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Higher-Order Clustering of the Transmembrane Anchor of DR5 Drives Signaling

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SUMMARY

Receptor clustering on cell membrane is critical in the signaling of many immunoreceptors and this mechanism has previously been attributed to the extracellular and/or the intracellular interactions. Here, we report an unexpected finding that for death receptor 5 (DR5), a receptor in the tumor necrosis factor receptor superfamily, the transmembrane helix (TMH) alone in the receptor directly assembles a higher-order structure to drive signaling, and that this structure is

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AUTHOR CONTRIBUTIONS L.P., T.-M.F., S.C., H.W., and J.J.C. conceived of the study. L.P., L.Z., and Q.F. prepared samples for NMR and in vitro assembly studies. W.C. performed the OG-label assay. L.P., L.Z., Q.F., Z.L., and J.J.C. collected and analyzed NMR data and/or determined the structures. A.P., L.Z., and L.P. performed membrane partition analysis. T.-M.F., W.Z., L.P., C.Q., S.C., H.W., and J.J.C. performed and/or analyzed functional experiments. J.J.C., H.W., and L.P. wrote the paper and all authors contributed to editing of the manuscript.

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ACCESSION NUMBERS

The atomic structure coordinate and structural constraints have been deposited in the Protein Data Bank (PDB), accession numbers 6NHW (WT) and 6NHY (mutant). The chemical shift values have been deposited in the Biological Magnetic Resonance Data Bank (BMRB), accession numbers 30553 (WT) and 30554 (mutant).

DECLARATION OF INTERESTS

The authors declare no conflict of interests.

SUPPLEMENTAL DATA

Supplemental Information includes seven figures and two tables and can be found with this article online at XXXX

inhibited by the unliganded ectodomain. Nuclear magnetic resonance structure of the TMH in bicelles shows distinct trimerization and dimerization faces, allowing formation of dimer-trimer interaction networks. Single TMH mutations that disrupt either trimerization or dimerization abolish ligand-induced receptor activation. Surprisingly, proteolytic removal of the DR5 ectodomain can fully activate downstream signaling in the absence of ligand. Our data suggest a receptor activation mechanism in which binding of ligand or antibodies to overcome the pre-ligand autoinhibition allows TMH clustering and thus signaling.

In Brief

Unlike traditional receptor clustering mediated by intracellular and extracellular domain oligomerization, the transmembrane domain alone of some TNF family receptors can form higher order structures competent to drive signaling, basally inhibited by the unliganded ectodomain.

INTRODUCTION

Members of the tumor necrosis factor receptor superfamily (TNFRSF) can be specifically activated to induce death of many cancer cells or regulate proliferation and activation of immune cells (Ashkenazi and Dixit, 1998; Boldin et al., 1995; Nagata and Golstein, 1995; Rieux-Laucat et al., 1995; Watts, 2005). There are genuine needs for in-depth understanding of the mechanism by which these receptors are activated, as many of them are targets for antibody-based activation for cancer therapy, e.g., the immune cell co-stimulators 4-1BB, OX40, and BCMA of the TNFRSF in immune-oncology (Cooper et al., 2002; Hatzoglou et al., 2000; Rogers et al., 2001), as well as death receptor 5 (DR5, also known as TRAIL receptor 2) in tumor killing by the extrinsic pathway of caspase activation (Ashkenazi, 2008; Chaudhary et al., 1997; Sheridan et al., 1997).

Receptors in the TNFRSF are Type I transmembrane (TM) proteins with an extracellular domain (ECD) composed of multiple cysteine rich domains (CRDs), a transmembrane helix (TMH), and an intracellular region that specifically interact with signaling adaptors such as Fas associated death domain (FADD), TNFR1-associated DD (TRADD), or TNFR-associated factor (TRAFs) (Baker and Reddy, 1998). Early functional and structural studies on TNFR1 and Fas have painted an overall picture of receptor triggering by trimerization (Vanamee and Faustman, 2018; Wajant, 2002). The binding of the trimeric ligand, in which neighboring protomers of the ligand interact with a receptor ECD, causes the receptor to trimerize. It is presumed that ECD trimerization allows subsequent clustering of the intracellular domains that recruits and activates downstream signaling proteins. In the case of Fas, structural studies of its intracellular DD in complex with FADD suggested formation of a higher-order oligomeric signaling complex (Esposito et al., 2010; Wang et al., 2010).

