Supplemental Information

Structural Basis and Functional Role
of Intramembrane Trimerization
of the Fas/CD95 Death Receptor

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Figure S1. Purification and Reconstitution of Fas-TM Domains (related to Figure 1)

(A) Reverse phase HPLC purification of mouse Fas-TM from CNBr-cleaved trpLE-Fas-TM on Zorbax SB-C3 column with a gradient from 0 – 50% acetonitrile in 5% isopropanol and 0.1% TFA. The product was verified by SDS-PAGE and Mass Spectrometry.

(B) The same as in (A) but for purifying the human Fas-TM.

(C) The 1D $^1$H NMR spectrum of a reconstituted Fas-TM sample in bicelle at 600 MHz showing the molar ratio of DMPC to DHPC to be $\sim 0.5$.

(D) An illustration of Fas-TM trimer in bicelle showing the relative sizes of Fas-TM trimer and the $q = 0.5$ bicelle.
Figure S2. NMR Spectra of Mouse and Human Fas-TM Trimers in Bicelles (related to Figure 2)
(A) The 2D $^1$H-$^{13}$C HSQC (28 mM constant time) recorded at 800 MHz showing the assignments of methyl group resonances of the mouse Fas-TM trimer in bicelles.

(B) The same spectrum as in (A) for the human Fas-TM trimer in bicelles.

(C) Residue-specific strips taken from the 3D $^{15}$N-separated NOESY-TROSY-HSQC spectrum (NOE mixing time = 300 ms) recorded at 800 MHz using the mouse Fas-TM sample containing 50% ($^{15}$N, $^2$H)-labeled peptide and 50% (15% $^{13}$C)-labeled peptide. The crosspeaks in the aliphatic regions are intermonomer NOEs between the backbone amide and the sidechain methyl protons.

(D) The same as in (C) showing intermonomer NOEs for the human Fas-TM trimer in bicelles.
Figure S3. WT and Mutant Fas Proteins were Localized on Plasma Membrane (related to Figure 5)

The WT and mutant Fas-mCherry overexpressed HeLa cells were imaged using fluorescence microscopy. Scale bars, 5 µm.
Figure S4: Example Histograms of FRET between Fas transmembrane Mutants (related to Figure 6)

The indicated Fas CFP and YFP fusion proteins and a control TNFR1 fusion protein were cotransfected into 293T cells and FRET assayed by collecting fluorescence in the YFP channel after excitation of CFP by flow cytometry. Histograms of FRET are shown for cells gated to express comparable levels of CFP and YFP.
Figure S5. Expression of TM Mutant Fas Proteins on the Plasma Membrane (related to Figure 6)

(A) Representative plots of flow cytometric detection of Fas surface expression and FasL binding of RapoC2 Fas deficient Jurkat cells transfected with the indicated Fas-YFP fusion protein constructs.

(B, C) Relative efficiency of surface Fas (n=8 from 3 experiments) (B) and FasL binding (C) on gated YFP positive RapoC2 cells (n=6 from 2 experiments). * = p<0.05, ** = p<0.005, *** = p<0.0005 by Mann-Whitney test compared to the average of WT Fas expression in each experiment. MFI: mean fluorescence intensity.
Table S1. Relative Intensities of Monomer, Dimer, and Trimer Bands in SDS-PAGE Analyses of Assembly Stability of Fas-TM and Mutants\textsuperscript{a,b} (related to Figures 3 and 4)

### Mouse Fas-TM mutations\textsuperscript{c}

<table>
<thead>
<tr>
<th>(%)</th>
<th>WT</th>
<th>T174A</th>
<th>T174R</th>
<th>V177A</th>
<th>I180V</th>
<th>I180A</th>
<th>P181A</th>
<th>L182A</th>
<th>V183A</th>
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<tr>
<td>Trimer</td>
<td>88.3±4.6</td>
<td>82.6±5.7</td>
<td>8.0±3.1</td>
<td>84.2±8.7</td>
<td>83.5±6.5</td>
<td>9.7±3.3</td>
<td>5.4±3.0</td>
<td>86.5±7.4</td>
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<td>Dimer</td>
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<td>Monomer</td>
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<td>8.5±5.8</td>
<td>83.9±5.4</td>
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### Human Fas-TM mutations\textsuperscript{d}

