Intramembrane cleavage of TREM2 is determined by its intrinsic structural dynamics

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Abstract (147 words, 1027 characters)

Sequence variants of the microglial expressed TREM2 (triggering receptor expressed on myeloid cells 2) are a major risk factor for late onset Alzheimer's disease. TREM2 requires a stable interaction with DAP12 in the membrane to initiate signaling, which is terminated by TREM2 ectodomain shedding and subsequent intramembrane cleavage by γ -secretase. To understand the structural basis for the specificity of the intramembrane cleavage event, we determined the solution structure of the TREM2 transmembrane helix (TMH). Due to the presence of a charged amino acid in the membrane region the TREM2-TMH adopts a kinked structure with increased flexibility. Charge removal leads to TMH stabilization and reduced dynamics, similar to its structure in complex with DAP12. Strikingly, these dynamical features perfectly correlate with the site of the initial γ -secretase cleavage event. These data suggest an unprecedented cleavage mechanism by γ -secretase where flexible TMH regions are identified to initiate substrate cleavage.

Single transmembrane helices remain a major challenge for structure determination approaches since they are highly flexible and therefore not suitable for crystallization. Solution NMR offers the advantage to study these dynamic domains in different membrane mimetics in solution and to obtain

detailed information about their dynamical features. Increasing evidence shows that these hydrophobic membrane spanning helices are more than just membrane anchors but often also involved in transmembrane signal transduction¹. Furthermore, intramembrane complex formation with adaptor molecules and homo-oligomerization as well as regulated intramembrane proteolysis are crucial events in the context of intracellular signaling, transcriptional regulation of gene expression, and degradation of membrane proteins, respectively. Regulated intramembrane proteolysis is also key for amyloid β -peptide ($A\beta$) generation², the major component of amyloid plaques invariably found in all Alzheimer's disease (AD) brains³. Here, the β -amyloid precursor protein (APP) is first cleaved by the β -site APP cleaving enzyme (BACE1)⁴, which removes the bulk of the ectodomain. γ -Secretase⁵, then generates $A\beta$ from the membrane-retained APP C-terminal fragment (CTF) by a number of sequential, approximately 3 amino acids spaced cleavages, initiated at the so-called ε -cleavage site located very close to the C-terminal end of the TMH⁶. Numerous type-I oriented γ -secretase substrates have been identified in the past^{7,8}. Like for APP, sheddases such as BACE1 or members of the ADAM (A Disintegrin and metalloproteinase domain-containing protein) family generate an N-terminally truncated substrate small enough to enter the γ -secretase complex^{9,10}. However, it is still unclear how γ -secretase selects its substrates, since not every protein with a small enough ectodomain can be cleaved by γ -secretase¹¹. Thus, in addition to membrane orientation and size of the ectodomain other molecular signatures must exist allowing interaction with the γ -secretase complex and recruitment to the active site^{12,13}. Unlike soluble proteases γ -secretase and other intramembrane cleaving enzymes usually do not recognize their substrates via short linear sequence motifs¹⁴. Rather they more likely rely on largely unknown intrinsic structural features of the transmembrane domain which provide flexibility enabling the protease to unfold the transmembrane helix and to cleave the peptide bonds. Indeed, mutations that affect transmembrane domain flexibility in the cleavage site region of APP can markedly alter γ -secretase cleavage¹³. Likewise, flexibility via a naturally occurring hinge in the TMH as in the case of APP can also be critical for γ -secretase cleavage¹⁵. Recent structural studies of γ -secretase in complex with APP or Notch1 substrates suggest that unfolding of the cleavage region is caused by formation of a hybrid β -strand between substrate and enzyme^{16,17}. Such major structural

changes may be facilitated by intrinsic dynamics of the substrate transmembrane domain. Despite these recent insights, a clear intrinsic structure/function (i.e. cleavage) relationship has not been established for γ -secretase substrates. It has therefore remained unclear which properties of their TMHs determine whether they will be identified as a substrate and cleaved by the enzyme.

We now used the triggering receptor expressed on myeloid cells (TREM) 2 as a substrate to investigate if and how structural flexibility of the transmembrane helix (TMH) could be involved in substrate recognition by γ -secretase. TREM2 is a type I membrane protein, which within the brain is exclusively expressed in microglia¹⁸. Large genome wide analyses revealed sequence variants specifically within TREM2, which strongly increase the risk for several neurodegenerative diseases¹⁹⁻²¹. Apparently, these sequence variants prevent activation of microglia upon neuronal insults, by locking them in a homeostatic state^{22,23}. Loss of TREM2 function is associated with reduced proliferation, survival, phagocytosis and lipid sensing of myeloid cells, as well as brain wide reduced energy metabolism and cerebral blood flow²⁴⁻²⁶. TREM2 requires association with the disulfide-linked homo-dimeric signaling partner DNAX activating protein of 12 kDa (DAP12) or DAP10 upon ligand activation for downstream signaling^{27,28}. Association of DAP12 with partner proteins is mediated by a salt bridge between their TMHs in the membrane, as previously shown for DAP12 in complex with the natural killer (NK) cell-activating receptor NKG2C²⁹. In TREM2, a lysine residue is located within the TMH and its homozygous mutation has been connected to various diseases, like Nasu-Hakola disease³⁰.

TREM2-mediated signaling is terminated by shedding of the extracellular domain by ADAM 10 or 17 producing soluble TREM2 (sTREM2) and a membrane-tethered C-terminal fragment (TREM2-CTF)^{31,32}, which subsequently undergoes regulated sequential intramembrane proteolysis³³ (**Fig. 1a**). This degradation process is initiated at the C-terminal end of the TMH at the so-called ϵ -site. Since γ -secretase activity leads to TREM2-CTF degradation, its inhibition enhances complex formation between TREM2-CTF and DAP12³³. In turn, this interaction leads to a stabilization of the TREM2-CTF³⁴ even without γ -secretase inhibition. Apparently, DAP12 and the TREM2-CTF must dissociate to enable further cleavage by γ -secretase^{33,34}. Cleavage of the TREM2-CTF by γ -secretase results in the release of the TREM2 intracellular domain (TREM2-ICD)^{2,33} and consequently in the

degradation of the membrane retained CTF. Complete degradation of the CTF is crucial to prevent inhibition of DAP12 that would encompass productive full-length-dependent TREM2 signaling.³³ The structural and dynamical features of a substrate TMH that are recognized and decoded by γ -secretase are still poorly defined. By solving the structure of the TREM2-TMH, we found that a region of lower structural order nearby a membrane-located charged residue is recognized and site-specifically cleaved by γ -secretase. Reduction of the intrinsic dynamics of TREM2-TMH by charge removal results in an altered cleavage site that is located at the end of the more stable α -helical secondary structure. Thus, we suggest that TREM2 cleavage by γ -secretase is regulated by an unprecedented mechanism based on the identification of flexible and less ordered regions.