More recently, we showed that the Fas TMH forms a trimer with a proline-containing signature sequence that allows for close van der Waals (VDW) contacts between the protomers (Fu et al., 2016). Disruptive mutations for TMH trimer formation severely attenuated Fas ligand (FasL)-induced signaling (Fu et al., 2016) and several of these mutations are oncogenic in human (Gronbaek et al., 1998; Lee et al., 2000). These results suggest that TMH trimerization positions the Fas intracellular DD in the right arrangement

to cluster and form the so-called death inducing signaling complex (DISC) with FADD and Caspase-8 (Driscoll, 2014). In contrast, none of the mutations that disrupted the TMH trimer had any effect on receptor self-association in the absence of FasL, suggesting that the TMH associates differently in the pre-ligand state of the receptor or does not participate in pre-ligand association (Fu et al., 2016). In this regard, previous studies suggested that the N-terminal CRD1 of TNFR1, Fas and other TNFRSF family members form a pre-ligand association domain (PLAD) and that a signaling defective mutant receptor with an intact PLAD dominant negatively inhibits signaling by the wild-type (WT) receptor (Chan, 2007; Chan et al., 2000; Siegel et al., 2000b).

The molecular mechanism for TNFRSF signaling remains intriguing, especially with respect to the TMH. First, while existing models propose that ligand- and antibody-induced clustering at the ECD activates intracellular signaling (Vanamee and Faustman, 2018), how can different ligands such as trimeric TNF family members and dimeric NGF and antibodies with potentially different clustering geometries all position the TMH for activation is unexplained. Second, while the proline motif found to mediate TMH trimerization in Fas is present in many members of the TNFRSF such as TNFR1, DR3, DR4, and CD40, it is absent in several other members such as DR5, OX40, and 4-1BB (Table S1). Instead, the TMHs of DR5 and OX40, for example, contain the GXXXG motif known for mediating TMH dimerization, not trimerization (MacKenzie et al., 1997; Trenker et al., 2015). Third, the oligomeric state of pre-ligand receptor association has been debated between dimeric (Naismith et al., 1995; Vanamee and Faustman, 2018) and trimeric (Valley et al., 2012) although there is not yet direct evidence for either form.

In this study, we performed structural and functional investigation on the DR5 TMH (DR5^{TMH}) to address the above issues while potentially uncovering previously unrecognized roles of TMH in receptor signaling. We determined the nuclear magnetic resonance (NMR) structure of DR5^{TMH} in the lipid-bilayer environment of small bicelles, which surprisingly showed distinct surfaces for both dimeric and trimeric interactions, via the GXXXG motif and a non-proline motif respectively. On the cell membrane, these interfaces together should allow DR5 to form a supramolecular dimer-trimer network to mediate higher-order clustering and signaling. Further cell based functional studies revealed that the ECD is not required for DR5 signaling; the intrinsic dimer-trimer network of TMH alone is both necessary and sufficient to drive DR5-mediated cell death, while unliganded ECD inhibits TMH oligomerization. These results challenge the view that ligand-induced extracellular clustering initiates the signaling, but rather suggest the postulation that an important role of ligand binding is to overcome ECD autoinhibition on the TMH. This hypothesis provides a unified explanation for the long-standing question on how both dimeric and trimeric binders activate TNFRSF signaling because unlike specific positioning, relieving autoinhibition may not require a specific clustering geometry. Furthermore, structure-based dimer-specific mutagenesis of the TMH, but not trimer-specific mutagenesis, significantly affects pre-ligand receptor association, which provides direct evidence not only for a dimeric pre-ligand state but also for participation of TMH in this process as well.

