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β-Site Amyloid Precursor Protein Cleaving Enzyme 1(BACE1) Regulates Notch Signaling by Controlling the Cleavage of Jagged 1 (Jag1) and Jagged 2 (Jag2) Proteins

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Background: We set out to determine whether Jag1 and Jag2 are natural substrates of BACE1.
Results: BACE1 cleaves Jag1 efficiently but cleaves Jag2 weakly, whereas ADAM10 cleaves both Jag1 and Jag2.
Conclusion: Altered BACE1 activity can regulate Jag1 signaling activity by controlling the level of its membrane-anchored form.
Significance: BACE1 appears to regulate specific Notch signaling activity by differential cleavage of Jag1 and Jag2.

BACE1 is a type I transmembrane aspartyl protease that cleaves amyloid precursor protein at the β-secretase site to initiate the release of β-amyloid peptide. As a secretase, BACE1 also cleaves additional membrane-bound molecules by exerting various cellular functions. In this study, we showed that BACE1 can effectively shed the membrane-anchored signaling molecule Jagged 1 (Jag1). We also mapped the cleavage sites of Jag1 by ADAM10 and ADAM17. Although Jag1 shares a high degree of homology with Jag2 in the ectodomain region, BACE1 fails to cleave Jag2 effectively, indicating a selective cleavage of Jag1. Abolished cleavage of Jag1 in BACE1-null mice leads to enhanced astrogenesis and, concomitantly, reduced neurogenesis. This characterization provides biochemical evidence that the Jag1-Notch pathway is under the control of BACE1 activity.

The activity of β-Site amyloid precursor protein (APP)[2] cleaving enzyme 1 (BACE1) is elevated in the brains of Alzheimer disease patients (1–6). This increased BACE1 activity enhances the production of β-amyloid (Aβ) peptides, which are 40- to 42-amino acid peptides released from APP that are prone to form aggregates upon aberrant accumulation (7). Aβ is the major component of amyloid plaques and is widely considered to be a primary contributor to Alzheimer disease pathogenesis. Alzheimer disease mouse models deficient in BACE1 produce negligible levels of Aβ and exhibit no amyloid plaques (8–10), indicating that the effective inhibition of BACE1 can reduce Aβ generation. For this reason, BACE1 is being actively pursued as a therapeutic target for Alzheimer disease therapy.

BACE1 is a type I transmembrane aspartyl protease (1–5) and sheds membrane-bound cellular substrates (11). Recent studies have demonstrated that BACE1 can cleave type I and III neuregulin 1 (Nrg1) as well as its family member neuregulin 3 and that abrogated cleavage of Nrg1 is linked to hypomyelination and schizophrenia-like behaviors in BACE1-null mice (12–15). Various studies have also shown that BACE1 cleaves other type I transmembrane proteins, such as the β subunit of voltage-gated sodium channel proteins (16), the neural cell adhesion molecule close homolog of L1 (CHL1) (17–19), and vascular endothelial growth factor receptor 1 (VEGFR1) (17). Using proteomic approaches, additional membrane-bound proteins have been suggested to be substrates of BACE1 (18, 19).

By examining brains of BACE1-null mice during early development, we demonstrated that BACE1 controls the balance of hippocampal astrogenesis and neurogenesis (20). During early development, the differentiation of radial glial stem cells in the BACE1-null mouse subgranular zone favors astrogenesis, whereas neurogenesis is reduced. The underlying molecular mechanism associated with this finding is enhanced Notch signaling activity because the level of Notch intracellular domain is elevated. Notch is a single-pass transmembrane protein that is normally located on the cell surface. The interaction between Notch on one cell and its membrane-bound ligand, belonging to the DSL (Delta/Serrate/Lag-2) family, on neighboring cells induces the release of the signaling molecule Notch intracellular domain (21). In BACE1-null hippocampi, elevated Notch correlates with higher levels of full-length protein of the Notch ligand Jagged 1 (Jag1). This suggests that Jag1 is a potential substrate of BACE1.

In this study, we aimed to determine whether Jag1 is an authentic substrate of BACE1 and whether other members of the DSL family can also be shed naturally by BACE1. By mapping the BACE1 cleavage sites and conducting site-directed mutagenesis assays, we confirmed that Jag1 is a natural substrate of BACE1. Interestingly, Jag2, a close homolog of Jag1, appeared to be a better substrate for γ-secretase, but it was less effectively cleaved by BACE1. Because Notch signaling is important for various cellular functions ranging from cell fate specification to angiogenesis, differential processing of Jag1 and Jag2 by BACE1 may regulate specific Notch signaling activity by controlling the levels of full-length Jag1 and Jag2.

EXPERIMENTAL PROCEDURES

Cell Lines, Animal Models, and Reagents—HEK293 cells were used to establish a stable cell line expressing HA-tagged

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[2] The abbreviations used are: APP, amyloid precursor protein; Aβ, amyloid β; DSL, Delta/Serrate/Lag-2; ctf, C-terminal fragment; DLL, Delta-like ligand; ADAM, A Disintegrin And Metalloproteinase.

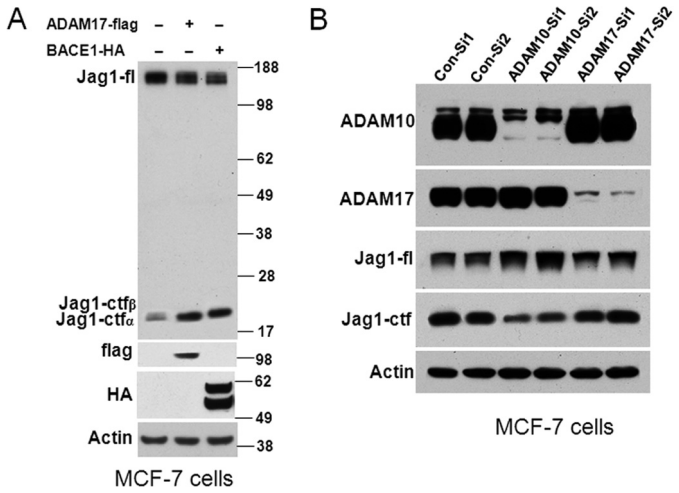

FIGURE 1. **Cleavage of Jag1 by α- or β-secretase.** *A*, cultured human breast cancer MCF7 cells were transfected with BACE1-HA or ADAM17-FLAG plasmid DNA for 48 h. Protein lysates were analyzed by Western blotting with an antibody specific to the C terminus of Jag1. Two C-terminal fragments cleaved by BACE1 (Jag1-ctf$_\beta$) or by ADAM17 (Jag1-ctf$_\alpha$) were visible under this specific condition. *B*, MCF7 cells were treated with the indicated siRNA duplexes for 48 h to knock down ADAM10 or ADAM17, and protein lysates were prepared for Western blot analysis with an antibody specific to the Jag1 C terminus. Enhanced full-length Jag1 was more evident in cells with ADAM10 knocked down, suggesting cleavage of Jag1 by ADAM10. *Con*, control.

