 cells were grown and maintained in DMEM with 10 % BCS. Cells were transfected by calcium phosphate in BBS (HEK293 and NIH3T3). PS1/2dKO cells were transfected using Fugene-6 (Roche).

CSL-reporter assays
NIH 3T3 cells or PS1/2dKO cells (12 well plates) were transfected with 1 µg total DNA containing 100 ng or 400 ng (PS1/2dKO) tester DNA, 200 ng 4xCSL-Luc, 20 ng cmv-βgal and carrier DNA for 12-16 hrs, re-fed and analyzed 40-48 hrs after transfection as described (37). Luciferase assays were also performed with Notch-RL constructs using the DualGlow or the Renilla Luciferase assay system (Promega). Transfections were normalized using a β-gal reporter assay system (Tropix).

Western Blot and Immunoprecipitation analysis
Whole cell lysates were prepared by directly adding Laemmli SDS sample buffer (+10 mM DTT) to PBS washed cells. For bacterial lysates freshly inoculated o/n cultures were normalized for OD (OD595), grown for 2 1/2 hrs at 37°C, centrifuged at 9000 rpm and resuspended in SDS sample buffer and boiled. Western blotting was performed as previously described (29) and detected by chemiluminescence (Pierce). Antibodies used are mouse anti-Myc 9E10, AN-1 (1:2000), rabbit anti-mNotch1Val1744 (Cell signaling, 1:1000), rabbit anti-Renilla Luciferase (1:1000, Chemicon), rabbit anti-MBP (1:10000,
Cell Signaling), rabbit anti-CAT (1:5000, Invitrogen) and rabbit anti-HA (1:1000, Covance, Babco).

For immunoprecipitation HEK293 cells (6-well, 25x 10e5) were transfected with a total of 2.0 ug DNA containing 100 ng DNA of tester 6MT(Flag or 6HA) and 100 ng reporter RL tagged construct (carrier DNA added to 2 µg). 40-48 hrs after transfection, cells were washed twice with ice-cold PBS and lysed in Co-IP buffer containing 200 mM KCl, 25 mM Heps (pH7.4), 20 mM NaF, 1% Igepal and 0.2 mM EGTA. Co-IP buffer was supplemented with complete protease inhibitor cocktail (containing EDTA, Roche), Leupeptin, Aprotinin and AEBSF. For immunoprecipitations in the presence of Ca2+ all solutions contained 1.8 mM CaCl2, EGTA was omitted and EDTA-free protease inhibitor cocktail was used instead (Roche).

Cells were lysed in 500 µl Co-IP buffer (29) immunoprecipitated with 1 µg anti-Myc antibody (9E10, Sigma); 1 µg rabbit anti-HA or M2-Flag beads (50 µl slurry, Sigma) as described. Antibody was recovered after 1hr incubation with protein A (CL-4B, 50 % slurry, Sigma). For post-lysis experiments singly transfected cells were mixed immediately after lysis and processed in parallel. Following three washes in Co-IP buffer and 2 washes in PBS, beads were resuspended in Co-IP buffer. Triplicate measurements of supernatants (3 x 20 µl) and IPs (3 x 20 µl) were assayed using the Renilla luciferase assay system (Promega) essentially as described by the manufacturer.

**Surface biotinylation**

For biotinylation of surface proteins cells were washed in ice-cold PBS++ (1mM MgCl2 and 0.1 mM CaCl2), and biotinylated with 0.5-1.0 mg/ml NHS-sulfo biotin (Pierce) for 20 min at 4°C. Cells were lysed in Co-IP buffer divided into equal aliquots and incubated with or without Streptavidin agarose (Vector) for 1 hr at 4°C and the biotinylated fraction was retrieved after centrifugation and repeated washing of beads in Co-IP buffer. The supernatant was used for Myc immunoprecipitation as described above.
RL measurements were also taken directly after lysis of cells to determine total amount of RL expression in the sample. For RL assays, beads were resuspended in Co-IP buffer and for western analysis beads were resuspended directly into Laemmli buffer under reducing conditions (10 mM DTT).
**Results**

**Active and Inactive Notch proteins are monomeric.**

We reasoned that if dimerization was an important component in regulation of Notch signaling, active and inactive Notch molecules should differ in their dimerization/oligomerization state. We previously reported that engineered oligomerization of the Notch ectodomain inhibited the metalloprotease dependent extracellular cleavage (S2) and monomeric forms of such proteins allowed S2 cleavage to occur (29). Likewise, Fibroblast growth factor receptor (FGFR)-Notch fusion proteins are constitutively cleaved (and thus active); addition of the ligand FGF promotes receptor dimerization and reduces their activity by about 30% (40). Similarly, placement of the an extracellular dimerization domain or a dimer-forming TMD inhibited processing of a TMD-galVP16 fusion protein in *Drosophila* (20). None of these studies, however, established the intrinsic ability of Notch proteins to autodimerize. To rigorously test whether inactive Notch proteins interacted with each other, we adapted a quantitative co-immunoprecipitation method developed by Taipale et al., (38). This method employs fusion proteins with the Renilla Luciferase (RL) gene; the degree of association between the tagged protein and an interacting partner was directly measured by RL activity recovered after immunoprecipitation (IP) with the partner (Figure 1C) corrected for remaining RL activity in the supernatant (Figure 1B). This specific association was presented as fold over non-specific interactions measured with a non-interacting control, the 12 pass transmembrane receptor mPatched1-RL (mPtc; Figure 1D).