Results

TREM2-TMH adopts a charge-induced kinked α -helical structure

For our *in vitro* and structural studies, the TREM2-TMH could be produced in high yields as a fusion protein with GB1 and purified in dodecyl-phosphocholine (DPC) micelles. This yielded a homogenous preparation as judged by SEC (**Fig. S1a** and **b**) that was suitable for further structural investigations. Next, using a U-²H, ¹³C, ¹⁵N, -labeled TREM2-TMH (aa 161-206) sample in 300 mM DPC micelles, backbone resonance assignments were obtained for 88% or all non-proline residues using a set of 3D-triple resonance experiments at 37 °C (**Fig. 1b**). Estimation of the secondary structure content using secondary ¹³C α and ¹³C β chemical shifts yielded an α -helical secondary structure between residues 174 and 198, corresponding to an α -helical content of 50% (**Fig. 1c**), in excellent agreement with the results obtained by CD spectroscopy (**Fig. 1d**). However, the magnitude of the secondary chemical shift indicating α -helix propensity decreases toward the C-terminal end of the helix. Thus, we moved on and determined the structure of TREM2-TMH using nuclear Overhauser effect (NOE) NMR spectroscopy (NOESY). For most parts of the α -helix of TREM2, a typical pattern could be observed in the NOESY where cross-peaks arise from amide protons of sequential neighbors (**Fig. 1e**). However, a 3-residue stretch ranging from A190-A192 at the C-terminal half of the TM helix did not show sequential NOE contacts and exhibited poor signal intensity, indicative of enhanced dynamics and lack of regular α -helical secondary structure. Thus, in the calculated structure of

TREM2-TMH a pronounced kink in the TM helix appears followed by a short C-terminal helix whose orientation relative to the longer N-terminal TM helix is variable (**Fig. 1f**). Due to the fact that TREM2-TMH harbors a charged lysine residue at position 186 (K186) that is located in the hydrophobic transmembrane region, we anticipate that this energetically unfavorable location is a key factor that determines its conformation and dynamics. In order to rule out the effect of a non-planar surface of a detergent micelle, we were able to insert the TREM2-TMH into di-myristoyl-glycero-phosphocholine/di-myristoyl-glycero-phosphoglycerol (DMPC/DMPG, 3:1) phospholipid nanodiscs assembled with MSP1D1ΔH5^{35,36} (**Fig. S1c,d**) and obtained high-quality 2D NMR spectra that enabled backbone resonance assignments (**Fig. S2a**), as well the acquisition of 3D-NOESY spectra (**Fig. S2c**). In this lipid bilayer environment, in comparison to DPC micelles, TREM2-TMH shows the same chemical-shift-derived secondary structure content (**Fig. S2b**) and an identical NOE pattern, where no contacts can be observed within the amino acid stretch between residues 190 and 192 (**Fig. S2c**). In line with these observations, a comparison of 2D-[¹H,¹⁵N]-TROSY spectra of TREM2-TMH wt in DPC micelles and DMPC/DMPG nanodiscs shows only minor chemical shift perturbations (**Fig. S2d**). The observed spectral changes are most likely caused by the slightly different electronic environment provided by DPC micelles or DMPC/DMPG lipids, and not by changes in the structure of the TMH. We further investigated the influence of the membrane thickness on the NMR spectra by using nanodiscs assembled with palmitoyl-oleyl-glycero-phosphocholine/palmitoyl-oleyl-glycero-phosphoglycerol (POPC/POPG, 3:1), that forms a thicker membrane as compared to DMPC. As shown in **Fig. S2e**, the 2D-[¹H,¹⁵N]-TROSY spectra of TREM2-TMH wt in these different nanodisc systems are almost identical, providing further evidence that the structure of TREM2-TMH in DPC micelles represents the biologically relevant conformation.

TREM2-TMH structure is stabilized by binding to DAP12 or by charge removal

Next, we used NMR spectroscopy to monitor the complex formation between TREM2-TMH and the dimeric signaling protein DAP12³⁷ as well as the effect of charge removal in TREM2-TMH (K186A) (**Fig. 2a,b**). Upon complex formation, DAP12 induced marked chemical shift perturbations in the 2D spectrum of ²H,¹⁵N-labeled TREM2-TMH that are located at the C-terminal end of the

transmembrane helix, i.e. in the unfolded kink region (**Fig. 2c**). Addition of mutant DAP12 lacking its negative charge (D50A) to isotope-labeled TREM2-TMH did not lead to chemical shift perturbations (**Fig. S3**), confirming that the interaction between K186 in TREM2 and D50 in DAP12 is governing complex formation. In order to monitor the structural effects of DAP12 binding on TREM2-TMH, we determined the solution structure of isotope-labeled TREM2-TMH in complex with unlabeled DAP12 using NMR chemical shift information and NOESY experiments. In line with the previous finding that DAP12 binding leads to TREM2-CTF stabilization³⁴, we here were able to show that this interaction leads to the formation of a continuous α -helix ranging from residues 174 to 198 without the previously kinked region (**Fig. 2d**). The structure of the TREM2-TMH in the DAP12-bound conformation was used to obtain a structural model of the complex with DAP12. In the structure of DAP12 in complex with the transmembrane helical region of NKG2C²⁹, the main interaction between DAP12 and NKG2C is a salt bridge between Asp50 and Lys89. Since DAP12 is a type I and NKG2C a type II transmembrane protein, the interaction takes place in an antiparallel manner in this case. In contrast, TREM2 is a type I transmembrane protein and thus binds to DAP12 in a parallel orientation. Thus, DAP12 appears to be capable of recognizing partner proteins in both orientations. The structural information on the binding site in DAP12 deduced from its complex structure with NKG2C²⁹ was utilized for NMR-data driven docking calculations of the TREM2-DAP12 complex using the HADDOCK webserver³⁸. For TREM2-TMH, NMR chemical shift perturbations (**Fig. 2c, upper panel**), were used to define its interface with DAP12. The complex structure that showed the best HADDOCK score and the correct parallel orientation of the two proteins was used for further visualization. In this complex structural model TREM2 and DAP12 form the expected salt bridge between K186 in TREM2 and D50 in DAP12 (**Fig. 2e**), leading to charge neutralization and TREM2-TMH helix stabilization. Since these studies clearly show that the positive charge within the TREM2-TMH is essential for the interaction with DAP12 and its structural instability, we designed a K186A variant of TREM2-TMH that can serve as a mimic of the neutralized DAP12-bound form. Indeed, a comparison of the 2D- $[^1\text{H}, ^{15}\text{N}]$ -TROSY spectra of the wild-type versus the K186A variant (**Fig. 2b**) indicates pronounced chemical shift differences. The magnitude and the overall pattern of the chemical shift perturbations along the protein sequence (**Fig. 2c, lower panel**) are very similar to the

CSPs detected upon complex formation with DAP12 (**Fig. 2c, upper panel**). CD spectroscopy with TREM2-TMH K186A indicated an increase in α -helical secondary structure content as compared to the wild-type protein (**Fig. 2f**). Thus, charge removal is most likely resulting in a similar structure of TREM2-TMH as in the complex with DAP12. NOE-based structure determination (**Fig. 2g**) of the K186A variant demonstrates that indeed a straight α -helix between residues 174 and 198 is present, without the kink that was observed in the wild-type scenario (**Fig. 2h**). Since this helix spans the membrane in a straight geometry, its C-terminal residues (196-198) are located outside the membrane, leading to reduced α -helix propensity, as probed by weaker sequential amide NOE contacts.