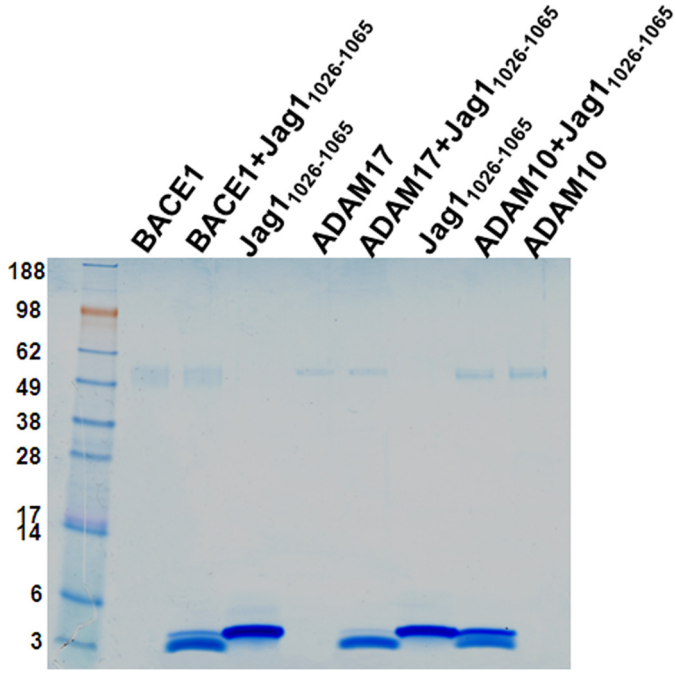

FIGURE 2. **Cleavage of a Jag1 synthetic peptide by α- or β-secretase.** A 40-amino acid peptide corresponding to a partial sequence (residues 1026–1065) of Jag1 was synthesized. This purified peptide was incubated with recombinant BACE1, ADAM10, or ADAM17 in an *in vitro* assay. The reaction mixture was separated by SDS-PAGE and visualized by Coomassie Blue staining. The parent peptide Jag1$_{1026-1065}$ was processed by BACE1, ADAM10, or ADAM17.

BACE1 (named HM cells) and were maintained by continuing selection with the antibiotic G418, as described previously (22). Maintenance and genotyping of BACE1-null mice have been described previously by Cai *et al.* (8). All experimental protocols were approved by the Animal Care and Use Committee at the Cleveland Clinic in compliance with the guidelines established by the Public Health Service Guide for the Care and Use of Laboratory Animals.

Generation of Expression Constructs—Rat Jag1 is highly conserved with human (96% homology) and mouse Jag1 (99% homology) at the amino acid level. The rat Jag1 cDNA expression construct (a gift from Drs. Raphael Kopan and Mary Blandford, Washington University, St. Louis) was used to make Jag1 mutants by site-directed mutagenesis according to the manual provided with the QuikChange^{TM} site-directed mutagenesis kit (Stratagene). Plasmid pRK5F-ADAM17 (A Disintegrin And Metalloproteinase) deposited by Dr. Rik Derynck, was purchased from Addgene.

Western Blot Analysis—Protein extraction from mouse brain homogenates or cells was performed according to procedures described previously (20). Equal amounts of protein were resolved on a NuPAGE BisTris gel (Invitrogen) and transferred onto nitrocellulose membranes (Invitrogen). Western blot analyses were reacted with primary antibodies (Jagged1 (1:200) and Jagged 2 (1:500), Santa Cruz Biotechnology; HA antibody, 1:1000, Roche; anti-actin in 1:10,000 and anti-FLAG in 1:1000, Sigma) and HRP-conjugated secondary antibodies using standard protocols.

Proteolytic Cleavage of Notch Ligands—For *in vitro* cleavage of Notch ligands such as Jag1 and Jag2, recombinant proteins or synthetic peptides were used routinely to incubate with purified recombinant BACE1, ADAM17, or ADAM10 in a protease assay, as described previously (23). For mapping Jag1 cleavages, the recombinant rat Jagged 1 Fc chimera, covering the entire extracellular domain of Jag1, was purchased from R&D Systems, Inc. 1 μg of the Jag1-Fc recombinant protein was first tested by incubation with 1 μg of purified recombinant BACE1, ADAM17, or ADAM10 for ~16 h. Peptides covering the potential BACE1 cleavage site in Jag1 and Jag2 were synthesized, and HPLC-purified peptides were subsequently used for mapping assays similar to the above description. The reaction mixtures were separated on SDS-PAGE, followed by standard protein staining for initial inspection. The processing products were further analyzed by HPLC or MALDI-TOF assays (24). For cultured cells, the rat Jag1 expression construct (a gift from the laboratory of Dr. Raphael Kopan) was transfected in HM cells or together with either empty vector or the BACE1-HA or ADAM17-FLAG expression construct in HEK293 cells for 48 h. Protein lysates were prepared for Western blot analysis according to standard procedures.

RESULTS

Jag1 Is Shed by ADAM10, ADAM17, and BACE1—Previous studies suggest that Jag1 is effectively cleaved by ADAM17 (25, 26). In this study, the human breast cancer MCF7 cell line, which endogenously expresses detectable levels of Jag1, was transfected with either HA-tagged BACE1 or FLAG-tagged ADAM17 to determine potentially altered cleavage of Jag1. An antibody specific to the C terminus of Jag1 detected endogenous levels of Jag1 full-length protein as well as two differentially migrating C-terminal fragments (ctf) that were barely separable on the gel (Fig. 1*A*). We found that increased expression of either BACE1 or ADAM17 reduced the level of full-length