RESULTS

DR5 TMH Contains both Trimer and Dimer Interfaces

We first investigated TMH assembly of DR5 using NMR and biochemistry protocols as similarly performed previously for the Fas TMH (Fu et al., 2016). DR5 has a domain organization similar to that of Fas; it contains an ECD composed of three CRDs, a TMH, and an intracellular DD (Figure 1A). While both ECD and DD are homologous between DR5 and Fas, the TMHs of the two death receptors share no recognizable sequence similarity. For NMR studies, we used a DR5 fragment from human isoform 1, containing residues 208 – 242, which encompasses the common predicted TMH of both isoforms (Figure S1A). The purified fragment, designated DR5^{TMH}, was reconstituted into bicelles with [lipid]/[detergent] ratio (q) of ~0.55 (Figures S1B and S1C). At this q , the estimated diameter of the bilayer region of the bicelle is ~49 Å (Glover et al., 2001; Sanders et al., 1994) and the proteins are in near lipid-bilayer environment (Piai et al., 2017b). We found that the unreconstituted DR5^{TMH} migrated in SDS-PAGE primarily as a monomer (theoretical MW 3.6 kDa; apparent MW ~5 kDa) while bicelle-reconstituted DR5^{TMH} appeared in the gel as a trimer without crosslinking (apparent MW ~16 kDa) (Figure S1D). The WT DR5^{TMH} bicelle sample generated high quality NMR spectra (Figure S1E), indicating the TMH was well folded in bicelles, followed by successful backbone assignment of DR5^{TMH} (Figure 1B).

Next, an isotopically mixed sample containing 1:1 mixture of (¹⁵N, ²H)-labeled DR5^{TMH} and (15% ¹³C)-labeled DR5^{TMH} was used to exclusively detect inter-monomer nuclear Overhauser enhancements (NOEs) between the amide protons (H_N) of the deuterated monomers and the aliphatic protons of the fully-protonated monomers. This experiment provided direct evidence of inter-monomer contacts in DR5^{TMH} homo-oligomers. Unlike Fas^{TMH}, however, which showed inter-monomer NOEs for only one face of the TMH (Fu et al., 2016), DR5^{TMH} showed too many NOEs on all sides of the helix that were not possible to fit with any trimer structures (Figure 1C). Among these NOEs, two were characterized to be from the backbone H_N of the two glycines (G213, G217) in the GXXXG motif to the H_α of the same glycines on the opposite chain, respectively, indicating close glycine packing that is the structural hallmark of GXXXG-mediated TMH dimerization. Collectively, these NOE data suggested that both dimer and trimer interfaces existed in DR5^{TMH} oligomers in bicelles, and that an obvious way to deconvolute the dimer- and trimer-specific NOEs was to generate a G217Y mutation in the GXXXG motif.

High quality NMR spectra of the DR5^{TMH} G217Y mutant suggest its well-folded structure, and its backbone was also assigned (Figures 1D and S2). Indeed, upon introducing the G217Y mutation, about half of the inter-monomer NOEs vanished, including GXXXG motif associated NOEs (Figures 1E and 2A–C), and the mutant still migrated in SDS-PAGE as a trimer (Figure 2D). The mutant results suggested that the single mutation in the GXXXG motif has removed the dimer interface but not the trimer interface. But, the exact oligomeric state of the WT and mutant DR5^{TMH} were still unclear, because both appeared in SDS-PAGE as trimers (Figure 2D), whereas minimally hexamers were expected for the WT having both dimeric and trimeric interactions. Since SDS can be disruptive for certain TM