First, we asked whether Notch proteins with the RL fused to their carboxy termini (Figure 1A) interact with the Notch partner, RBPjk. When co-expressed in HEK293 cells, N1ICV-RL proteins efficiently immunoprecipitated with Flag-tagged RBPjk (Figure 1D) as did N1ICV-RL produced from the membrane-tethered, extracellular domain deleted Notch (N1ΔE-RL). As expected, this latter interaction was blocked by adding γ-secretase inhibitor (DAPT) to the growth medium or when using a N1ΔE mutant (V1744G) that is cleaved less efficiently by γ-secretase. Intact Notch proteins are not γ-secretase substrates in the absence of ligand, and they too interacted poorly with RBPjk. Finally, we assayed the ability of constitutively active N1ΔE-RL proteins to activate a Notch
reporter construct composed of four tandem CSL-binding sites (4xCSL-Luc; (41); Figures 2B, 6C). These control experiments confirmed that Notch RL fusion proteins behave as their Myc-tagged counterparts and that RL permits quantitative analysis of Notch-partner interactions.

Intact Notch and N₁LNR molecules lacking the extracellular EGF repeats are inactive in signaling (Figure 1A; (29)); the current models would thus predict they are oligomeric. Mutation of a cysteine pair (CC1682SS) can activate Notch signaling in flies (28) and also activate N₁LNR (29). Additional activating mutations in Notch proteins which map to the juxtamembrane region include S1597N (42) and A1695T (24). The current models predict that these molecules are monomers and that these mutations affect the ability of these molecules to dimerize. To determine if these mutations affected the oligomerization state of Notch proteins they were introduced into the mouse N₁LNR proteins and found that although far inferior to N₁ΔE, this resulted in a 2-3 fold greater reporter activity compared to the parental N₁LNR-RL protein (Figure 3A). Further biochemical analyses indicated that all mutants are expressed to a similar extent, undergo furin (S1) cleavage with equal efficiency (Figure 2B) and all show similar cell surface expression as determined by surface biotinylation (not shown). Myc immunoblot analysis of wild type and mutant LNR-6Myc tagged fusion proteins shows all constructs undergo efficient furin-cleavage (TMIC) but only mutant (CC>SS, S>N, A>T) undergo the proteolytic cleavages consistent with S2 cleavage and S3 cleavage (compare N₁ΔE control and CD4ΔIgΔE). Introducing the AV>VH mutation at S2 reduces both furin-cleavage and S2 cleavage in LNRCC>SS proteins. S3 cleavage (detectable using a neo-epitope specific antibody recognizing the free amino terminus of NICD, Val1744; Figure, 2B) and transcriptional activity of these proteins correlated well. Activity and S3 cleavage could be reduced or blocked with the γ-secretase inhibitor DAPT (Figure 2A).

To test if oligomerization state of N₁LNR variants varied in correlation with their activity, extracts from cells transfected with LNR-RL and LNR-6MT wild type proteins or harboring the activating CC>SS or S1597N mutation were subjected to Myc-IP. Recovered luciferase activity from LNR-RL and mPtc1-RL in anti-Myc immunoprecipitates was comparable (Figure 3). This could indicate that LNR proteins
failed to interact with each other. However, IP experiments are normally conducted in buffers lacking calcium (Ca\(^{2+}\)) which is required for folding of the LNR domain \textit{in-vitro} (43,44). Repeating the experiments in the presence of physiological amounts of Ca\(^{2+}\) confirmed that active and inactive LNR proteins fail to specifically associate with each other (see below and data not shown.).