TREM2-TMH dynamics is mediated by interactions with the headgroups and can be abolished by mutation

Since conformational flexibility plays a crucial role in the recognition of intramembrane protease substrates, we decided to investigate the nanosecond to picosecond dynamics of TREM2-TMH wild-type as well as the K186A variant. For this, we recorded T_1 , T_2 and $\{^1\text{H}\}, ^{15}\text{N}$ -heteronuclear NOE experiments at 950 MHz and 600 MHz proton frequencies and were able to obtain a generalized order parameter (S^2) for all observed backbone amide resonances in each protein variant (**Fig. 3a,b**). S^2 can adopt values between zero and one, indicative of unrestricted or completely rigid dynamics, respectively^{39,40}. The observed pattern of S^2 values along the TREM2-TMH wt sequence correlates very well with the presence of secondary structure elements. S^2 values of around 0.9 for the ordered first half of the TMH, of around 0.6 for the unstructured kink region and 0.7 for the C-terminal helix can be extracted. These data suggest that the first half of the TMH is rigid, the kink region highly dynamic and the C-terminal helix again more rigid (**Fig. 3a**). The same set of experiments were recorded for the K186A variant. In contrast to the wild-type scenario, the entire amino acid sequence of the transmembrane part of the TREM2-TMH K186A (residues 174-195) shows order parameters of ~ 0.9 , indicating a rigid and continuous helix without flexible loop regions (**Fig. 3b**). In order to characterize the molecular basis of the observed differences in dynamics, we performed molecular dynamics simulations of TREM2-TMH wt and K186A inserted into a phospholipid bilayer membrane composed of POPC and POPG lipids, a native-like phospholipid composition. **Figs. 3c and 3d** show

snapshots of the two simulations at various time points, as indicated in each figure. We used a starting structure from the calculated NMR ensemble with linear helix geometry in both cases. During the simulation, the positive charge of the K186 side-chain in the wild-type TREM2-TMH is moving toward the phosphate moieties in the phospholipid headgroups leading to a tilt in the helix orientation relative to the membrane surface. Once the K186 side chain is in contact with the phosphate, the amino acids at the C-terminus are pushed outside the hydrophobic region of the bilayer, leading to the experimentally observed kink in helix geometry. However, in order to reproduce the formation of the kink by MD, by far more extended simulation times would be required. Since we have experimental data on the final state of the TMH, we were mostly interested to observe the contact formation between K186 and the headgroups. In contrast, the MD trajectory for the K186A variant is very stable with only minor tilting or translational motions. Due to the missing charge in the latter case the helix is stably inserted in the membrane, in agreement with the observed NMR data.

Position of TREM2-TMH in the membrane

The MD simulations of wild-type TREM2-TMH suggest that the C-terminal part of the helix is positioned outside the hydrophobic region of the membrane. In order to experimentally prove this finding, we performed paramagnetic relaxation enhancement (PRE) experiments with the TREM2-TMH in DPC micelles using the free radical agent 16-doxyl-stearic acid (16-DSA). This radical leads to NMR line broadening in protein regions that are located in the hydrophobic region of the detergent micelle, which can readily be observed by a strong decrease in signal intensity. **Fig. 4a** shows the intensity ratio of the amino acid residues in TREM2-TMH in the presence of reduced (radical quenched by ascorbic acid) or oxidized 16-DSA for the wild-type and K186A proteins. In the wild-type protein, the signal intensity ratio is strongly reduced within the transmembrane region (residues 174-189) but adopts higher values in the kink region and in the C-terminal helix. This pattern suggests that the first helix is indeed a transmembrane helix whereas the kink region and the C-terminal helix are located outside the membrane. For the K186A variant (red bars in **Fig. 4a**) the observed intensity ratios are markedly altered in comparison to the wild-type protein. In this case, there is a drop in signal intensity ratio within the entire helical secondary structure (residues 173-196), indicative of a stable

membrane-location of this region. In comparison to the wild-type protein, signal intensity ratios are markedly reduced for the C-terminal half of the TMH. This picture can be corroborated by analysis of NOE-contacts (up to 6 Å) between the backbone amide resonances of the two TREM2 variants and selected regions of the DPC molecule (**Fig. 4b**). For the wild-type protein, NOE contacts to the hydrocarbon region of DPC can be detected for the first helix, confirming that this part is embedded in the membrane. However, these contacts are completely missing for residues in the kink region and can only be faintly observed for the second helix. Furthermore, amino acid residues located in the kink region give rise to a pronounced exchange peak with water, indicating solvent accessibility and a lack of hydrogen bonds in this region. For the K186A variant, NOE contacts to the methyl and methylene moieties of DPC can be detected throughout the entire helical region, corroborating a transmembrane location of the entire helix. Using these data, a model of the membrane position of both protein variants can be constructed (**Fig. 4c**). TREM2-TMH-wt contains a transmembrane region between residues 174 and 189, followed by a kink and an α -helical stretch that is located on the surface of the membrane. The two tryptophan side chains at positions 194 and 198, located on the same side of the C-terminal helix serve as anchors in the membrane (**Fig. S4**). In contrast, TREM2 K186A adopts a straight helical conformation without a kink with both tryptophan side chains buried inside the membrane (**Fig. S4**). The K186 side chain amino moiety translocates towards the phosphate head group region of the membrane, thus pushing the C-terminal end of the helix outside of the membrane. This movement induces a kink right outside the membrane region and the C-terminal half of the helix rearranges itself to a surface-attached position. In the K186A variant, due to a lack in electrostatic attraction with the lipid head groups, the entire helix is stably located inside the membrane.