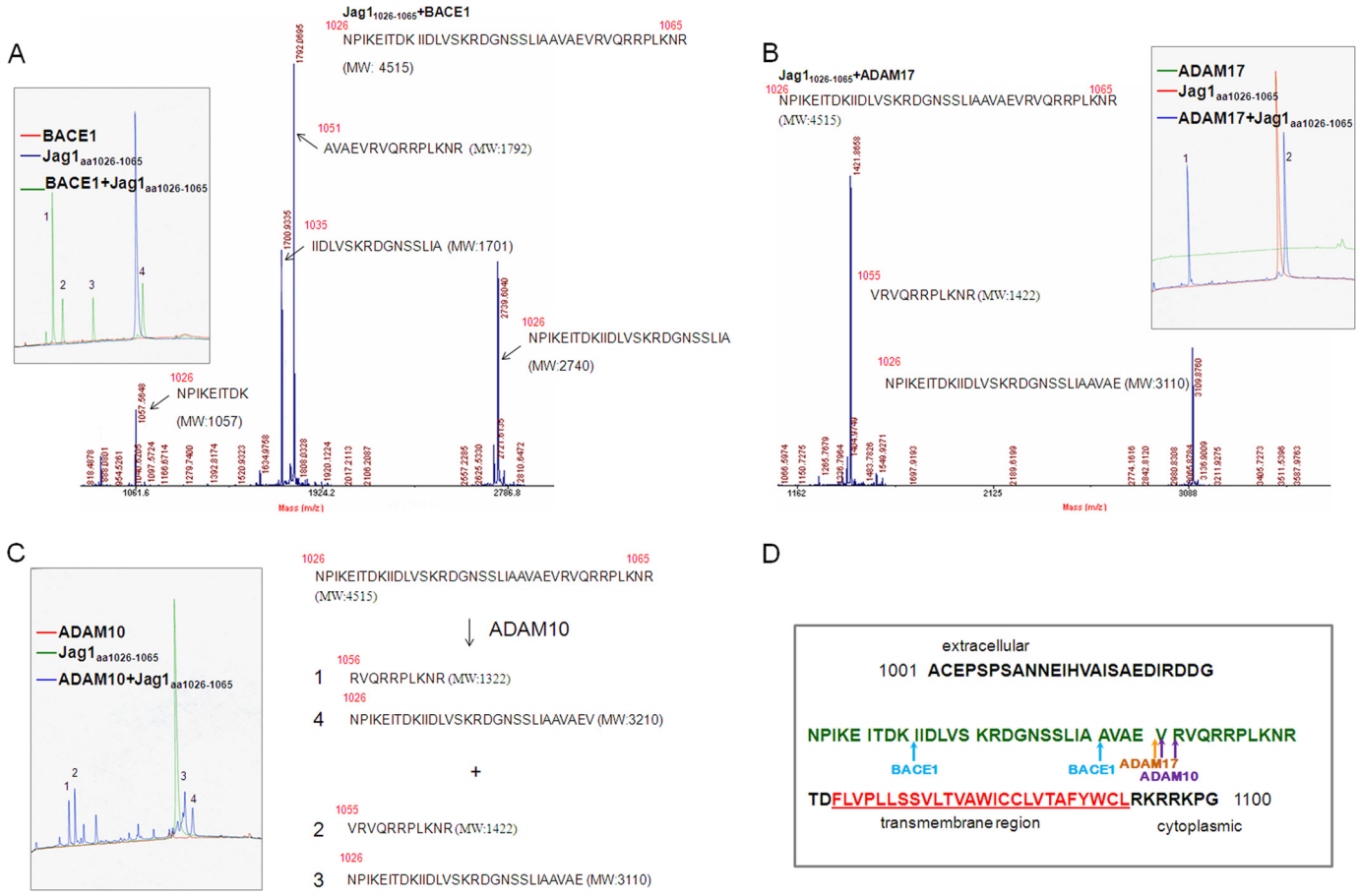

FIGURE 3. **Mapping of Jag1 cleavage sites.** *A*, the peptide Jag1$_{1026-1065}$ was cleaved by BACE1 in a reaction mixture, which was subjected to HPLC separation. Either the entire mixture or each individual peak fraction was analyzed by MALDI-TOF mass spectrometry. The molecular weight of each peptide, purified by HPLC as shown in the *inset*, matches the corresponding mass peak. Two cleavage sites (Lys^{1034}-Ile^{1035} and Ala^{1050}-Ala^{1051}) were identified. *B*, the same peptide cleaved by ADAM17 produced two peaks on HPLC separation, and the reaction mixture was analyzed similarly by MALDI-TOF mass spectrometry. One cleavage site (Glu^{1054}-Val^{1055}) was identified. *C*, the peptide Jag1$_{1026-1065}$ was incubated with ADAM10, and the reaction mixture was separated by HPLC. Multiple peaks were observed by HPLC separation, and the major peaks, designated *1–4*, contained peptides with molecular weights that matched MALDI-TOF profiles. *D*, sequence illustration of the individual cleavages of Jag1 by each protease. The entire sequence covers Jag1 from residues 1001–1100, and the transmembrane domain is shown in *underlined red letters*. The peptide Jag1$_{1026-1065}$, used for proteolytic cleavage, is shown in *green letters*.

Jag1 but increased the level of Jag1-ctfs (Fig. 1*A*). Increased expression of BACE1 preferentially elevated the slower migrating band, which was designated Jag1-ctf$_\beta$. We designated the faster migrating band Jag1-ctf$_\alpha$.

We have shown previously that full-length Jag1 is increased in BACE1-null mice (20). In this study, we examined whether full-length Jag1 is altered when ADAM17 or ADAM10 is down-regulated. MCF7 cells were used for specific RNA interference experiments. We found that treatment with either ADAM10 or ADAM17 siRNA duplexes altered the cleavage of Jag1 (Fig. 1*B*). Knocking down ADAM10 appeared to be more potent in elevating full-length Jag1 and reducing Jag1-ctf than knocking down ADAM17 (Fig. 1*B*). Thus, it appears that ADAM10 is more effective at cleaving Jag1 in MCF7 cells than ADAM17.

Mapping the Cleavage of Jag1 by BACE1—To validate the cleavage of Jag1 by BACE1 and by α-secretase (ADAM10 and ADAM17), we synthesized several short peptides, including the one spanning residues 1026–1065 (Jag1$_{1026-1065}$). These peptides potentially contained the putative cleavage sites on the basis of our initial sequence analysis. We incubated each peptide with recombinant BACE1, ADAM10, or ADAM17 in our *in vitro* enzymatic assays, as described previously (14, 24). Our results showed that BACE1, ADAM10, and ADAM17 cleave Jag1$_{1026-1065}$ similarly (Fig. 2), suggesting that the cleavage sites are likely in close proximity.

Further separation of BACE1-cleaved Jag1$_{1026-1065}$ by HPLC revealed four separated peaks (Fig. 3*A*, *inset*), suggesting the presence of two potential BACE1 cleavage sites in this peptide. A mapping analysis of cleaved peptides in the enzymatic reaction as well as each peak by MALDI-TOF mass spectrometry confirmed that BACE1 cleaved Jag1$_{1026-1065}$ at two sites (Fig. 3*A*). One cleavage site was located between residues Lys^{1034} and Ile^{1035} and the other was located between Ala^{1050} and Ala^{1051}.

For ADAM17 cleavage, one site was detected because two peaks were found in the HPLC separation profile (Fig. 3*B*). Further MALDI-TOF mass spectrometry analysis showed that the cleavage site is located between residues Glu^{1054}-Val^{1055}. Interestingly, ADAM10 cleaves Jag1$_{1026-1065}$ at two neighboring sites: Glu^{1054}-Val^{1055} and Val^{1055}-Arg^{1056} (Fig. 3*C*). The first site is identical to the ADAM17 cleavage site, which is 13 residues away from the transmembrane domain and only four residues downstream of the Ala^{1050} and Ala^{1051} site (Fig. 3*D*). These data show that the BACE1 and ADAM cleavage sites are in close proximity. These mapping results are consistent with

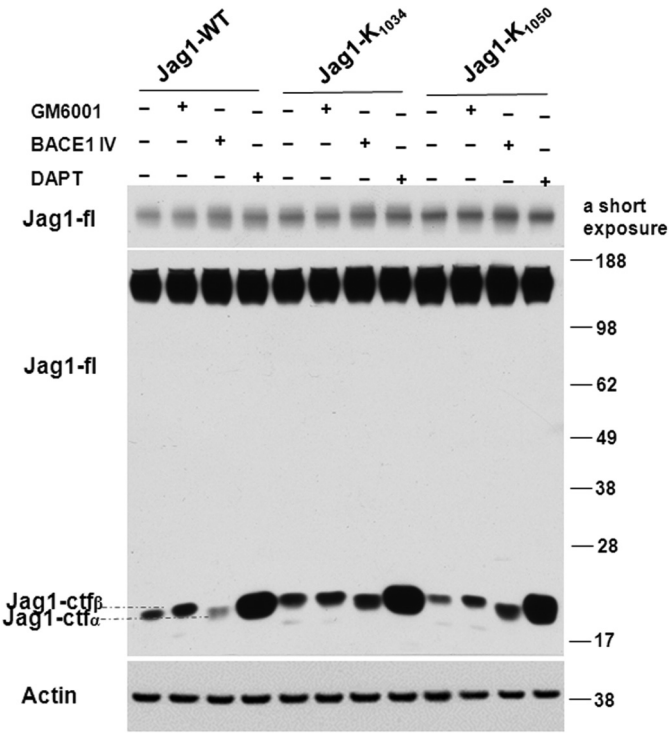

FIGURE 4. **Cleavage of Jag1 mutants by BACE1.** Jag1 mutants (Jag1-Lys^{1050}; Ala^{1050}-Ala^{1051} → Lys^{1050}-Lys^{1051}; Jag1-Lys^{1034}; Lys^{1034}-Ile^{1035} → Lys^{1034}-Lys^{1035}) were generated by site-directed mutagenesis and were expressed in HM cells, which were established by overexpressing BACE1 in HEK293 cells. A BACE1 inhibitor (BACE IV), α-secretase inhibitor (GM6001), and *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (*DAPT*, a γ-secretase inhibitor) were used to inhibit the corresponding proteases. *Dashed red lines* distinguish Jag1-ctf$_\beta$ from Jag1-ctf$_\alpha$. Two barely separable Jag1-ctf$_\alpha$ bands were observed when cells were treated with BACE1 inhibitor, which is consistent with the two identified ADAM10 cleavage sites in Jag1.

the observation that BACE1- and ADAM17-cleaved fragments are not readily resolved on an SDS gel, as shown in Fig. 1*A*.