One limitation of these experiments and those performed in \textit{Drosophila} (20) lies in the use of truncated Notch proteins. To determine the oligomerization state of inactive full length Notch proteins, we co-expressed RL-tagged N\(_1\)FL-RL with N\(_1\)FL-6Myc. To identify potential dimerization-mediating domains within Notch proteins N\(_1\)FL-RL was also co-expressed with N\(_1\)LNR, N\(_1\)\(\Delta\)E, N\(_1\)ICV or the isolated N\(_1\) TMD (N\(_1\)\(\Delta\)ICE, see below and Figure 1). Only immunoprecipitation with N\(_1\)FL-6Myc recovered N\(_1\)FL-RL to a greater degree than with mPtc1-RL (Figure 4C), indicating specific homodimerization of Notch1 full length molecules only. Since N\(_1\)LNR failed to associate with N\(_1\)FL-RL (Figure 4) or with itself (Figure 3), we concluded that the EGF repeats were necessary for this interaction. In order to investigate whether the extracellular domain was also sufficient for this interaction, we co-expressed N\(_1\)FL-RL with a chimeric molecule containing the entire Notch extracellular domain fused to the FGFR1-TMD and intracellular domain (N\(_1\)FGFR1). N\(_1\)FL-RL and N\(_1\)FGFR1-6Myc interact, confirming that the Notch EGF domain is necessary and sufficient to mediate these homotypic interactions (Figure 4D). The Notch1 receptor contains 36 EGF repeats of which 21 potentially bind Ca\(^{2+}\) (cbEGF, (45)). We repeated the experiments in Ca\(^{++}\) containing buffers and obtained similar results (Supplementary Figure S1).

We observed that the N\(_1\) EGF repeats also mediate heterodimerization between N\(_1\)FL-RL and the mammalian Notch 2 receptor (N\(_2\)FL) as well as with the Notch ligand Jagged 1(Figure 4D, Supplementary Figure S2 and (46)). Thus, the highly conserved EGF repeats appear to mediate both homotypic and heterotypic interactions. To further validate the specificity of these interactions, additional control experiments were performed. First, we determined that the interaction we observed between full-length Notch molecules occurred in cells and not during the IP procedure by performing post-lysis mixing experiments as controls. Under these conditions, no dimers were identified
indicating that heterodimers between N1FL-RL and N1FL-6MT can only form and be recovered when both are co-expressed in vivo. Second, no N1FL-RL proteins could be immunoprecipitated by the Myc antibody in the absence of N1FL-6MT indicating specificity of the immunoprecipitation. Third, we could not detect any significant association of Notch with a series of control Myc-tagged proteins including FGFR1-Myc, C99APP-Myc, CD4Δlg3-4ΔE-Myc (Supplementary Figure S1 and not shown), indicating the associations were not mediated through the Myc epitope. Next we examined whether the interaction between N1 EGF repeats was confined to the highly conserved EGF repeats of Notch proteins and their ligands or whether it extended to unrelated EGF repeat-containing proteins as well. We co-expressed N1FL-RL with an HA tagged Low Density Lipoprotein Receptor (hLDLR) (47) that has similar Ca\(^{++}\)-binding-EGF repeats (45). We found that hLDLR preferentially associated with mPtc1-RL (Supplementary Figure S2). Finally, to exclude the possibility that truncated Notch proteins could not interact with full length Notch proteins because they were targeted to different membrane subdomains, we also co-expressed RL equivalent forms of the truncated proteins (e.g. N1ΔE-RL with N1ΔE-6MT, N1LNR-RL with N1LNR-6MT; Figure 1A). The RL-tagged proteins failed to form homotypic interactions in the absence of the EGF repeats (i.e., they interacted with each other as efficiently as they interacted with mPtc-1; Figure. 3 and not shown).

If all surface full length Notch proteins are dimeric, our results would be consistent with the notion that intact Notch molecules are kept inactive by dimerization. To determine the fraction of dimers at the surface, we biotinylated surface proteins and measured RL activity recovered by Myc immunoprecipitation before or after depletion of surface protein from the same lysate. If all the dimers we observed were at the cell surface, depletion of biotinylated surface proteins from the lysate (with streptavidin - agarose) prior to IP should greatly reduce the recovery of Notch dimers compared to non-biotinylated extracts (experimental scheme in Figure 4E). Instead, we found that the majority (81-88 %) of Notch dimers survive depletion and are thus intracellular. Only a fraction (0.94 to 1.90 %) is present at the cell surface. In contrast, N1FL-RL monomers were 3.6 to 7.0 fold more abundant at the cell surface than dimers, yet they were not
cleaved and did not interact with RBPjk (Figure 1). The observation that most surface Notch proteins are inactive in a monomeric state is in agreement with the observation that changes in oligomerization state cannot discriminate between active (ΔE and LNR mutants) and inactive (LNR, FL) molecules.