Intramembrane cleavage of TREM2 by γ -secretase at structurally dynamic regions

To investigate if intrinsic structural dynamics of the TREM2 TMH determine substrate cleavability, we sought to identify the initial γ -secretase cleavage site of TREM2 releasing the intracellular domain (ICD) by the ϵ cleavage. To allow isolation of sufficient amounts of the ICD and to prevent its rapid degradation we made use of our previously established cell-free *in vitro* γ -secretase assay⁴¹. We stably expressed the recombinant TREM2 CTF with its N-terminus at amino acid 158 corresponding to the

ADAM10/17 cleavage site^{31,32} in human embryonic kidney (HEK) 293 cells. To allow immunoaffinity-isolation of the ICD we added an HA-tag to the C-terminus as well as two proline residues to inhibit rapid degradation⁴² (**Fig. 5a**). Isolated membranes of HEK293 cells stably expressing the TREM2 CTF were incubated for 16 hours to allow *de novo* formation of the TREM2 ICD in the presence and absence of the γ -secretase inhibitor L-685,458⁴³ at 37 °C. Immunoprecipitation of the soluble fraction using an anti-HA antibody revealed γ -secretase dependent generation of the TREM2 ICD (**Fig. 5b**). Immunoprecipitated ICDs were subjected to mass spectrometry as described previously⁴². This revealed a major peak at 5596,20 Da, which was absent at 4 °C and reduced upon treatment with L-685,458 (**Fig. 5c**). This peak corresponds to a major ϵ -cleavage site after amino acid 192 within the TMH (**Fig. 5c, e, f**). Interestingly, the K186A mutation, which strongly stabilizes the α -helical structure of the TREM2 TMH, abolishes cleavage after amino acid 192 and shifts the ϵ -cleavage to a new site after amino acid 195 and to a lower extent to 193 (**Fig. 5d-f**). Strikingly, the site of intramembrane cleavage of wild type TREM2 correlates well with the observed enhanced dynamics between amino acids 189 and 193 (**Fig. 3a**). Furthermore, introduction of the K186A mutation, which reduces structural dynamics within that region (**Fig. 3b**) completely abolished intramembrane proteolysis after amino acid 192 and shifted the cleavage site to the structurally dynamic region immediately C-terminal of the stabilized α -helical TMH at position 195 and to a lower extent at position 193. Thus, structurally dynamic regions appear to be preferred sites for substrate cleavage by γ -secretase in the TREM2 case.

Discussion

The aim of this study was to gather structural and dynamical information of the TREM2 TMH and ask whether and how this could be linked to the initial ϵ cleavage by γ -secretase. We employed an interdisciplinary approach combining NMR structural analyses and biophysical methods with membrane-based γ -secretase cleavage assays followed by mass spectrometry to precisely determine the cleavage sites. Owing to a lack in sequence conservation in γ -secretase substrates¹⁴ it has been unclear which features allow γ -secretase to select its substrates. In general, structures of proteases in complex with their substrates show that substrates need to be present in an extended conformation at

the protease active site⁴⁴. For γ -secretase substrates, backbone hydrogen bonds at the C-terminal end of their transmembrane helix must be opened to allow the protease active site getting access to their scissile bonds. First insights into how this unfolding of a substrate TMH might occur have only recently been obtained for γ -secretase. Structures of γ -secretase in complex with Notch1¹⁷ or APP¹⁶ substrate fragments showed that the cleavage region requires to be actively unfolded, which is energetically compensated by the formation of a stabilizing hybrid β -strand composed of a peptide stretch in the substrate between the cleavage site and the C-terminus as well as regions of the PS1 NTF and CTF in γ -secretase in vicinity to the active site.

Despite these atomic details, the question remains how substrate unfolding is achieved by the enzyme. As shown for the rhomboid protease GlpG and the archaeal presenilin homolog PSH, TMH unfolding can be induced by interaction of the substrate with the protease⁴⁵. For these proteases, the ability of the TMH of the substrate Gurken to adopt a 3_{10} -helical conformation is key for the unfolding step when the substrate contacts the protease⁴⁵. Intrinsic substrate TMH properties that provide conformational flexibility such as the di-glycine hinge near the middle of the APP TMH have also been shown to play an important role for cleavage⁴⁶. Although hydrogen-deuterium exchange experiments showed that the cleavage site region in APP is generally stable with respect to local helix flexibility⁴⁶⁻⁴⁹, MD simulations suggested that changes in bending and rotational motions, i.e. in the global flexibility, mediated by this hinge are critical for correct presentation of the cleavage site domain to the active site⁴⁶.

As found here for TREM2, an unfolded region manifested by a lower order parameter can also be intrinsically present in a substrate TMH. A critical amino acid in TREM2-TMH is the positively charged K186, that is located within the hydrophobic interior of the membrane and which is required for complex formation with DAP12 (**Fig. 2**). Without DAP12, the presence of an unpaired charge in the membrane leads to the formation of a partially unwound helical region with increased conformational dynamics. In order to mimic the DAP12-bound TREM2-TMH conformation, a single K186A point mutation is sufficient for straightening of the TMH while leaving a few C-terminal residues (>A195) more flexible (**Fig. 3**). γ -Secretase cleavage occurs directly in the central unfolded stretch in TREM2-TMH wt, whereas replacement of K186 by alanine shifts the cleavage site to the C-

terminal end of the TMH of TREM2, where the stable and rigid α -helical secondary structure of the TMH terminates. Since our *in vitro* cleavage assays have been performed in POPC-rich membrane fractions, whereas the structural information was mainly obtained in DPC micelles, we compared the TREM2-TMH wt spectra in POPC nanodiscs and DPC micelles (**Fig. S2**) and could show that the structure of TREM2-TMH is identical in all investigated membrane mimetics. The structure dependent shift of the γ -secretase cleavage may suggest that the TREM2-TMH, once it has entered the substrate-binding cavity of γ -secretase, may be able to slide vertically in a piston-like movement within the binding groove until it reaches a position where local unwinding of the C-terminal end of the TMH is energetically feasible by the protease (**Fig. 6a**). The resulting register of the substrate within the protease then simply determines which scissile peptide bond is attacked by its active site residues, as sketched for the TREM2-TMH wt and K186A variant in **Fig. 6b and 6c**. Thus, our data suggest that γ -secretase is able to sample substrate TMHs according to their local stability and inherent flexibility. Such TMH sampling may explain the observation that helix-destabilizing diglycine mutations introduced between the γ - and ϵ -sites increase the cleavage efficiency and processivity of γ -secretase¹³. Using chemical unfolding experiments, we indeed observe that TREM2-TMH K186A is more stable than the wild-type helix ($\Delta G = -18.8$ kJ/mol vs $\Delta G = -14.9$ kJ/mol) (**Fig. 6d, e**). This difference is due to the presence of 1-2 additional hydrogen bonds in the K186A variant considering an energetic contribution of a single hydrogen bond in proteins of ~ 2 -4 kJ/mol⁵⁰. This is in excellent agreement with the contiguous α -helical structure determined by NMR. A comparison of the amino acid sequences of TREM2-TMH with Notch1 and APP (**Fig. 6f**) shows that the α -helical amino acid stretches located C-terminal of the γ -secretase cleavage site of free APP⁵¹ and Notch1⁵² are shorter than for TREM2. These amino acids are succeeded by polar and charged residues that are located outside of the membrane. Thus, local unfolding of the C-terminal end of the TMH of APP and Notch1 by γ -secretase, as observed in cryo-EM structures^{16,17} is most likely energetically less demanding. In comparison, the C-terminal end of TREM2-TMH is 3-4 amino acids longer, which results in a shift of the cleavage site by three residues if the TREM2-TMH is stabilized and straightened up by the K186A mutation. Only if the kink is present, as observed in the wild-type protein, cleavage occurs exactly at this destabilized region. The longer helical secondary structure in TREM2-TMH is required for

binding to DAP12 (**Fig. 2**) and the two tryptophan residues together with the presence of a positive charge (K186) lead to a surface-attached position of the C-terminal end of the TMH and the formation of a kink region. Of particular interest is that the cleavage site in Notch1 is preceded by small and polar residues (GCG motif), further facilitating local unfolding by the enzyme. In APP, a threonine residue is located at the identical position as the cysteine in Notch1 or a serine residue in TREM2. In the APP case, another polar threonine residue is located at the same position inside the membrane as the charged lysine residue in TREM2, which might also contribute to its destabilization.