Mutagenesis of the BACE1 Cleavage Site in Jag1—It should be noted that BACE1 cleaves APP at two sites (β and β′ sites), which are separated by 10 amino acids and are recognized as APP-C99 and C89 fragments on Western blot analyses (23, 27–30). Our *in vitro* mapping assays suggest the presence of two presumable BACE1 cleavage sites in Jag1, separated by 16 amino acids. To definitively establish whether BACE1 cleaves Jag1 at these two sites, we conducted site-directed mutagenesis by individually changing the residues Lys^{1034}-Ile^{1035} to Lys^{1034}-Lys^{1035} (designated Jag1-Lys^{1034}) and Ala^{1050}-Ala^{1051} to Lys^{1050}-Lys^{1051} (designated Jag1-Lys^{1050}) as well as creating a double mutant at both sites (designated as Jag1dKK) in the WT Jag1 expression construct. Mutating the BACE1 site to Lys-Lys has been shown to reduce the cleavage by BACE1 in the APP case (30).

We have shown that inhibition of Jag1-expressing cells with GM6001, a pan-inhibitor of ADAM, increased the intensity of Jag1-CTF$_\beta$ because of additional BACE1 cleavage of Jag1 after α-secretase activity was inhibited (Fig. 1). Comparing the Jag1-CTF$_\beta$ from protein lysates expressing mutant constructs, we noticed differential patterns (Fig. 4). As expected, GM6001 treatment of WT Jag1 expression cells could weakly elevate the Jag1-ctf$_\beta$ level (Fig. 4). GM6001 treatment of cells expressing the Jag1 Jag1-Lys^{1034} construct also showed a small increase in the Jag1-ctf$_\beta$ level, suggesting no major impact on BACE1 processing in this mutant Jag1. However, GM6001 treatment of cells expressing Jag1-Lys^{1050} (mutated at the Ala^{1050}-Ala^{1051} site) showed barely any increase in the Jag1-ctf$_\beta$ level, indicating that Jag1-Lys^{1050} was not processed effectively by BACE1. If the Lys^{1034}-Ile^{1035} site is a weak BACE1 cleavage site, impaired cleavage at the Ala^{1050}-Ala^{1051} site in Jag1-KK1 should favor BACE1 cleavage at the Lys^{1034}-Ile^{1035} site, and a slower migrating band would appear. In fact, we did not observe an additional BACE1-cleaved Jag1-CTF$_\beta$ in Jag1-Lys^{1050} expression (Fig. 4), suggesting that cleavage at the Lys^{1034}-Ile^{1035} site is not readily accessible by BACE1 in cells.

When BACE1 activity was inhibited by compound BACE1 IV, we found that Jag1-CTF$_\alpha$ migrated relatively faster, consistent with the expectation of a slightly smaller size of Jag1-ctf$_\alpha$. Treatment of cells with the γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester enhanced the accumulation of Jag1-CTF fragments (two bands were visible with light exposure), confirming that Jag1 is a substrate of γ-secretase (31, 32).

It is clear that Lys-Lys mutations in Jag1 were not sufficient to completely suppress BACE1 cleavage and that additional mutagenesis will be required to identify a null mutation. Nevertheless, our results from these *in vitro* mapping and mutagenesis experiments confirmed that Jag1 is a natural substrate of BACE1, which mainly cleaves Jag1 at the Ala^{1050}-Ala^{1051} site.

BACE1 Ineffectively Cleaves Jag2—In mammalian genomes, Jag1 is one of five membrane-anchored ligands belonging to the DSL family that can interact with Notch on the cell surface to initiate Notch signaling (21). To determine whether BACE1 cleaves other Jag1 family members, we first compared their sequences in the ectodomain region. Surprisingly, there was divergence ~40 residues upstream of the transmembrane domain from each DSL member (Fig. 5*A*). The only exception is that residues surrounding the BACE1-cleavable Ala^{1050}-Ala^{1051} site in Jag1 and Jag2 showed relatively high homology (Fig. 5*A*). Residues in Jag1 and Jag2 in this region showed no homology with that of Delta-like ligand (DLL) members.

To determine whether Jag2 is a suitable substrate of BACE1, we synthesized a Jag2 peptide covering residues 1054–1072 (Jag2$_{1054-1072}$). We found that BACE1 failed to cleave this peptide under the same assay conditions for the Jag1 peptide (data not shown). However, the peptide Jag2$_{1054-1072}$ could be processed weakly when higher concentrations of purified recombinant BACE1 (4 μg instead of 1 μg) were used for the *in vitro* enzymatic reaction. As shown in Fig. 5*B*, the cleavage site was located between Lys^{1062} and Ala^{1063}, comparable with the site in Jag1 (Fig. 5*A*).

For Jag2 cleavage by α-secretase, we noted that ADAM10 effectively cleaved the Jag2$_{1054-1072}$ peptide at the predicted site (Fig. 5*D*). Contrary to our expectation, this same peptide was barely cleaved by ADAM17 under conditions similar to Jag1 cleavage site mapping (data not shown). We inferred that the slight difference of sequences surrounding the EV sites (AV<u>A</u>EV<u>R</u>V to AV<u>T</u>EV<u>K</u>V) might be sufficient to suppress cleavage of Jag2 by ADAM17.

To further verify these cleavage levels, we examined processing of Jag2 in transfected HEK-293 cells. We noted that C-ter-