**Isolated TMDs of γ-secretase substrates are dimeric, not monomeric**

A second prediction of the model is that dimeric TMDs cannot be utilized by iCLiPs (20); TMDs of Type I proteins should thus be monomeric when not forced to dimerize by the extracellular domain. Moreover, while oligomerization may not normally play a role in Notch regulation, mutations that abolish Notch S3 proteolysis may do so by enhancing oligomerization of its TMD. To address both predictions we assayed the self-association of wild type and mutant TMDs from γ-secretase substrates in the plasma membrane of *Escherichia coli* with the TOX-CAT system developed by Russ and Engelman (33). Briefly, dimerization is inferred from the activation of TOXR, a transcription activator, which is anchored to the plasma membrane through fusion with a TMD of interest. TMD-mediated dimerization of TOXR will activate transcription from the cholera toxin promoter leading to chloramphenicol-acetyltransferase (CAT) expression and hence antibiotic resistance. To ascertain correct insertion of the fusion protein within the plasma membrane, the carboxy-terminal sequences of the tested TMD are fused to the Malto Binding Protein (MBP). Correct plasma membrane insertion of the fusion proteins was assessed by the ability of the TMD fusion proteins to complement maltose deficiency in the *MalE* deficient strain *NT326* (Figure 5B). Fusion proteins lacking a TMD were expressed but did not permit growth under restrictive conditions (-TMD, Figure 5B, C). All TMD fusion proteins supported growth in the presence of maltose as the only carbon source. In accordance, immunoblotting of bacterial extracts with an anti-MBP antiserum demonstrated that all contained the expected 65 kDa TOXR-TMD-MBP fusion product (Figure 5B, C). The characterized GpA TMD and its monomeric mutant GpA (G83I) TMD served as controls (33). G83I TMD was only able to weakly activate the expression of CAT compared to the GpA TMD (Figure 5B). As an additional control, constructs expressing the FGFR1 TMD were also tested; they too
activated CAT expression weakly. Surprisingly, we found that the Notch1 TMD elicited significant expression of CAT in comparison to the reference monomer G83I TMD (Figure 5B; (33)).

If dimerization contributed to or detracted from TMD recognition or cleavage by γ-secretase, mutations that abolish PS-dependent cleavage might elicit changes in the dimerization state of substrate TMDs. To test this we analyzed the effect of most TMD mutations shown in Figure 6A on dimerization of the Notch 1 TMD. We found no obvious difference between mutations that completely abolished PS cleavage or transcriptional activity (i.e. GCGV>LLFF, V1744G and V1744L) and those that had no effect on activity (i.e. V1744A, shown in Figure 5 and V1740L) compared to wild type N1 (N1) (Figure 6B). To assess the oligomerization state of TMDs from additional PS substrates, we subjected the TMDs of the mammalian N2, N3 and N4 receptors and APP to the TOX-CAT test. All showed robust CAT expression when compared to the monomeric TMDs of GpA or FGFR1 (Figure 5C). We observed slight differences in the propensity to dimerize among the Notch TMDs, with the Notch2 TMD consistently being the strongest dimer. A slight reduction in CAT expression was observed between N1ΔQLH (N1TMD lacking the QLH residues) relative to N1, however the significance of this remains unclear. Moreover, others using the same assay have demonstrated dimerization of the ErbB4 TMD (48). Thus, contrary to the notion that γ-secretase substrate TMDs are monomeric we find that all can self-associate in this assay.

Changes in self-association motifs do not affect γ-secretase cleavage

It has been shown that PS-dependent cleavage of Notch in Drosophila does not critically depend on the primary amino-acid composition within the TMD (Struhl and Adachi 2000). Furthermore, no significant homology or motif has emerged as more bona fide γ-secretase substrates are being identified (table1 in (13)), yet Val1744 mutations impact Notch proteolysis in mammalian cells. To identify additional sequences within the Notch1 TMD that could impact its utilization as a substrate, we first substituted residues at or in close proximity to the S3 cleavage site (Figure 6A) in the context of a signaling molecule (N1ΔERL). We compared the ability of all N1ΔE TMD mutants to activate
4xCSL-Luc. The previously characterized G, K and L substitution for Val1744 reduced cleavage of N1,ΔE in a range of 30-80% of wild type, V1744G being the weakest and V1744L being the strongest, at the Notch DNA concentration used here (Figure 6C). Alanine at V1744A was tolerated, only modestly affecting cleavage (Figure 6B) and transcriptional activity. Replacing the entire GCGV1744 sequence to LLFF completely abolished activity as reported previously (37,49).

While helix breaking residues such as glycine are critical in the TMDs of substrates cleaved by other intramembranous cleaving enzymes (50,51) the V1744G substitution in the Notch1 TMD attenuates cleavage. We introduced several additional mutations to further test the role of helix-breaking residues in Notch cleavage while leaving Val1744 intact (underlined below and in Figure 6A). Replacement of both Gly in the sequence GCGV with Ala, a residue known to promote helix formation (ACAV), did not markedly affect reporter activation, suggesting it was still a substrate for γ-secretase (Figure 6C). Substitution of these glycines (an important component within the dimerization motif in GpA) with alanines did not affect dimerization of N1 in the TOX-CAT assay (Figure 5B and not shown).