Since cleavage at the initial ϵ -site will lower the overall substrate stability, the energy cost of helix unwinding will be markedly decreased after this first cleavage event. For the substrate APP, this mechanism can explain the multiple downstream cleavage events observed for γ -secretase.

Taken together, as exemplified here for the TREM2-TMH, our study suggests that sampling for intrinsically flexible regions in a TMH is one of the mechanisms allowing γ -secretase to initiate substrate cleavage.

Methods

Sample preparation

TREM2-TMH₁₆₁₋₂₀₆, TREM2-TMH_{K186A} & DAP12-TMH_{C33S} in pET21a were inserted into modified vectors and were produced in *E. coli* BL21 (DE3) cells as fusion protein with the protein GB1 harboring an N-terminal His₆-tag. Cells were grown at 37 °C and production was induced at an OD₆₀₀ of 0.7 by the addition of 1 mM IPTG. Cells were shaken for 3-4 hours at 37 °C, harvested by centrifugation at 6000 g and stored at -80 °C until further use. To produce [*U*-²H, ¹³C, ¹⁵N]-labeled TREM2-TMH, bacteria were grown in M9 medium supplemented with 1 g/L [98% ¹⁵N]-NH₄Cl and 2 g/L [98% ²H, ¹³C]-glucose in 99% D₂O (Eurisotope or Sigma-Aldrich). The bacterial pellet was dissolved in lysis buffer (20 mM Tris pH 8.0, 100 mM NaCl, 1 protease inhibitor tablet (Roche Complete), 0.5 mM EDTA) and incubated with lysozyme for 30 min. After cell breakage by sonication (30 min, 30% amplitude, 10 min total duration) DNaseI was added, together with 5 mM MgCl₂ and incubated for 30 min on ice. The detergent n-dodecylphosphocholine (DPC, Fos-Choline-12) was added to a final concentration of 1.5% (w/v) and the mixture was incubated on a roller shaker

for 1.5 hours, supplemented with additional 0.9 M NaCl and incubated for 30 more minutes. Insoluble material was removed by centrifugation (40,000 g, 30 min, 4 °C) and the supernatant was applied to 5 mL Ni-NTA resin (GE-Healthcare, equilibrated with 20 mM Tris, pH 8.0, 100 mM NaCl, 0.1 % DPC) using a gravity flow column (Biorad) and incubated with the resin for one hour at 4°C. After washing with 10 column volumes (CV) with the same buffer + 10 mM imidazole, the protein was eluted using 400 mM Imidazole (5 CV) and loaded onto a HiLoad 16/600 Superdex 200 pg size exclusion column (GE Healthcare). The main peak was collected, supplemented with 25 U thrombin protease and incubated overnight at 37 °C. The enzymatic digestion was verified by SDS-PAGE and the protein finally purified using reverse Ni-NTA chromatography. The flow-through was collected and loaded onto a HiLoad 16/600 Superdex 200 column for buffer exchange to NMR buffer (20 mM NaPi, pH 7.0, 50 mM NaCl, 0.5 mM EDTA, 0.1% DPC). The protein concentration was determined by UV/Vis spectroscopy using absorption at 280 nm and a calculated extinction coefficient ϵ_{280} of 11000 M⁻¹ cm⁻¹. For NMR, the final DPC concentration was adjusted to 200 mM.

Nanodisc assembly

Nanodisc assembly has been conducted according to previous protocols^{35,36,54}. A typical assembly reaction is described below: 1 mL of 600 μ M MSP1D1 Δ H5^{35,36}, 0.5 mL of a 300 μ M solution of ²H, ¹⁵N-TREM2-TMH-GB1 in 0.1 % DPC, 0.36 mL of a 50 mM DMPC solution in 100 mM cholate, 0.12 mL of a 50 mM DMPG solution in 100 mM cholate were mixed and diluted with 3 mL MSP-Buffer (20 mM Tris pH 7.5, 100 mM NaCl, 0.5 mM EDTA) including 0.05 % DPC to a final volume of 5 mL with final concentrations of 120 μ M MSP1D1 Δ H5, 3.6 mM DMPC, 1.2 mM DMPG, 30 μ M ²H, ¹⁵N-TMH-GB1. After incubating the mixture for 1 h at room temperature, the nanodisc assembly reaction was dialyzed twice against 5 L MSP buffer in a 20 kDa cut-off dialysis tube. To yield the loaded nanodiscs the assembly was added to 3 mL of Ni-NTA resin in a gravity flow column and incubated for 1 h at room temperature while shaking. The Ni-NTA elution fraction was concentrated in a centrifugal device (30 kDa MWCO, Millipore) to a final volume of 1 mL and purified with an ÄKTA Pure system on a S200a column. A homogeneous peak was obtained and concentrated to ~2 mL. 25 units of thrombin were added and incubated overnight at 37°C. The mixture was then

purified via reverse Ni-NTA and the buffer was changed to NMR buffer (see above) using either a NAP-5 desalting column (GE Healthcare) or by S200a size exclusion chromatography.

Circular dichroism (CD) spectroscopy

For CD spectroscopy, we used 15 μ M of TREM2-TMH and the K186A variant in 20mM NaPi, pH 7.0, 10 mM NaCl, 0.1% DPC. Spectral measurements in the range of 190-260 nm were carried out with a Jasco J-715 spectropolarimeter at 20 °C in a 1 mm path-length quartz cuvette (Hellma Analytics). The fractional helicity (FH), representing the content of α -helical secondary structure, was calculated using the experimental mean residue weight (MRW) ellipticity at $\lambda = 222$ nm (θ^{exp}) and the corresponding values for the fully helical or fully unfolded conformation (θ^h and $\theta^u = -36000$ and -3000 deg cm² dmol⁻¹, respectively) with the following equation: $FH = \frac{\theta_{\lambda}^{exp} - \theta_{\lambda}^u}{\theta_{\lambda}^h - \theta_{\lambda}^u}$ (Ref⁵⁵).