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<th>(%)</th>
<th>WT</th>
<th>C178R</th>
<th>L180F</th>
<th>P183L</th>
<th>I184V</th>
<th>I184A</th>
<th>P185A</th>
<th>L186A</th>
<th>I187A</th>
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<tr>
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<td>82.5±6.4</td>
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<tr>
<td>Dimer</td>
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<td>Monomer</td>
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<td>82.5±5.4</td>
<td>12.9±4.8</td>
<td>65.6±7.8</td>
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### Human Fas-TM Proline 183 mutations and recover mutations\textsuperscript{e}

<table>
<thead>
<tr>
<th>(%)</th>
<th>WT</th>
<th>P183L</th>
<th>P183A</th>
<th>P183L+L181V</th>
<th>P183L+I187V</th>
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<tr>
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<td>Dimer</td>
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<tr>
<td>Monomer</td>
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<td>82.9±5.3</td>
<td>62.1±5.9</td>
<td>64.5±6.4</td>
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\textsuperscript{a} Gel band intensity is quantified using the program IMAGEJ-V1.49 (Girish and Vijayalakshmi, 2004).

\textsuperscript{b} The band intensity is shown as mean ± SD calculated from 3 independent experiments.

\textsuperscript{c} Band intensities from SDS-PAGE shown in Figure 3B

\textsuperscript{d} Band intensities from SDS-PAGE shown in Figure 3D

\textsuperscript{e} Band intensities from SDS-PAGE shown in Figure 4C
### Table S2. Comparison of Single Point Energies of Different Fas-TM Mutants\(^a\) (related to Figure 4)

<table>
<thead>
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<th></th>
<th>Human Fas-TM</th>
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<th>Mouse Fas-TM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>P183L</td>
<td>V177L+V183I</td>
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<tr>
<td>Single point energy</td>
<td>-4246.287</td>
<td>-3981.197</td>
<td>-4314.373</td>
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\(^a\) The single point energies were computed using the software Schrodinger MacroModel, with the imbedded OPLS_2005 Force field. The dielectric constant was set to 1.0 for electrostatic treatment. The limitations of Van der Waals and short-range electrostatic interactions were smoothly truncated at 7.0 Å and 12 Å, respectively. The protein backbones were restricted with force constant 100 kJ/mol Å\(^2\). The side chains were allowed to move freely.
Supplemental Experimental Procedures

Protein Expression and Purification
The human and mouse Fas-TMs were expressed as a C-terminal in-frame fusion to the trpLE sequence with an N-terminal 9-His tag in the pMM-LR6 vector. Transformed *E. coli* BL21 (DE3) cells were spread on a LB-Agar plate and single colony was picked and inoculated into 1 ml LB medium. The overnight-cultured cells were spun down and inoculated into 1000 ml M9 minimal medium with one or more stable isotope labels in 2.0 L baffled flasks. Cultures were grown at 37 °C to O.D. ~0.6 at 600 nm and were induced overnight at 18 °C using 0.1 mM IPTG. Full deuteration of the peptide required growth in 99.9% D₂O with deuterated glucose (Cambridge Isotope Laboratories).