Next, we probed the role of putative self-association motifs within the TMD. Leucine zippers are well-known protein-protein interaction domains and have been implicated in dimerization of TMD sequences (52). We tested the effect of substituting the di-Leu motifs in LLFFVGCGVLLS with Alanine (AAFFVGCGVAAS), to maintain the helical structure of the TMD. Notch1 AAS mutants as well as the quadruple mutant behaved as wild type in 4xCSL activation assay with LLFF>AAFF mutant consistently showing a small increase in activity relative to the wild type. Finally, the V1744L substitution (VGCGV-VGCGL) may have enhanced the helical structure by generating a new interaction between V1740 and L1744. If this was the case, the reciprocal substitution, replacing V1740 with Leucine, should have an identical negative impact on proteolysis (i.e. VGCGV-LGCGV). However, we found that V1740L had no impact on the ability to activate reporter transcription. The activity of all these mutants was completely abolished in PS1/2 dKO cells (not shown) indicating that the luciferase activity was PS dependent. Collectively, these experiments could not support a role for
self-association in either positive or negative regulation of S3 cleavage nor could they establish a role for helix breaking or helix enhancing residues in regulation of Notch proteolysis by γ-secretase.

Phenylalanine scanning of the APP TMD generated shifts in scissile bond preferences by γ-secretase (53). We therefore asked whether the scissile bond cleaved in any of the mutants described above was indeed G1743-V1744, as it is in vivo (54-57). To identify the scissile bond we used an antibody recognizing the amino-terminal sequence VLLS exposed after γ-secretase cleavage. We found that in the mutants Val1740L and LLFF>AAFF, cleavage still occurred at Val1744. Surprisingly, in the GCGVLLS-ACAVLLS mutant the overall activity was unaffected, and NICD was generated (immunoblot with the AN-1 antibody) but we could not detect NICD with Val1744 antibodies (Figure 6B, long exposure). Detection of NICD with this antibody only occurs if the N-terminal sequence VLLS is preserved (58), suggesting perhaps a shift in the S3 cleavage site has occurred.

The Notch transmembrane domain is sufficient for γ-secretase cleavage.

To determine if proteins that might be associated with the Notch intracellular domain influenced the behavior of the mutant TMDs in our assay, we replaced the Notch 1 intracellular domain with a 6 Myc tag (N1ΔICE-6Myc). The residual extracellular domain contains only 25 residues including the S2 cleavage site and a signal sequence. Expression of N1ΔICE-6Myc in HEK 293 cells showed robust cleavage of the expected 27kDa protein into a faster migrating species (Figure 6D). Mutation of the Val1744 to the helix breaking Glycine (V>G) completely abrogated the appearance of this cleavage product. In addition, cleavage of N1ΔICE6Myc is completely abolished in cells lacking PS (PS1/2 dKO) and is recovered after reconstitution of PS1 expression in PS1/2 dKO cells (Figure 6E). Moreover, the same scissile bond generated in vivo is also generated in the minimal N1ΔICE substrate (Figure 6E, D). These results indicate that the transmembrane sequences of Notch 1 (with a possible contribution from the juxtamembrane region) was sufficient to act as a PS substrate and that sequences in the intracellular domain do not contribute to the characteristic cleavage by γ-secretase. However, we could not determine with certainty the self-association properties of N1TMDs in mammalian cells.
transfected with N1ΔICE molecules because of their non-specific association with control proteins (e.g. mPtc1-RL), most likely due to the hydrophobic nature of the TMD.
Discussion

Intramembrane proteolysis is emerging as a widespread and evolutionary conserved regulatory mechanism for a variety of signaling pathways. With the exception of Rhomboid substrates (59), all other I-CLiP require an ectodomain shedding event prior to intramembrane proteolysis of the substrate (18). In the regulation of Notch proteolysis, shedding of the extracellular domain is induced by ligand binding and carried out by a disintegrin metalloprotease (29,49,60). Similarly, ectodomain processing of APP by γ-secretase requires cleavage of APP by BACE, a transmembrane aspartyl protease (61), or by the disintegrin metalloprotease TACE or ADAM10 (62,63). It has been proposed that the role of this shedding event is to sever the extracellular domain thus removing an auto-inhibitory domain (e.g., the LNR), inhibiting I-CLiP cleavage. In the case of SPP and SREBP substrates this inhibitory domain is thought to be another helical TMD. Progressive shortening of the extracellular domain of chimeric Notch constructs in transgenic flies leads to an increase in γ-secretase cleavage (20), whereas forced dimerization of TMD substrates reduced proteolysis. Here, we examined two predictions made by prevailing models explaining the regulation of I-CliP activity: (i) TMDs of γ-secretase substrates are protected from proteolysis by virtue of their forced-association and (ii) following ectodomain shedding the TMDs will dissociate.