Chemical unfolding experiments

Protein stability was tested by guanidine hydrochloride (GuHCl) induced unfolding with denaturant concentrations up to 8 M. 15 μ M TREM2 TMH wt or K186A in 20mM NaPi, pH 7.0, 10 mM NaCl, 0.1% DPC was incubated for 30 min with increasing concentrations of GuHCl and the CD ellipticity was measured for a 30-second time interval at a wavelength of 209 nm. The raw data were normalized and fitted with an equation describing a two-state folding model⁵⁶.

NMR spectroscopy and structure calculation

NMR experiments were recorded at 37°C on Bruker AvanceIII spectrometers operating at 600, 900 and 950 MHz proton frequency equipped with cryogenic probes. For obtaining backbone resonance assignments and NOE distance restraints, TROSY-based 3D-triple resonance experiments⁵⁷ and a 3D-¹⁵N-edited-[¹H,¹H]-NOESY experiment were recorded in a non-uniform-sampled (NUS) manner⁵⁸. Schedules were prepared using Poisson-gap sampling⁵⁹. 3D-NUS spectra were reconstructed using the iterative soft threshold (IST) method⁶⁰ and processed with NMRPipe⁶¹. Spectral analysis was done with SPARKY4 (Goddard and Kneller, UCSF, 2004) and NMRFAM-SPARKY⁶². Backbone angles for structure calculations were predicted from chemical shift information using the program

TALOS+⁶³ and verified by specific NOE contacts. Structure calculations were done with Xplor-NIH⁶⁴ using standard protocols. The 20 structures with the lowest restraint violation energies were used to obtain structural statistics of the ensemble. Ramachandran map analysis was performed with the RAMPAGE webserver⁶⁵.

NMR data driven docking

Docking of the complex between TREM2-TMH and DAP12 was done with the HADDOCK2.2 webserver³⁸ using the herein determined structure of TREM2-TMH in its complex with DAP12 and the structure of DAP12 in complex with the transmembrane helical region of NKG2C²⁹. The core residues, in particular D50, that form the complex interface within DAP12 with NKG2C were defined as active (monomer1: V47, D50, T54, A58, V61; monomer2: V42, L43, I46, G49, L53, I57, A60), whereas surface neighbors were defined as inactive residues. For TREM2-TMH, the residues that showed large chemical shift perturbations in the NMR titration with unlabeled DAP12 were defined as active, and in particular the charged K186 residue (L176, L179, A180, F183, K186, I187, A189, A190, S191, L193, W194, A195, A197). After the calculations, the obtained structural clusters were selected based on the correct orientation of the binding partners and the overall docking score. The generation of the structural model of the complex between γ -secretase and TREM2-TMH was done by using the γ -secretase-Notch cryo-EM structure¹⁷ as a template and by replacing the amino acid sequence of Notch by the TREM2 sequence in the program Chimera⁶⁶. The exact position of the TREM2-TMH sequence in the binding groove of γ -secretase was defined with the experimentally determined cleavage site for the wild-type and the K186A case.

Paramagnetic relaxation enhancement (PRE) experiments

Purified and ²H, ¹⁵N-labeled TREM2-TMH and K186A protein samples in 20 mM NaPi, 50 mM NaCl, 0.5 mM EDTA, 200 mM DPC were mixed with the paramagnetic label 16-doxyl stearic acid (16-DSA) dissolved in the same buffer. The final 16-DSA concentration was 4 mM which results in approximately one 16-DSA molecule per DPC micelle (DPC aggregation number: ~50). The resulting ratio of protein to 16-DSA was 1:4. 2D-[¹H, ¹⁵N]-TROSY spectra were recorded with a recycle delay of 2 seconds with

96 complex points in the indirect ^{15}N dimension and 32 scans per increment. The free radical in the spin labeled fatty acid leads to distance-dependent line broadening of the amide resonances in the protein. The same experimental parameters were used for a reference experiment after the addition of 40 mM ascorbic acid (from a 1M stock in 20 mM NaPi, pH 6.5, 50 mM NaCl and 0.5 mM EDTA), which is quenching the free radical in the spin label. Signal intensity ratios of the amide resonances in the corresponding spectra can be calculated using the individual peak height values in the oxidized case (I_{ox}), i.e. containing a radical, and the reduced spectrum (I_{red}), i.e. after reduction of the free radical by ascorbic acid. The resulting values range between 0 and 1 indicating either close or no proximity to the spin labeled fatty acid hydrocarbon chain, respectively.

NMR relaxation experiments and data analysis

T_1 , T_2 relaxation rates as well as $\{^1\text{H}\}$ - ^{15}N -heteronuclear NOEs have been recorded in a fully interleaved manner at two magnetic field strengths (600 and 950 MHz proton frequency). For T_1 rates, 14 relaxation delays ranging from 100 ms to 1.8 s have been used, including three duplicates for error estimation. For the determination of T_2 rates we used 14 delays ranging from 16 to 192 ms. Heteronuclear NOE was measured with and without proton saturation (2s saturation time). For all 2D spectra, we recorded 1024 and 96 complex points in the ^1H and the ^{15}N dimension, respectively. Order parameters of TREM2-TMH have been extracted with the software Relax⁶⁷⁻⁶⁹ in fully automated fitting mode using relaxation data at two static magnetic fields (see above).

Molecular Dynamics Simulations

TREM2-TMH was inserted into a hexagonal box of 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-glycero-3-phosphoglycerol (POPG) lipid bilayer (3:1 ratio) using the CHARMM-GUI web server (<http://www.charmm-gui.org>)⁷⁰ in presence of 0.15 M KCl.

Equilibration of the system was done at 310 K in two phases with 3 cycles each. The force constants to fix the position of the protein and membrane were gradually reduced in each cycle. In the first phase, a timestep of 1 fs and a simulation time of 50 ps in each cycle were used whereas in the second phase a timestep of 2 fs and a simulation time of 200 ps for each cycle were used. Total equilibration time was

750 ps. MD simulations of up to 150 ns duration were carried out with the isothermal-isobaric ensembles at 310 K with the program NAMD⁷¹ in the absence of a transmembrane potential. Long-range electrostatic interactions were described using the particle-mesh Ewald method⁷². A smoothing function was applied to truncate short-range electrostatic interactions. Analysis and visualization of the obtained trajectories was done with VMD⁷³.