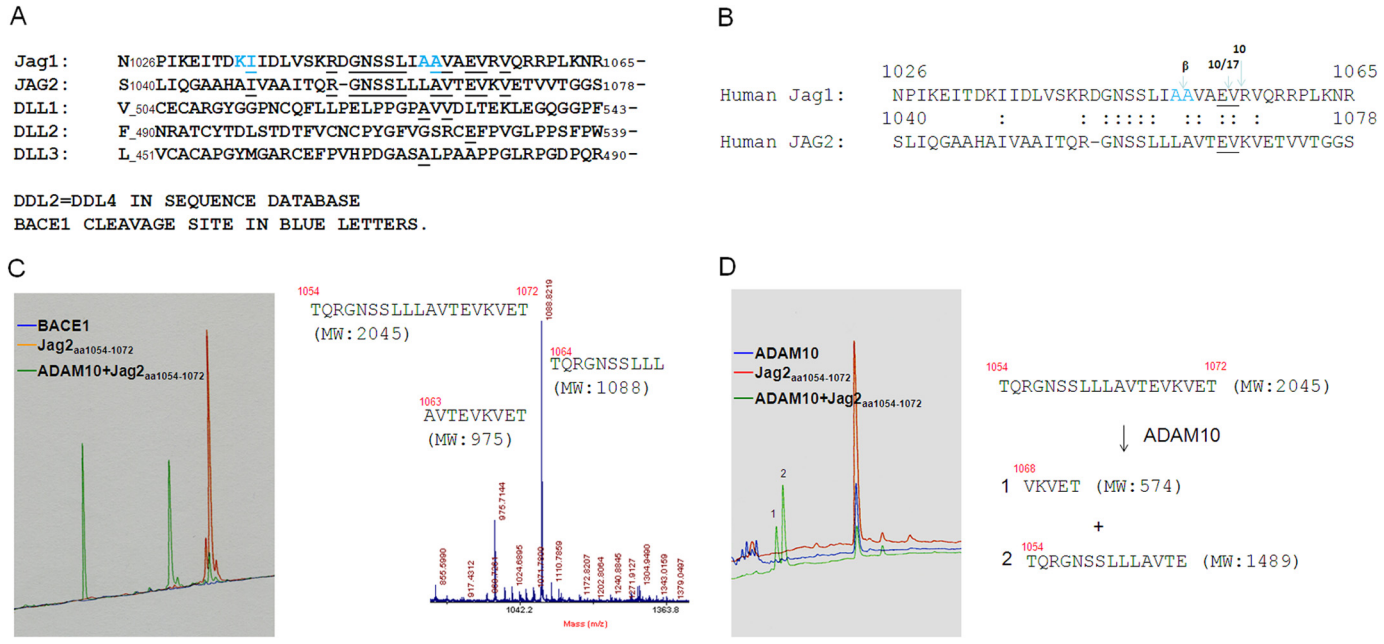

FIGURE 5. **Jag2 is efficiently cleaved by ADAM10.** *A*, alignment of ectodomain sequences from human Jag1, Jag2, and Delta-like proteins (*DLL1–3*). The identical residues between Jag1 and Jag2 are *underlined*. Potential BACE1 cleavage residues are shown by *blue letters*. *B*, the aligned Jag1 and Jag2 sequences show that both BACE1 cleavage sites in Jag1 were not identical with those in Jag2, whereas the EV site was identical between Jag1 and Jag2. *C*, a synthetic peptide $Jag2_{1054-1072}$ was incubated with recombinant BACE1 $Jag2_{1054-1072}$. BACE1 cleaves this peptide between Lys and Ala, as shown by HPLC separation and MALDI-TOF mass spectrometry. *MW*, molecular weight. *D*, cleavage of this same peptide by ADAM10 was summarized on the basis of separation by HPLC. Each peak was analyzed by MALDI-TOF mass spectrometry, and the molecular weight of the cleaved product is illustrated.

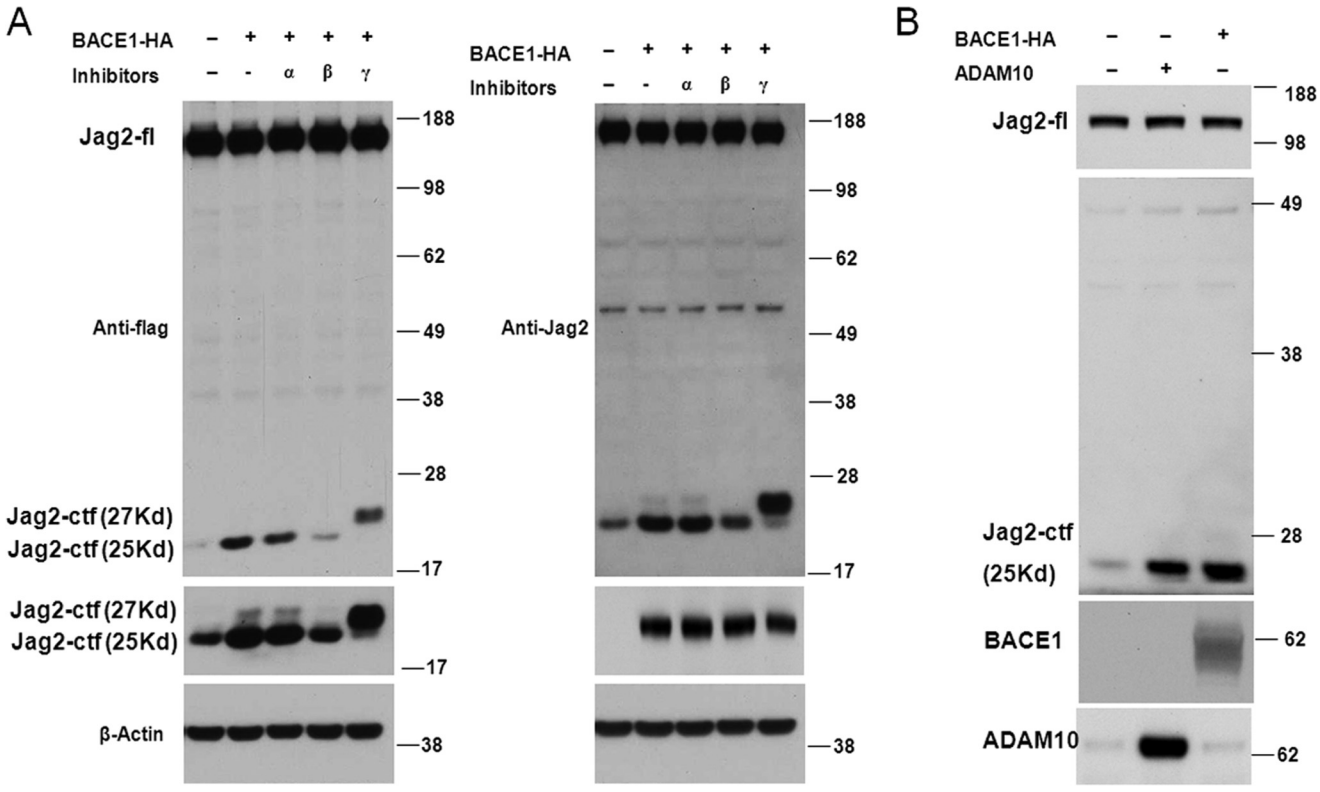

FIGURE 6. **Processing of Jag2 in cultured cells.** *A*, HEK293 cells were transfected with a C-terminal FLAG-tagged Jag2 expression construct, and stably expressing cells with high levels of Jag2 were transfected with BACE1-HA. BACE1 inhibitor IV (a BACE1 inhibitor), GM6001 (a pan-ADAM inhibitor), and *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (a γ-secretase inhibitor) were used for inhibiting the corresponding enzyme in cultured cells. Either anti-FLAG or anti-Jag2 was used for detecting Jag2 and its processing products. *B*, the same Jag2-expressing cell line was transfected with either BACE1 or ADAM10 for 48 h, and protein lysates were examined using an anti-Jag2 antibody.