Ectodomain shedding of Notch is not regulated through oligomerization

We established that the highly conserved EGF repeats in the Notch extracellular domain are necessary and sufficient to interact with itself as well as with other Notch family members and the Notch ligand Jagged1 (Figure 2, 4, Supplementary Figure S1 and (46)). Notch can act as a sensor for extracellular Ca²⁺ concentration in vivo (64) and coordination of Ca²⁺ ions is important in the folding of the EGF and LNR domain in vitro (43,45). Depletion of Ca²⁺ by EDTA from the culture medium from Notch expressing cells leads to their activation by dissociating the heterodimeric bond holding the Notch extracellular domain; this region may fold in a manner that obscures access to the S2 site (3). However, we found no role for Ca²⁺ ions in regulating homotypic interactions between the EGF repeats of Notch (Figure S1) suggesting perhaps that oligomerization
involves EGF repeats that do not bind Ca\textsuperscript{2+}. Intracellular association between Notch molecules and their ligands has been reported previously (46), but the association with Notch2 prompted us to test for specificity. We find no association between Notch and hLDLR, a protein containing multiple tandem EGF repeats (Figure S2). The role for homodimerization between different Notch molecules (Notch1 and Notch2) remains unclear.

The model predicts that inactive Notch proteins exist mostly in a dimeric state to keep the TMD from drifting apart and becoming subject to proteolysis. To our surprise, we found that Notch and APP all have a propensity to dimerize in the TOX-CAT assay. This is also the case for the TMDs of other γ-secretase substrates (ErbB4 (48), E-cadherin (65) and Syndecan-3 (66)). Notch dimers indeed form from both furin-cleaved (i.e. trans Golgi and cell surface) and uncleaved molecules (found in the ER, data not shown). However, using biotin/streptavidin depletion we established that only between ~1-2 % of all dimers reach the cell surface (Figure 4E). The role of dimerization in export from the ER (67,68) as well as retention of misfolded receptors in the ER (69) is well established; the large percentage of intracellular Notch dimers may reflect overexpressed proteins trapped in the ER (YHP, MV and RK, unpublished observations). Importantly, the majority of full-length Notch molecules at the cell surface are monomeric. These molecules are not ligand-bound and cannot signal, thus the simple notion that transition from dimer to monomer will suffice to render the protein a substrate for ectodomain shedding and subsequent γ-secretase cleavage is not correct.

100% of the inactive N\textsubscript{1}LNR and the active N\textsubscript{1}LNR mutants (e.g N1LNR\textsuperscript{ccc>ss}, N1LNR\textsuperscript{S>N}) are all monomers; and all require S2 cleavage prior to activation ((29) and our unpublished observations). The presence of inactive, monomeric Notch at the cell surface, the lack of detectable oligomerization in active (ΔE, and LNR mutants) and inactive (LNR, FL) molecules, and the self association of substrate TMD in the TOX-CAT assay therefore falsifies both of the main predictions of the current paradigm.

Alternatively, it has been proposed that the length of the extracellular domain could be the major determinant in the regulation of Notch extracellular cleavage (20,54). This too fails too explain the differences in activity since active and inactive N\textsubscript{1}LNR
molecules are of the exact same length. Therefore, regulation of the S2 extracellular cleavage is likely to involve structural changes that may be mimicked by mutations (24,42). The nature of these structural changes remains uncertain.

**The helical content of the Notch TMD**

It is accepted that helical substrate TMDs are resistant to cleavage by I-CliPs. Whereas Rhomboid (51), SPP (50) and SREBP (70) all require helix-breaking residues in the TMDs of their substrates to elicit intramembrane cleavage; we found no such requirement for γ-secretase. We have described several mutations at the S3 cleavage site that affect the activity of Notch proteins from mildly (V>G, V>K) to severely (GCGV>LLFF); of these the V>G substitution was predicted to enhance cleavage as it inserted a helix-breaking residue (Gly). Introduction of strong helix-promoting residues (V>A) do not seem to affect the activity of the Notch protein in mammalian cells. The V>L substitution impacts proteolysis but appears more severe due to rapid degradation of NICD due to an N-terminal Leucine (71). To investigate the possibility that we suppressed proteolysis by creating a helix-stabilizing pair (V1744L with V1740), we tested the reciprocal substitution (V1740L with V1744) but no impact on proteolysis was observed. Helix-breaking residues are found in the vicinity of the conserved Valine in vertebrate and *Drosophila* Notch and the *C.elegans* Notch receptor, Lin-12. Substitution of Glycine (GCGV) with the helix-promoting residue Alanine (ACAV) was predicted to reduce cleavage; the cleavage site may have shifted to another position (which remains to be determined) but no marked reduction in signaling capacity was observed. Thus, it appears that γ-secretase substrates differ from other I-CliP substrates in their tolerance to helix-forming residues and Notch1 differs from other γ-secretase substrates in its intolerance of helix-breaking residues at the P1’ site.