Generation and maintenance of stably transfected HEK293 Flp-In cell lines

For the determination of the initial ϵ cleavage within the human TREM2 TMH we generated a construct that essentially follows the construct design as previously described for neuregulin 1 type III⁴². In brief, the TREM2 signal peptide at the N terminus was followed by a FLAG tag, an IM linker sequence, the TREM2 CTF (residues 158-230), a GG linker, a HA tag, and a PP motif for ICD stabilization. The corresponding DNA was purchased from IDT and inserted into the pcDNA5/FRT/TO vector via HindIII (New England BioLabs) and XhoI (ThermoFisher Scientific) restriction sites. The K186A mutant (AAG>GCG) was generated by site-directed mutagenesis employing PfuTurbo DNA polymerase (Agilent Technologies). All constructs were verified by DNA sequencing (Eurofins Genomics). Transfection of HEK293 Flp-In cells (ThermoFisher Scientific) with the wildtype and mutant constructs was performed using lipofectamine 2000 according to the manufacturer's recommendations (ThermoFisher Scientific). Successful transfectants were selected using 100 μ g/ml hygromycin B (ThermoFisher Scientific) and stably transfected cell lines were generated as pools. Culturing of stable cell lines was performed as previously described²⁴.

***In vitro* γ -secretase assay**

The *in vitro* γ -secretase assay to generate TREM2 ICDs was essentially performed as previously reported⁴¹. Total protein concentrations of cell homogenates of the TREM2 CTF wild-type and mutant cell lines were determined using the BCA assay (Interchim) in order to properly adjust the volume of the buffer (150 mM sodium citrate pH 6.4, 1 mM EDTA, protease inhibitor mix (Sigma)) used for the assay. To allow generation of the ICD of TREM2, samples were incubated at 37°C overnight and inhibition of γ -secretase mediated ICD formation was performed using 1 μ M L-685,458 (Sigma, L1790).

In the last step, soluble (S100) and insoluble (P100) fractions were separated by ultracentrifugation (55,000 rpm; 60 min).

Determination of ϵ cleavage sites by IP/MS

Soluble fractions as derived from the *in vitro* γ -secretase assay were subjected to peptide alkylation as previously described⁴² to generate monomeric peptide. In the following, the 6-fold volume of IP/MS buffer (0.1 % n-octyl glucoside, 10 mM Tris-HCl, pH 8.0, 5 mM EDTA, and 140 mM NaCl) was added and samples were pre-cleared for 1 hour using protein G sepharose (GE Healthcare, 17-0618-01). Immunoprecipitation was carried out overnight at 4 °C using anti-HA agarose beads (Sigma, A2095). On the next day beads were washed three times with IP/MS buffer and two times with water. After the last washing step water was carefully sucked off and beads were stored at -20 °C until MS analysis. MALDI MS determination of γ -secretase cleavage sites was performed as previously reported^{12,31,42}.

Immunoblotting

The pH of soluble fractions as derived from the *in vitro* γ -secretase assay was adjusted to pH 8.0 using 1 M Tris-HCl pH 8.0 and EDTA was added to a final concentration of 4mM. Protease inhibitor mix (Sigma) was added additionally. The 6-fold volume of IP/MS buffer (0.1 % n-octyl-glucoside, 10 mM Tris-HCl, pH 8.0, 5 mM EDTA, and 140 mM NaCl) was added and all samples were subjected to immunoprecipitation overnight at 4 °C using anti-HA agarose beads (Sigma, A2095). Samples were washed tow times with IP/MS buffer and two times with water. After the last washing step water was carefully sucked off and beads were boiled in 2x SDS sample buffer at 95 °C for 10 minutes. For immunoblot analysis samples were loaded onto precast 10-20 % Tris Tricine gels (NOVEX, ThermoFisher Scientific). Upon gel electrophoresis, proteins were transferred onto a 0.2 μ m nitrocellulose membrane (GE Healthcare, 10600001). Membranes were boiled in PBS for 5 minutes upon protein transfer. TREM2 ICDs were detected using an anti-HA HRP-coupled antibody (clone 3F10, Roche, 1:250). As a positive control, we confirmed ICD generation from APP using a rabbit monoclonal C-terminal anti-APP antibody (Abcam, ab32136, 1:4000). Comparable amounts of enzymatically active γ -secretase and mature γ -secretase between different assay conditions were confirmed using mouse monoclonal anti-presenilin 1 (Biolegend, SIG-39194, 1 μ g/ml) and rabbit

polyclonal anti-nicastrin (Sigma, N1660, 1 µg/ml) antibodies. Samples for anti-presenilin 1 and anti-nicastrin immunoblots were incubated for 10 min at 65 °C in Laemmli buffer supplemented with 2M urea before loading onto Tris Tricine gels. The HRP-conjugated goat anti-mouse (1:10,000; Promega) and goat anti-rabbit IgG (1:10,000; Promega) antibodies were used as secondary antibodies. Analysis of immunoblotting was done using enhanced chemiluminescence technique (Pierce).

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Author contributions

A.S., K.S. B.B. and F.H. performed experiments and analyzed data. A.S., K.S., C.H. and F.H. designed research, H.S. contributed to data interpretation and A.S., K.S., H.S., C.H. and F.H. wrote the paper.

Competing Financial Interests

CH and KS collaborate with DENALI Therapeutics.

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Figure Legends

Fig. 1. Structural analysis of TREM2 transmembrane helix (TMH) in dodecyl-phosphocholine (DPC) micelles. (a) Proteolytic processing of TREM2. First, ADAM10 or 17 cleave off the ectodomain followed by sequential cleavage of the CTF by γ -secretase creating a p3-like extracellular peptide and the intracellular domain (ICD). Only an intact CTF can bind to DAP12. (b) 2D- $[^1\text{H}, ^{15}\text{N}]$ -TROSY spectrum of 400 μM ^2H , ^{13}C , ^{15}N -labeled TREM2-TMH (residues R161-H206) in DPC micelles at 37 °C. Assigned resonances are labeled. (c) Secondary chemical shifts plotted against the residue number indicates an α -helical region between residues 174 and 197. The used TREM2-TMH construct along with the predicted transmembrane helix content (aa 175-195) is also indicated. (d) Circular dichroism (CD) spectrum of TREM2-TMH in DPC micelles indicates an α -helical secondary structure content of ~50%. (e) 3D- ^{15}N -edited NOESY spectra show that the transmembrane helix is interrupted at residues 190-192. Dashed red lines indicate sequential amide NOE contacts that are characteristic for an α -helical secondary structure. (f) Final NMR-derived structural bundle of the 20 lowest-energy structures of TREM2 shows that the C-terminal end of the transmembrane helix can adopt various positions relative to the well-defined TMH. r.m.s.d.: root mean squared deviation; CTF: C-terminal fragment.