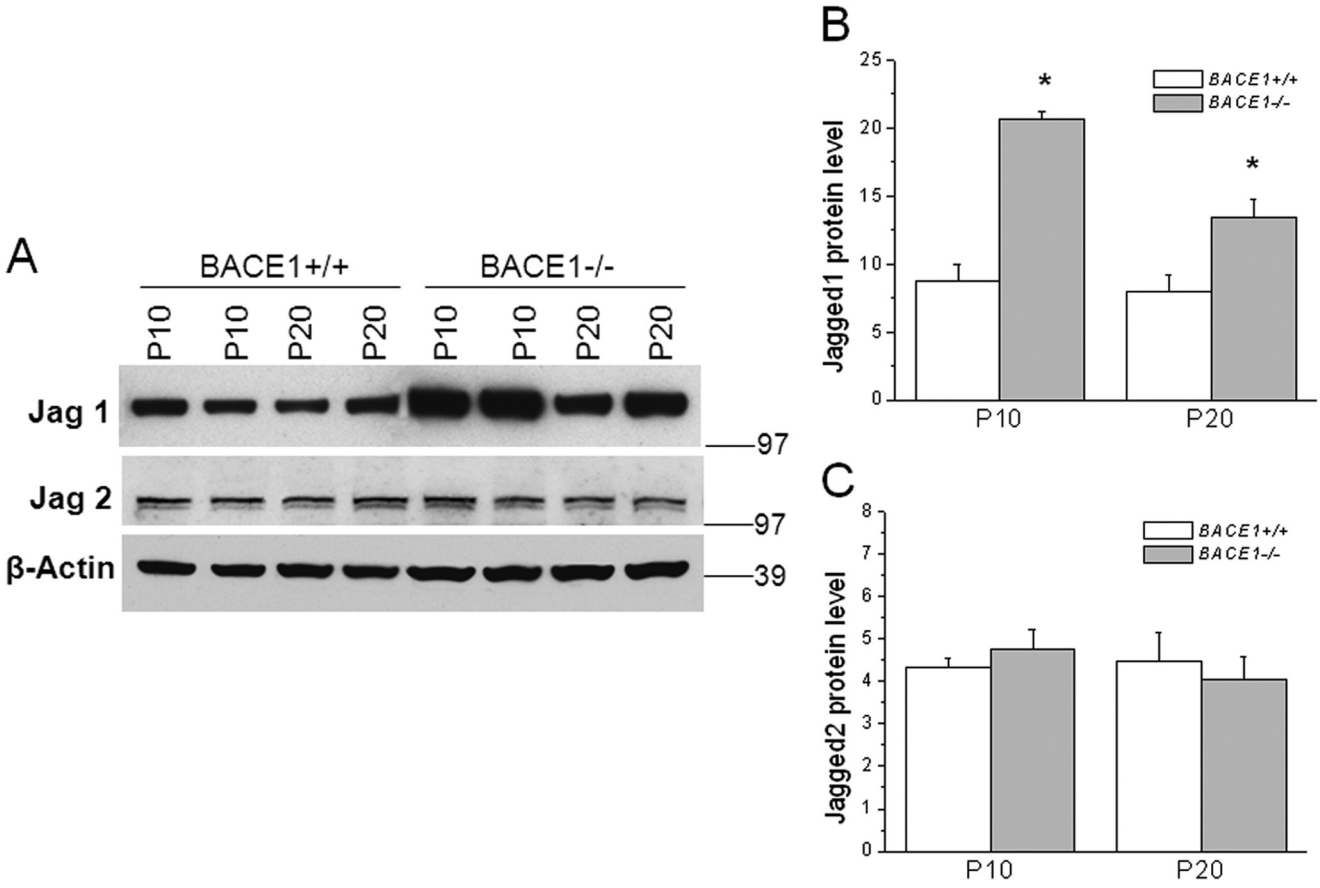

FIGURE 7. **Processing of Jag1 and Jag2 in brain lysates.** *A*, brain lysates from BACE1-null mice were used to examine the levels of Jag1 and Jag2. Jag1 levels were higher in BACE1-null mice compared with wild-type littermates, consistent with prior reports. On the other hand, levels of Jag2 were not significantly altered in BACE1-null brains. *B* and *C*, the relative levels of full-length Jag1 or Jag2 over the loading β-actin control.

minal Jag2 fragments were undetectable in transiently transfected HEK293 cells, although full-length Jag2 was highly expressed. To circumvent this, we established stable cell lines that expressed Jag2 in high abundance, as suggested in a prior publication (31). Overexpressed BACE1 clearly increased the C-terminal fragment (Jag2-ctf), which migrated near 25 kD. Its intensity was reduced when a BACE1 inhibitor was applied (Fig. 6*A*). We also overexpressed ADAM10 in the same Jag2-expressing cell line and showed enhanced Jag2-ctf (Fig. 6*B*). Similar to the cleavage of Jag1, BACE1-cleaved Jag2-ctf and ADAM10-cleaved Jag2-ctf were not separable on the gel because of their sites being in close proximity (Fig. 6). Longer exposure of the gel showed a weak band that migrated at 27 kD, which was accumulated significantly by the γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester. A prior study suggests that Jag2-ctf is a substrate that is processed by γ-secretase (31), and our result is consistent with this observation. Constant cleavage of Jag2-ctf by γ-secretase also explains why Jag2 is not readily detectable by routine Western blotting.

We also examined the levels of Jag2 in BACE1-null mouse brains. Consistent with the above biochemical characterization, the levels of full-length Jag2 in BACE1-null mouse cortices or hippocampi were not altered visibly compared with wild-type littermates (Fig. 7). This is in contrast to the noticeable increase in Jag1 levels in BACE1-null brains. Together, these data show that Jag1 is readily cleaved by BACE1 compared with Jag2 and that BACE1 may differentially regulate the Jag1-Notch and Jag2-Notch pathways because of this differential processing activity.

DISCUSSION

Jag1, a membrane-anchored signaling molecule, exerts its signaling activity by binding to its cognate Notch receptor (21). Mice deficient in Jag1 are embryonically lethal because of the failure of remodeling the embryonic vasculature (33). Mutations of Jag1 in humans are linked to the autosomal dominant disorder Alagille syndrome (34–36), which is characterized by intrahepatic cholestasis and abnormalities of the heart, eyes, and vertebrae as well as by a characteristic facial appearance. Because of the indispensable role of Jag1 during development, it is extremely important to understand how the activity of Jag1 is regulated specifically and why the loss of Jag1 is not compensated for by its DSL family members.

It has been demonstrated that paracrine interactions between membrane-anchored Notch ligands, such as Jag1, and Notch initiates subsequent cleavage of Notch by ADAM17, also known as TACE (TNF-α converting enzyme), and γ-secretase (37, 38). Presumably, the level of membrane-anchored Jag1 dictates its signaling activity. In this study, we provide biochemical evidence that BACE1 sheds Jag1 at the ectodomain and regulates the available levels of membrane-anchored, full-length

Jag1. According to a model of the Jag-Notch pathway, Jag1 signaling activity is likely up-regulated in BACE1-null mice because of abolished cleavage (20).

Two BACE1-cleavable sites were found when a long peptide was used in our *in vitro* enzymatic mapping. The additional cleavage of Lys^{1034}-Ile^{1035} was likely due to an *in vitro* enzymatic star activity. Such a star activity could be ruled out by examination of processing patterns in cultured cells. For example, BACE2 has been shown to cleave Swedish mutant APP peptide in an *in vitro* assay, but analyses of the C-terminal fragment ruled out this cleavage (39–41). By analyzing mutant Jag1, we showed that BACE1 would essentially cleave Jag1 on the Ala^{1050}-Ala^{1051} site. A previous report suggested that Jag1 is only cleaved by ADAM17 but not by ADAM10 (26). Our *in vitro* enzymatic mapping study shows that ADAM10 can cleave Jag1 at two neighboring sites, whereas ADAM17 cleaves Jag1 at only one site (Fig. 3*D*). Hence, all three proteases may coordinately process Jag1 to finely regulate the Jag1-Notch signaling activity.

Jag2 appears to be less efficiently processed by both BACE1 and α-secretase because the expected 27-K_d C-terminal fragment was not readily expressed under normal transiently transfected conditions (this study and Ref. 32), contrary to conventional transfection studies. This is likely due to the change of residues surrounding the cleavage sites. Prior assays showed that small changes of P and P′ residues in the BACE1 cleavage site can alter catalytic efficiency (23, 42, 43). Further systematic enzyme kinetic assays may verify this conjecture.