In conclusion, we are still left with the conundrum of monomeric substrate proteins (N1FL, N1LNR) that are bound to γ-secretase but are not proteolytic substrates (72,73) until after S2 cleavage (29,60). We present evidence that Notch signaling is not simply regulated by oligomeric changes in the extracellular and/or transmembrane domain, nor is the length of the extracellular domain regulating cleavage. While we cannot rule out that ligand binding may alter the oligomerization state of Notch, we
propose that Notch cleavage (and perhaps all γ-secretase substrates) is more likely to be regulated by structural changes induced upon ligand binding to Notch receptors. We propose that intact proteins cannot be transferred to the active site of γ-secretase where the helical TMD is relaxed. Activating mutations in the conserved region carboxy-terminal to the LNR repeats could act by enforcing a conformation that is competent to enter the active site. Conversely, helix-breaking mutations in the Notch TMD do not enhance cleavage because they may lock the transmembrane domain in the wrong conformation, preventing hydrolysis. Given that γ-secretase is the only known multi-protein I-CLiP that cleaves substrates multiple times within their TMDs, the mechanism controlling cleavage of its substrates may be unique among I-CLiPs. Similar experiments will have to be conducted with other I-CLiP substrates.
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Figure legends.

Figure 1. Validation of Notch Renilla luciferase (RL) as a quantitative IP assay. (A) Schematic representation of the fusion constructs used in this study. Proteins differ only in their carboxy-terminal tag (Myc, or Renilla luciferase (RL). (B-D) Association between Notch proteins and FLAG-RBPjk was determined by measurement of RL units which co-immunoprecipitated with FLAG-RBPjk. (B) Remaining RL activity in the supernatant after IP and (C) recovered RL activity from the Co-IP with FLAG-RBPjk. (D) RL units in IP relative to total (sup+IP) presented as fold over the non-specific association between FLAG-RBPjk and mPtc-1-RL, a protein that does not associate with Notch or RBPjk. Percent association was calculated by determining the ratio IP (RL)/IP (RL)+ SUP (RL). These values were expressed as fold over the non-specific association observed with the mPtc1 receptor in the same experiment.

Figure 2. Active and inactive Notch LNR signaling constructs. (A) Schematic representation of the truncated 6Myc and RL- N1LNR construct with the position of the activating mutations indicated (references in text). Although far inferior to N1∆E, signaling through LNR-RL mutants is 2-3 fold better than the parental protein and is also completely blocked by DAPT (A, B and not shown). (B) Biochemical analysis of proteolytic cleavage of 6Myc tagged LNR mutants.

Figure 3. Wild type and mutant LNR proteins are monomeric. (A) Renilla Luciferase activity in supernatant (B) and in Myc immunoprecipitates. (C) Activity is corrected for association with mPtc1-RL. Both inactive (LNR) and active (CC>SS; S>N) proteins are monomers.

Figure 4 Notch Full length proteins form dimers, but majority of surface Notch is monomeric. Self association was analyzed by co-transfecting cells with N1FL-RL (black bars) or mPtc1-RL (grey bars) with 6Myc tagged N1FL, N1LNR, N1∆E, N1∆ice or N1ICV (see Figure 1A). (A) RL activity in the supernatants, (B) in Myc immunoprecipitates and
(C) relative RL activity expressed as fold association over Ptc1. (D) The EGF domain is sufficient for self association: replacing the Notch TMD and intracellular domain with the corresponding domains of the FGF receptor (N1FGFR1-6Myc) maintains interactions with N1FL-RL. Shown is a representative experiment of triplicate transfections with average and s.d. (E) Scheme of experimental design (left), calculation (bottom) and results (right). Depletion of biotinylated surface proteins reveals that only a fraction of N1FL-RL dimers are at the surface (blue bar) whereas most of surface N1FL-RL are monomers (red bar). Data compiled from two independent experiments (see text for details).

**Figure 5.** TMD of γ-secretase substrates are constitutive dimers. (A) TMD substrates cloned into the TOX-CAT assay for dimerization, M1726V mutation in N1 shown. (B) Growth of fusion proteins in minimal medium (M9/Amp) and anti-MBP blot to indicate membrane insertion and expression; proteins without TMD (-TMD) are expressed MBP (lower panel shows anti-MBP immunoblot) but will not complement MalE deficiency. Lowest panel shows anti-CAT immunoblot detecting relative transcriptional activity (and thus, dimer formation) of fusion proteins. Note robust CAT expression in GpA wt but not in control (monomeric) GpAm mutant. (C) N1, N2, N3 and N4 TMD were tested along with control TMDs. N1ΔQLH is a N1TMD lacking the QLH residues, note some loss of CAT expression (compare B and C). The asterisk (*) indicates non-specific immunoreactivity.