For purification, inclusion bodies were collected by centrifugation at 8000 g after cell lysis by sonication, and were suspended in 6 M guanidine HCl, 50 mM Tris (pH 8.0), 100 mM NaCl, and 1% (v/v) Triton X-100. The fusion protein in the cleared inclusion-body suspension was batch-purified in 8 M urea solution using nickel affinity resins (Sigma-Aldrich). The nickel resins were washed with water and the fusion proteins were eluted with 70% (v/v) formic acid. The FAS-TM peptides were then released from the fusion protein by cleavage at the methionine position by cyanogen bromide (0.1 g/ml) in 70% (v/v) formic acid. The reaction mixture was dialyzed in water, lyophilized and loaded onto a Zorbax SB-C3 column (Agilent) in 90% (v/v) formic acid. Polypeptide fragments were separated using a gradient from 5% (v/v) isopropanol with 0.1% (v/v) trifluoroacetic acid to 50% (v/v) acetonitrile with 5% (v/v) isopropanol and 0.1% (v/v) trifluoroacetic acid. The fractions corresponding to pure FAS-TM peptide were identified by MALDI-TOF mass spectrometry and SDS-PAGE analysis.

Protein Reconstitution Into Bicelles
Lyophilized Fas-TM peptide (1-2 mg) was dissolved in hexafluoro-isopropanal (HFIP) with approximately 9 mg DMPC (protonated or deuterated), followed by drying of the solution under a nitrogen stream to achieve a thin film. The thin films were then dissolved in 3 ml of an 8 M
urea solution containing approximately 27 mg DHPC (protonated or deuterated), followed by dialysis against 20 mM sodium phosphate buffer (pH 6.8) to remove the denaturant. After dialysis, DHPC was added to adjust the ratio of DMPC:DHPC to approximately 1:2. The solution with reconstituted Fas-TM was concentrated using centicon. The final NMR sample contains ~ 1 mM Fas-TM (monomer), 60 mM DMPC, 120 mM DHPC, 20 mM sodium phosphate (pH 6.8), and 5% D$_2$O. The final DMPC/DHPC ratio was determined using $^1$H NMR spectrum (Figure S1C).

**NMR Spectroscopy**
All NMR spectra were collected at 30 °C on Bruker spectrometers operating at $^1$H frequency of 600 MHz or 800 MHz and equipped with cryogenic probes. The triple resonance and NOE experiments were recorded on Bruker spectrometers operating at 600 and 800 MHz, respectively. Sequence specific assignment of backbone chemical shifts was accomplished using 3 pairs of TROSY-enhanced triple resonance experiments (Salzmann et al., 1999), recorded using a ($^{15}$N, $^{13}$C, $^2$H)-labeled sample. The triple resonance experiments included HNCA, HN(CO)CA, HN(CA)CO, and HNCO. Protein side chain aliphatic and aromatic resonances were assigned using a combination of NOESYs including $^{15}$N-separated NOESY-TROSY-HSQC (150 ms NOE mixing time, $\tau_{\text{NOE}}$) and $^{13}$C-separated NOESY-HSQC ($\tau_{\text{NOE}}$ =120 ms). Specific stereo assignment of the methyl groups of valines and leucines were obtained from a 28 ms constant-time $^1$H-$^{13}$C HSQC spectrum recorded using a 15% $^{13}$C-labeled sample (Szyperski et al., 1992).

The above $^{15}$N-separated NOESY-TROSY-HSQC and $^{13}$C-separated NOESY-HSQC with short $\tau_{\text{NOE}}$ were used to assign local NOEs. The combination of local NOE restraints and backbone dihedral angles derived from chemical shifts accurately defined the helical region of the monomer. Intermonomer NOEs between protein backbone amide and side chain aliphatic protons were assigned using a sample that was reconstituted with a 1:1 mixture of ($^{15}$N, $^2$H)-labeled and (15%) $^{13}$C-labeled peptides. In this sample, the DMPC in the bicelles was also deuterated at the acyl chains. Recording a 3D $^{15}$N-separated NOESY-TROSY-HSQC ($\tau_{\text{NOE}}$ =
300 ms) with this sample allowed exclusive detection of NOE crosspeaks between the $^{15}\text{N}$-attached protons of one monomer and aliphatic protons of other monomers. The non-deuterated peptide was (15% $^{13}\text{C}$)-labeled for recording the $^1\text{H}$-$^{13}\text{C}$ HSQC spectrum as internal aliphatic proton chemical shift reference while providing stereospecific assignment of leucine and valine methyl groups (Szyperski et al., 1992).