helix assembly, we examined DR5^{TMH} oligomeric state using an oligomer-labeling technique (OG-label) that we developed recently (Chen et al., 2018). In this method, each of the protomers in the bicelle-reconstituted His-tagged DR5^{TMH} oligomer was labeled stoichiometrically with a TriNTA-tagged soluble crosslinkable protein (SCP) named GB1 via a His-NTA interaction. The SCPs were then crosslinked with an amine-reactive crosslinker (Lomant's reagent) and released from the DR5^{TMH} by EDTA for SDS-PAGE analysis (Figure 2E). The OG-label results clearly indicate that WT DR5^{TMH} in bicelles forms hexamers whereas the G217Y mutant only forms trimers (Figure 2F). The same analysis also showed that the V227Y mutation, which was later shown to affect neither trimeric nor dimeric interaction, is also hexameric, as the WT (Figure 2F).

Atomic Structure of a Minimal Dimer-Trimer Assembly of DR5^{TMH}

To define both the trimerization and dimerization interfaces in the TMH assembly, we first determined the trimer structure of the G217Y mutant (Table S2). The NOE data of isotopically mixed DR5^{TMH} mutant showed obvious inter-monomer NOEs at T219, A222, and I226 (Figure 2B), resulting from trimeric packing at these positions. In parallel, a control sample with only (¹⁵N, ²H)-labeled protein did not generate the above NOEs (Figure 2C), confirming that the inter-monomer NOEs have arisen from mixing of differently labeled protomers. The trimer structure shows three layers of hydrophobic interaction along the 3-fold axis, including interactions between T219 and V218, between A222 and V223, and between I226 and L225 (Figures 3A and S3). Single mutations that were predicted by the structure to disrupt trimerization, T219Y and A222Y, both led to dissociation of the trimer (Figure 3B). The NMR structure of the G217Y mutant has a similar mode of trimerization as that of Fas^{TMH} (Fu et al., 2016), but with less intimate associations along the symmetry axis due to the lack of the proline motif (Figure 3C).

Having identified the trimer-specific inter-monomer contacts in the G217Y mutant, we were able to unambiguously assign both dimer- and trimer-specific NOEs in WT DR5^{TMH} (Figure 2A) and determined the minimal hexamer structure of DR5^{TMH} in bicelles (Figures 4A, S4A and S4B). The trimeric assembly is essentially the same as that of the mutant. As expected, the dimerization interface mainly involves strong inter-monomer contacts at G213 and G217, although several other residues such as V220 and A221 appear to contribute as well with less tight packing (Figure 4B). The OG-label analysis in Figure 2F already showed that the G217Y mutation can completely break the dimeric interaction. We note that our NOE data is consistent with either dimer-of-trimer or trimer-of-dimer model, as either would fit the bicelle size we used. The trimer-of-dimer structure was also calculated using the same set of NOE restraints (Figure S4C). Further, the fact that only one set of NMR peaks was observed in the TROSY-HSQC spectrum (Figure 1B) is more consistent with a mixture of dimer-of-trimer and trimer-of-dimer in fast change.

To address the positions of the dimer- and trimer- forming residues in membrane, we performed TM partition analysis of the TMH using a paramagnetic probe titration (PPT) method developed previously for bicelle-reconstituted protein samples (Piai et al., 2017b). To simplify analysis, the trimeric mutant in bicelles was used because it only has one symmetry axis. Titration of the water-soluble paramagnetic probe Gd-DOTA outside the

bicelles provided residue-specific paramagnetic relaxation enhancement amplitudes (PRE_{amp}) (Figure S4D). Then, PRE_{amp} vs. *residue number* was converted to PRE_{amp} vs. *position* (Figure S4E) by translating residue number to the corresponding H_N position along the 3-fold axis in the mutant structure. Finally, the TMH position relative to the bilayer center was determined using “sigmoidal fitting” (Figure S4F). The results indicate that the trimer-forming residues are mostly in the core of the bilayer whereas the GXXXG motif is in the lipid headgroup region (Figure 4C).