Fig. 2. Interaction of TREM2-TMH with the signaling partner DAP12. Overlay of 2D- $[^1\text{H}, ^{15}\text{N}]$ -TROSY spectra of ^2H , ^{15}N -labeled TREM2-TMH in DPC micelles (black) and in complex with (a) unlabeled DAP12 (red) or (b) TREM2-TMH lacking its positively charged lysine residue (K186A, red). (c) Chemical shift perturbations (CSP) between the two spectra in panels (a) or (b). The α -helical secondary structure as determined for wild-type TREM2-TMH is indicated above and the position of the K186A mutation is labeled in green. (d) NOE-derived lowest-energy structure of TREM2-TMH in complex with disulfide-bridged dimeric DAP12 shows one continuous α -helix. (e) The experimental structure of TREM2-TMH was docked to a previously determined structure of DAP12 in complex with NKG2C²⁹. The positive charge in K186 is engaged in a salt bridge with D50 of DAP12. This charge complementarity together with hydrophobic interactions stabilize the complex and lead to a straight α -helical conformation in TREM2-TMH. D50 of the second DAP12 monomer is not in direct

contact with TREM2, similar to the DAP12-NKG2C complex²⁹. (f) For TREM2-K186A-TMH a ~7% increase in α -helical secondary structure can be detected by CD spectroscopy. (g) A continuous α -helical secondary structure in the kink region (aa 189-192) was probed by sequential amide NOE contacts in TREM2-K186A-TMH. Red dots indicate the chemical shift of intra-residual (i) amide resonance and red broken lines the inter-residual connectivity ($i\pm 1$). (h) Removal of the positive charge (K186A) leads to a very similar structure of the TREM2-TMH as in its complex with DAP12. The CSP values color coded onto the NOE-derived structures in each case (e, h) show similar patterns (color coding according to the dotted lines in panel (c), i.e. lower line: mean CSP value, upper line: mean + standard deviation). The location of the K186A mutation is indicated by a green sphere. Spheres shown in panels e and h represent residues with CSP values above the mean value plus one standard deviation.

Fig. 3. Dynamics of TREM2-TMH wild-type and the K186A variant. (a,b) Generalized order parameter S^2 for backbone amide resonances derived from NMR relaxation experiments with wild-type TREM2-TMH (a) and the K186A variant (b), indicative of motions in the nanosecond to picosecond time scale. S^2 can adopt values between 1 (rigid) and 0 (unrestricted motion). (c,d) Molecular dynamics simulations of TREM2-TMH wt (c) and K186A (d) in a POPC/POPG (3:1) bilayer provide information on the stability of helix insertion into a lipid bilayer membrane. Since K186 is initially located in the hydrophobic membrane, its positively charged side chain is pulled towards the negatively charged phosphates in the lipid head group region leading to a pronounced tilt in helix orientation in the nanosecond time scale and on a μ -second to milli-second time scale to a kink in the C-terminal half of the α -helix, as probed by NMR line broadening. In contrast, in the K186A variant the transmembrane helix is stably inserted within the bilayer.

Fig. 4. Membrane position of TREM2-TMH and the K186A variant. (a) Paramagnetic relaxation enhancement (PRE) with a free-radical-modified stearic fatty acid in DPC micelles. Low values indicate location inside whereas values close to one suggest a location outside the detergent micelle. Orange bars: TREM2-TMH wt, green bars: K186A variant. The N-terminal residues of TREM2-TMH-wt are located deeper inside the micelles as in the K186A case, whereas the C-terminal end is positioned at the outside for wild-type and inside for the K186A variant. Negative bars indicate missing assignments. (b) NOE-cross peaks between backbone amides of TREM2-TMH wt (top) or the K186A variant (bottom) and selected regions of DPC (see color coding of the structure of the DPC molecule on the right). The K186A variant shows strong contacts to the hydrophobic aliphatic regions of the detergent throughout the transmembrane helix whereas no such contacts can be seen at the kink site of the wild-type helix, indicating a location outside of the micelle for this part. (c) Model of the position of TREM2-TMH wt and K186A within the membrane. The amino acids in the kink region are exposed to the solvent in the wild-type case but buried in the membrane for the K186A variant.

Fig. 5. Identification of γ -Secretase cleavage sites in TREM2 wild-type and the K186A variant. (a) Schematic overview of the *in vitro* γ -secretase assay to determine the ϵ -cleavage sites of TREM2. For more details see Materials and Methods. (b) Immunoblot detection of ICD accumulation of both TREM2 wildtype and the K186A mutant mediated by γ -secretase cleavage upon overnight incubation of membrane preparations at 37 °C. ICD formation is blocked by 1 μ M of the transition state analogue L-685,458. *In vitro* generation of the ICD of APP (AICD) was used as a positive control. Immunoblot detection of mature nicastrin and the presenilin 1 NTF, which results from presenilin endoproteolysis necessary to activate γ -secretase⁵³, proves comparable amounts of γ -secretase in the individual assays. (c) MALDI MS determination of the ϵ -cleavage site of the wt TREM2 CTF reveals ϵ -cleavage C-terminal of A192. A minor peak at slightly higher molecular weight that could not be assigned is indicated by an asterisk. (d) MALDI MS determination of the ϵ -cleavage site of TREM2 CTF K186A reveals major and minor ϵ -cleavage sites C-terminal of A195 and L193, respectively. Two peaks that could not be assigned are indicated by an asterisk. (e) Schematic representation of the ϵ -cleavage sites of TREM2 CTF wildtype (top) and K186A (bottom). Residue 186 is indicated in bold. The predicted

transmembrane domain is boxed in orange and green for TREM2 TMH wt and the K186A mutant, respectively. (f) Table listing the calculated and observed masses of both wildtype and mutant ICDs as determined by MALDI MS. Note that molecular weights are increased by 58 Da due to alkylation of C213.

Fig. 6. Cleavage specificity of TREM2-TMH by γ -secretase is governed by its local helix stability.

(a) Structural model of the complex of TREM2-TMH wild-type and γ -secretase obtained by docking guided by the cryo-EM structure of the γ -secretase-NOTCH complex¹⁷. Protein regions in γ -secretase that occlude the substrate binding site have been removed for clarity. (b) TREM2-TMH wild-type in the active site of γ -secretase in a register that would lead to cleavage between residues A192 and L193 (marked by spheres in red and purple), as detected by mass spectrometry (see **Fig. 5**). The K186 residue in TREM2 is also shown as well as the catalytically relevant residues in γ -secretase (D385 and D257). In TREM2-TMH K186A, the register of the helix moves upwards leading to positioning of A195 and A196 in the active site (c). The C-terminal end of the helix needs to be unwound by γ -secretase, thus, the intrinsic stability of the helical substrate is a critical determinant of the cleavage position. (d, e) Chemically induced unfolding of TREM2-TMH wild-type (d) and the K186A variant (e) demonstrates that the less dynamic variant is more stable than the wild-type protein. (f) Amino acid sequence comparison of TREM2-TMH wild-type with Notch1 and APP. α -helical secondary structure elements in the free form of each protein are labeled by grey boxes. Positively charged residues are marked by blue and polar residues close to the cleavage site (indicated by the comma mark) by yellow boxes. Red boxes indicate the unfolded sequence stretches in the bound state preceding the cleavage site. The secondary structure of the substrate helices (or derived from the structural model for TREM2-TMH) in complex with γ -secretase is shown above.

Figure 1

Figure 2

Figure 3

Figure 5

Figure 6