The NMRPipe (Delaglio et al., 1995) and XEASY (Bartels et al., 1995) software were used for data processing and spectral analysis, respectively. TALOS+ was used for predicting backbone dihedral angles from chemical shifts (Shen et al., 2009).

**Structure Calculation**

Structures were calculated using the program XPLOR-NIH (Schwieters et al., 2003). The monomer structure was first derived in XPLOR-NIH using intramonomer NOE-derived local distances and backbone dihedral restraints derived from chemical shifts using the TALOS+ program (Shen et al., 2009). A total of 10 monomer structures were calculated using a standard simulated annealing (SA) protocol. Three copies of the lowest-energy monomer structure were used to construct an initial model of the trimer using intermonomer NOE restraints collected from the mixed-labeled sample. For each intermonomer restraint between two adjacent monomers, three identical distance restraints were assigned respectively to all pairs of neighboring monomers to satisfy the condition of C3 rotational symmetry. Then, using a SA protocol in which the bath was cooled from 1000 to 20 K, the trimer was refined against the complete set of NOE restraints (including intramonomer and intermonomer distance restraints) and dihedral restraints. The NOE restraints were enforced by flat-well harmonic potentials, with the force constant ramped from 25 to 50 kcal/mol Å$^{-2}$ during annealing. For the defined helical regions, backbone dihedral angle restraints ($\Phi = -60^\circ$, $\Psi = -40^\circ$) were applied, all with a flat-well ($\pm10^\circ$) harmonic potential with force constant ramped from 15 to 30 kcal/mol rad$^{-2}$. A total of 75 structures were calculated and 15 low energy structures were selected as the structural ensemble. Ramachandran plot statistics for the structure ensemble, calculated using PROCHECK (Laskowski et al., 1993), are as follows: most favored (92.8% for mouse
Fas-TM and 91% for human Fas-TM), additionally allowed (5.4% for both mouse and human Fas-TM), generously allowed (1.8% for mouse Fas-TM and 3.6% for human Fas-TM) and disallowed (0.0% for both mouse and human Fas-TM).

**Cell Death Assays**

Hela cells were transfected with different plasmids using lipofectamine 2000 according to the standard protocol. Then, cells were harvested and stained by Annexin V-FITC and propidium iodide (PI). The cell counting was carried out using Cellometer (Nexcelom Bioscience) and data was processed through FCS Express 4 software. Both apoptotic and necrotic cells were treated as dead cells. For Fas ligand induced cell death assays, 8 x 10^6 cells were transfected with 25 µg of plasmid DNA expressing full-length Fas (either WT or harboring a TM mutation) via electroporation (BTX ECM 830, Harvard Apparatus). 24 hr post-transfection, cells were purified via Ficoll isolation prior to incubation with increasing levels of isoleucine-zipper fused FasL (FasL-LZ) for 16-18 hr, 37 °C. YFP-positive cells were analyzed for cell death using annexin-V and Live/Dead stain as previously described (Ramaswamy et al., 2011). For experiments testing the dominant inhibitory ability of the TM mutants, Rapo C2 cells were co-transfected with a WT full-length Fas construct fused to ECFP and truncated Fas construct lacking the death-domain (ΔDD, amino acids 211-317) fused to EYFP, either WT or harboring a TM mutation as described above, and transfected cells were stimulated with graded amounts of FasL for 18 hr and apoptosis assayed as above. Specific cell death was calculated as previously described (Ramaswamy et al., 2011) and statistical comparisons were made with a two tailed t-test or Mann-Whitney non-parametric statistics when n < 9 or the data did not appear to be normally distributed (Prism 6, Graphpad software).
Supplemental References