We demonstrated recently that BACE1 regulates astrogenesis and neurogenesis via control of the Jag1-Notch pathway (20). In mouse brains, during early development, Jag2 is expressed by postmitotic differentiating neurons and activates the Jag2-Notch pathway (44). We showed that the level of full-length Jag2 in brain lysates from BACE1-null mice was not altered significantly. This is consistent with our postulation that Jag2 is less effectively processed by endogenous levels of BACE1. Despite this inefficient cleavage, we argue that cleavage of Jag2 by BACE1 may contribute to certain biological effects under specific conditions that include lowered ADAM10 activity.

Because the ectodomain sequences of Delta-like proteins (DLL1, DLL2, and DLL3) are completely divergent from the similar region of Jag1/Jag2, it is difficult to predict whether BACE1 cleaves these DLL proteins, and future mapping studies will be required for a definitive answer. Nevertheless, our results suggest that BACE1 can selectively regulate Jag-Notch pathways by cleaving certain members of Notch ligands.

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REFERENCES

1. Vassar, R., Bennett, B. D., Babu-Khan, S., Kahn, S., Mendiaz, E. A., Denis, P., Teplow, D. B., Ross, S., Amarante, P., Loeloff, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lile, J., Jarosinski, M. A., Biere, A. L., Curran, E., Burgess, T., Louis, J. C., Collins, F., Treanor, J., Rogers, G., and Citron, M. (1999) β-Secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* **286,** 735–741
2. Yan, R., Bienkowski, M. J., Shuck, M. E., Miao, H., Tory, M. C., Pauley, A. M., Brashier, J. R., Stratman, N. C., Mathews, W. R., Buhl, A. E., Carter, D. B., Tomasselli, A. G., Parodi, L. A., Heinrikson, R. L., and Gurney, M. E. (1999) Membrane-anchored aspartyl protease with Alzheimer's disease β-secretase activity. *Nature* **402,** 533–537
3. Hussain, I., Powell, D., Howlett, D.R., Tew, D. G., Meek, T. D., Chapman, C., Gloger, I. S., Murphy, K. E., Southan, C. D., Ryan, D. M., Smith, T. S., Simmons, D. L., Walsh, F. S., Dingwall, C., and Christie, G. (1999) Identification of a novel aspartic protease (Asp 2) as β-secretase. *Mol. Cell Neurosci.* **14,** 419–427
4. Sinha, S., Anderson, J. P., Barbour, R., Basi, G. S., Caccavello, R., Davis, D., Doan, M., Dovey, H. F., Frigon, N., Hong, J., Jacobson-Croak, K., Jewett, N., Keim, P., Knops, J., Lieberburg, I., Power, M., Tan, H., Tatsuno, G., Tung, J., Schenk, D., Seubert, P., Suomensaari, S. M., Wang, S., Walker, D., Zhao, J., McConlogue, L., and John, V. (1999) Purification and cloning of amyloid precursor protein β-secretase from human brain. *Nature* **402,** 537–540
5. Lin, X., Koelsch, G., Wu, S., Downs, D., Dashti, A., and Tang, J. (2000) Human aspartic protease memapsin 2 cleaves the β-secretase site of β-amyloid precursor protein. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 1456–1460
6. Yang, L. B., Lindholm, K., Yan, R., Citron, M., Xia, W., Yang, X. L., Beach, T., Sue, L., Wong, P., Price, D., Li, R., and Shen, Y. (2003) Elevated β-secretase expression and enzymatic activity detected in sporadic Alzheimer disease. *Nat. Med.* **9,** 3–4
7. Selkoe, D. J., Yamazaki, T., Citron, M., Podlisny, M. B., Koo, E. H., Teplow, D. B., and Haass, C. (1996) The role of APP processing and trafficking pathways in the formation of amyloid β-protein. *Ann. N.Y. Acad. Sci.* **777,** 57–64
8. Cai, H., Wang, Y., McCarthy, D., Wen, H., Borchelt, D. R., Price, D. L., and Wong, P. C. (2001) BACE1 is the major β-secretase for generation of Aβ peptides by neurons. *Nat. Neurosci.* **4,** 233–234
9. Luo, Y., Bolon, B., Kahn, S., Bennett, B. D., Babu-Khan, S., Denis, P., Fan, W., Kha, H., Zhang, J., Gong, Y., Martin, L., Louis, J. C., Yan, Q., Richards, W. G., Citron, M., and Vassar, R. (2001) Mice deficient in BACE1, the Alzheimer's β-secretase, have normal phenotype and abolished β-amyloid generation. *Nat. Neurosci.* **4,** 231–232
10. Roberds, S. L., Anderson, J., Basi, G., Bienkowski, M. J., Branstetter, D. G., Chen, K. S., Freedman, S. B., Frigon, N. L., Games, D., Hu, K., Johnson-Wood, K., Kappenman, K. E., Kawabe, T. T., Kola, I., Kuehn, R., Lee, M., Liu, W., Motter, R., Nichols, N. F., Power, M., Robertson, D. W., Schenk, D., Schoor, M., Shopp, G. M., Shuck, M. E., Sinha, S., Svensson, K. A., Tatsuno, G., Tintrup, H., Wijsman, J., Wright, S., and McConlogue, L. (2001) BACE knockout mice are healthy despite lacking the primary β-secretase activity in brain: implications for Alzheimer's disease therapeutics. *Hum. Mol. Genet.* **10,** 1317–1324
11. Yan, R., Han, P., Miao, H., Greengard, P., and Xu, H. (2001) The transmembrane domain of the Alzheimer's β-secretase (BACE1) determines its late Golgi localization and access to β-amyloid precursor protein (APP) substrate. *J. Biol. Chem.* **276,** 36788–36796
12. Willem, M., Garratt, A. N., Novak, B., Citron, M., Kaufmann, S., Rittger, A., DeStrooper, B., Saftig, P., Birchmeier, C., and Haass, C. (2006) Control of peripheral nerve myelination by the β-secretase BACE1. *Science* **314,** 664–666
13. Hu, X., Hicks, C. W., He, W., Wong, P., Macklin, W. B., Trapp, B. D., and Yan, R. (2006) Bace1 modulates myelination in the central and peripheral nervous system. *Nat. Neurosci.* **9,** 1520–1525
14. Hu, X., He, W., Diaconu, C., Tang, X., Kidd, G. J., Macklin, W. B., Trapp, B. D., and Yan, R. (2008) Genetic deletion of BACE1 in mice affects remyelination of sciatic nerves. *FASEB J.* **22,** 2970–2980
15. Savonenko, A. V., Melnikova, T., Laird, F. M., Stewart, K. A., Price, D. L., and Wong, P. C. (2008) Alteration of BACE1-dependent NRG1/ErbB4 signaling and schizophrenia-like phenotypes in BACE1-null mice. *Proc. Natl. Acad. Sci. U.S.A.* **105,** 5585–5590
16. Kim, D. Y., Carey, B. W., Wang, H., Ingano, L. A., Binshtok, A. M., Wertz, M. H., Pettingell, W. H., He, P., Lee, V. M., Woolf, C. J., and Kovacs, D. M. (2007) BACE1 regulates voltage-gated sodium channels and neuronal activity. *Nat. Cell Biol.* **9,** 755–764