**Figure 6.** The impact of cleavage site mutations targeting putative dimerization motifs in Notch TMD. (A) Schematic diagram depicting N1ΔE-RL and N1ΔICE. Both contain a signal peptide (SP), 16 residues at the extracellular cleavage site (encompassing S2) and the Notch TMD with the Val1744 cleavage site underlined. The N1 intracellular domain is replaced by a 6Myc tag to generate N1ΔICE. Indicated below are the Val1744 cleavage site and the surrounding residues that have been modified in one or both
backbones. (B) Anti-AN-1 immunoblot showing S3 cleavage (arrow) in wild type N₁ΔE-RL and mutants. (C) 4xCSL-Luc reporter assay monitoring the effect of TMD mutations, results corrected for background activity of the reporter in the absence of transfected Notch proteins. (D) Anti Myc immunoblotting showing efficient cleavage of wild type but not mutated (V>G) N₁Δice (~28 kDa) in HEK293 cells. NICD detected by Val1744 immunoblot (Lower panel). (E) N₁ΔICE transfected in PS1/2dKO fibroblasts. * Indicates non-specific immunoreactivity.
Supplemental Figures

**Figure S1.** Effect of Calcium on dimerization. (A) Association of N₁FL proteins was determined in the presence or absence of 1.8 mM Ca²⁺ (physiological concentration). Fold association over non-specific association with Ptc1 in a representative experiment, performed in triplicate, is shown (except NFL/NFL + Ca²⁺ performed in duplicate). Controls indicating the specificity of the interaction are: # - post lysis mixing of singly transfected cells indicating association occurs in cells and not during lysis, ## - no significant Renilla luciferase activity in anti-Myc IP of N₁FLRL and Ptc1RL co-transfected cells. (B) N₁FL proteins associate with self or ligand (JAG1) but not with control transmembrane proteins. Shown is a representative experiment with fold over Ptc1 association of N₁FL with FGFR1, C99 APP or CD4ΔIg³-⁴ΔE. (C) N₁FL proteins heterodimerize with N₂FL proteins through the EGF repeats. Cells were co-transfected with N₁FL (LNR or ΔE) RL and N₂Flag. Shown is representative experiment (performed in triplicate) corrected for non-specific Ptc1 association.

**Figure S2.** EGF repeats mediate specific dimerization of Notch (A-C) N₁FLRL was tested for its ability to immunoprecipitate with LDLR, a highly related EGF repeat-containing protein. No specific association could be found. (A) Renilla luciferase activity in the supernatant and (B) Renilla luciferase activity recovered in anti-HA immunoprecipitates. For unknown reasons HA immunoprecipitation consistently retrieved fewer N-N dimers than with anti-Myc but did not affect the association with JAG1. Ptc1 seems to have a higher affinity for LDLR than LDLR has for Notch1.
**A**

![Graph showing 4xCSL Luc / bgal levels](Image)

| Condition          | 4xCSL Luc / bgal |
|--------------------|------------------|
| ΔE                 | 5000             |
| ΔE + DAPT          | 4000             |
| wt                 | 1000             |
| C>S                | 500              |
| A>T                | 0                |
| S>N                | 0                |

**B**

![Western Blot analysis](Image)

**DAPT**

- WT: -
- CC>SS: -
- CC>SS: +
- AV>VH: +
- A>T: +
- S>N: +
- ΔE: -
- ΔEV>K: +

**anti-Myc**

- TMIC
- S2
- S3

**anti-Val1744**

**anti-Val1744(l)**
A. Renilla units in SUP:

B. Renilla units in N1FL-6MT IP:

C. N1FL/Notch interaction:

D. N1FL/EGF repeats interaction:

E. Add Biotin:

1. Notch at the surface: 
   \[
   \text{[(sAv+)/}(0.5 \times \text{Total RL})] 
   \]

2. Dimer at the surface:
   \[
   \text{[(IP(sAv-)]-[IP(sAv+)}}/(0.5 \times \text{Total RL})] 
   \]

3. Monomer at the surface:
   \[
   [\text{(1)-(2)}] 
   \]
A

|   | mN1   | mN2   | mN3   | mN4   | APP   | FGFR1 | GpAwt | GpAm  |
|---|-------|-------|-------|-------|-------|-------|-------|-------|
|   | OLHL\textsuperscript{V}YVA\textsubscript{A}AAAF\textsubscript{V}LLFFV\textsubscript{GCGV}LLS | L\textsubscript{YLL}LAVAV\textsubscript{VII}LL\textsubscript{F}ILL\textsubscript{GVIMA} | L\textsubscript{PPLL}LV\textsubscript{AGAV}\textsubscript{FLLL}IIIF\textsubscript{ILGVMVA} | L\textsubscript{CSPVV}GV\textsubscript{LLL}ALG\textsubscript{ALL}V\textsubscript{VQLI} | G\textsubscript{AI}IIG\textsubscript{L}M\textsubscript{VGV}\textsubscript{VIAT}V\textsubscript{IVIT}\textsubscript{LVML} | IIYCTGAFL\textsubscript{I}ISCMLG\textsubscript{SVIIYK} | L\textsubscript{IIIFGV}M\textsubscript{A}GV\textsubscript{IGTI}LI | L\textsubscript{IIIFGVMAI}IV\textsubscript{IGTI}LI |

B

![Image of protein expression results for different constructs](image)

C

![Image of protein expression results for different constructs](image)
Ectodomain shedding and intramembrane cleavage of mammalian notch proteins is not regulated through oligomerization
Marc Vooijs, Eric H. Schroeter, Yonghua Pan, Mary Blandford and Raphael Kopan

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