17. Cai, J., Qi, X., Kociok, N., Skosyrski, S., Emilio, A., Ruan, Q., Han, S., Liu, L., Chen, Z., Bowes Rickman, C., Golde, T., Grant, M. B., Saftig, P., Serneels, L., de Strooper, B., Joussen, A. M., and Boulton, M. E. (2012) β-Secretase (BACE1) inhibition causes retinal pathology by vascular dysregulation and accumulation of age pigment. *EMBO Mol. Med.* **4,** 980–991
18. Kuhn, P. H., Koroniak, K., Hogl, S., Colombo, A., Zeitschel, U., Willem, M., Volbracht, C., Schepers, U., Imhof, A., Hoffmeister, A., Haass, C., Roßner, S., Bräse, S., and Lichtenthaler, S. F. (2012) Secretome protein enrichment identifies physiological BACE1 protease substrates in neurons. *EMBO J.* **31,** 3157–3168
19. Hemming, M. L., Elias, J. E., Gygi, S. P., and Selkoe, D. J. (2009) Identification of β-secretase (BACE1) substrates using quantitative proteomics. *PLoS ONE* **4,** e8477
20. Hu, X., He, W., Luo, X., Tsubota, K. E., and Yan, R. (2013) BACE1 regulates hippocampal astrogenesis via the Jagged1-Notch pathway. *Cell Rep.* **4,** 40–49
21. Kopan, R., and Ilagan, M. X. (2009) The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell* **137,** 216–233
22. He, W., Lu, Y., Qahwash, I., Hu, X. Y., Chang, A., and Yan, R. (2004) Reticulon family members modulate BACE1 activity and amyloid-β peptide generation. *Nat. Med.* **10,** 959–965
23. Tomasselli, A. G., Qahwash, I., Emmons, T. L., Lu, Y., Leone, J. W., Lull, J. M., Fok, K. F., Bannow, C. A., Smith, C. W., Bienkowski, M. J., Heinrikson, R. L., and Yan, R. (2003) Employing a superior BACE1 cleavage sequence to probe cellular APP processing. *J. Neurochem.* **84,** 1006–1017
24. Luo, X., Prior, M., He, W., Hu, X., Tang, X., Shen, W., Yadav, S., Kiryu-Seo, S., Miller, R., Trapp, B. D., and Yan, R. (2011) Cleavage of neuregulin-1 by BACE1 or ADAM10 protein produces differential effects on myelination. *J. Biol. Chem.* **286,** 23967–23974
25. Boyer-Di Ponio, J., Wright-Crosnier, C., Groyer-Picard, M. T., Driancourt, C., Beau, I., Hadchouel, M., and Meunier-Rotival, M. (2007) Biological function of mutant forms of JAGGED1 proteins in Alagille syndrome: inhibitory effect on Notch signaling. *Hum. Mol. Genet.* **16,** 2683–2692
26. Parr-Sturgess, C. A., Rushton, D. J., and Parkin, E. T. (2010) Ectodomain shedding of the Notch ligand Jagged1 is mediated by ADAM17, but is not a lipid-raft-associated event. *Biochem. J.* **432,** 283–294
27. Liu, K., Doms, R. W., and Lee, V. M. (2002) Glu11 site cleavage and N-terminally truncated Aβ production upon BACE overexpression. *Biochemistry* **41,** 3128–3136
28. Qahwash, I., He, W., Tomasselli, A., Kletzien, R. F., and Yan, R. (2004) Processing amyloid precursor protein at the β-site requires proper orientation to be accessed by BACE1. *J. Biol. Chem.* **279,** 39010–39016
29. Huse, J. T., Liu, K., Pijak, D. S., Carlin, D., Lee, V. M., and Doms, R. W. (2002) β-Secretase processing in the *trans*-Golgi network preferentially generates truncated amyloid species that accumulate in Alzheimer's disease brain. *J. Biol. Chem.* **277,** 16278–16284
30. Fluhrer, R., Capell, A., Westmeyer, G., Willem, M., Hartung, B., Condron, M. M., Teplow, D. B., Haass, C., and Walter, J. (2002) A non-amyloidogenic function of BACE-2 in the secretory pathway. *J. Neurochem.* **81,** 1011–1020
31. Ikeuchi, T., and Sisodia, S. S. (2003) The Notch ligands, Delta1 and Jagged2, are substrates for presenilin-dependent "γ-secretase" cleavage. *J. Biol. Chem.* **278,** 7751–7754
32. LaVoie, M. J., and Selkoe, D. J. (2003) The Notch ligands, Jagged and Delta, are sequentially processed by α-secretase and presenilin/γ-secretase and release signaling fragments. *J. Biol. Chem.* **278,** 34427–34437
33. Xue, Y., Gao, X., Lindsell, C. E., Norton, C. R., Chang, B., Hicks, C., Gendron-Maguire, M., Rand, E. B., Weinmaster, G., and Gridley, T. (1999) Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. *Hum. Mol. Genet.* **8,** 723–730
34. Oda, T., Elkahloun, A. G., Pike, B. L., Okajima, K., Krantz, I. D., Genin, A., Piccoli, D. A., Meltzer, P. S., Spinner, N. B., Collins, F. S., and Chandrasekharappa, S. C. (1997) Mutations in the human Jagged1 gene are responsible for Alagille syndrome. *Nat. Genet.* **16,** 235–242
35. Li, L., Krantz, I. D., Deng, Y., Genin, A., Banta, A. B., Collins, C. C., Qi, M., Trask, B. J., Kuo, W. L., Cochran, J., Costa, T., Pierpont, M. E., Rand, E. B., Piccoli, D. A., Hood, L., and Spinner, N. B. (1997) Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. *Nat. Genet.* **16,** 243–251
36. Yuan, Z. R., Kohsaka, T., Ikegaya, T., Suzuki, T., Okano, S., Abe, J., Kobayashi, N., and Yamada, M. (1998) Mutational analysis of the Jagged 1 gene in Alagille syndrome families. *Hum. Mol. Genet.* **7,** 1363–1369
37. Schroeter, E. H., Kisslinger, J. A., and Kopan, R. (1998) Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature* **393,** 382–386
38. De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J. S., Schroeter, E. H., Schrijvers, V., Wolfe, M. S., Ray, W. J., Goate, A., and Kopan, R. (1999) A presenilin-1-dependent γ-secretase-like protease mediates release of Notch intracellular domain. *Nature* **398,** 518–522
39. Farzan, M., Schnitzler, C. E., Vasilieva, N., Leung, D., and Choe, H. (2000) BACE2, a β-secretase homolog, cleaves at the β site and within the amyloid-β region of the amyloid-β precursor protein. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 9712–9717
40. Yan, R., Munzner, J. B., Shuck, M. E., and Bienkowski, M. J. (2001) BACE2 functions as an alternative α-secretase in cells. *J. Biol. Chem.* **276,** 34019–34027
41. Sun, X., He, G., and Song, W. (2006) BACE2, as a novel APP θ-secretase, is not responsible for the pathogenesis of Alzheimer's disease in Down syndrome. *FASEB J.* **20,** 1369–1376
42. Turner, R. T., 3rd, Loy, J. A., Nguyen, C., Devasamudram, T., Ghosh, A. K., Koelsch, G., and Tang, J. (2002) Specificity of memapsin 1 and its implications on the design of memapsin 2 (β-secretase) inhibitor selectivity. *Biochemistry* **41,** 8742–8746
43. Grüninger-Leitch, F., Schlatter, D., Küng, E., Nelböck, P., and Döbeli, H. (2002) Substrate and inhibitor profile of BACE (β-secretase) and comparison with other mammalian aspartic proteases. *J. Biol. Chem.* **277,** 4687–4693
44. Nelson, B. R., Hartman, B. H., Ray, C. A., Hayashi, T., Bermingham-McDonogh, O., and Reh, T. A. (2009) Acheate-scute like 1 (Ascl1) is required for normal Delta-like (Dll) gene expression and notch signaling during retinal development. *Dev. Dyn.* **238,** 2163–2178