Propagation of Dimerization and Trimerization Mediates Higher-Order Assembly of DR5

The estimated diameter of ~ 50 Å for the bilayer region of the bicelles used in DR5^{TMH} reconstitution for NMR studies would be able to fit either a dimer-of-trimer of the TMH or a trimer-of-dimer. However, in the absence of size restraints on the cell surface, having both trimeric and dimeric interfaces suggest that DR5^{TMH} is able to form higher-order clusters in membrane (Figures 4D and 4E). Indeed, superimposing one dimer in a trimer-of-dimers structure with a dimer in another trimer-of-dimers structure did not create any steric hindrance, but rather generated multiple trimer-of-dimers (Figure 4D) that can mediate formation of a patch of dimer-trimer TMH network on membrane (Figure 4E).

TMH Clustering is Required for DR5 Signaling

From the structure and mutagenesis data above, we identified a set of single-point mutations critical for addressing which TMH oligomerization is needed for DR5 activation, whether it is trimerization, dimerization, or both. These mutations are the dimer-breaking G217Y, the trimer-breaking T219Y or A222Y, and the control V227Y that has no effect on dimerization or trimerization. Moreover, the NMR structure explained why the mutation V227Y did not affect DR5^{TMH} oligomerization (Figure 2F) because V227 is not located in the dimer or trimer interface (Figure 5A). We tested these mutants with two independent experiments. The soluble DR5 ligand, known as TNF-related apoptosis-inducing ligand (TRAIL), was used to induce DR5-mediated apoptosis. In one case, the WT and mutants were transiently expressed in DR5-deficient BJAB cells in the form of C-terminal EGFP fusion proteins, and Caspase-8 activity was monitored for TRAIL-induced receptor activity. Transfection efficiency was 40–60% for all DR5 variants (Figure S5A) and the receptors were mainly expressed on the cell surface (Figure S6A). In another case, stable HEK293T cell lines expressing the WT and mutants were developed to achieve more controlled protein expression, and the receptor-mediated apoptosis was evaluated in the form of cell viability measurement. Cell surface expression levels were also similar among the DR5 variants according to anti-DR5 antibody immunostaining (Figure S5B). In both assays, the V227Y mutant, whose TMH assembly was not affected, showed the same signaling activity as the WT (Figures 5B and 5C). The estimated EC_{50} of TRAIL in these two experiments is 60–100 ng/ml. In contrast, single mutations that disrupt either dimeric (G217Y) or trimeric (T219Y, A222Y) interaction as well as the double mutation (G217Y/A222Y) that disrupts both interactions all abolished the receptor activity in these assays (Figures 5B and 5C). The TRAIL-induced signaling assays strongly indicated that both trimeric and dimeric interactions of the TMH are essential to DR5 signaling. While the need for TMH trimerization in receptor activation was expected from the study of Fas (Fu et al., 2016), the

role of TMH dimerization is likely exerted by mediating higher-order receptor clustering, a concept that has not been previously attributed to the TMH.

A previous study showed that crosslinking TRAIL could greatly augment agonistic activity toward DR5 (Nair et al., 2015). This result led us to test whether crosslinking the TRAIL can compensate for the defect of the G217Y mutant to form higher-order clusters. For this experiment, we used TRAIL with N-terminal FLAG tag (FLAG-TRAIL) and anti-FLAG IgG to crosslink the TRAIL trimers. In this case, the antibody should provide the dimeric interactions that the G217Y mutant lacks. Indeed, for both transiently expressed G217Y mutant in DR5-deficient BJAB cells and stably expressed mutant in the stable HEK293T cells, crosslinking TRAIL with antibody rescued the inability of the mutant receptor to signal (Figures 5D and 5E), lending further support for the role of TMH dimerization in mediating higher-order receptor clustering.

To gather more direct evidences of TMH-mediated receptor clustering, we used confocal microscopy to examine ligand-induced DR5 clustering on the cell surface and the effect of TM mutations on cluster formation. In this experiment, the C-terminus of DR5 was fused to the enhanced green fluorescent protein (EGFP) (designated DR5-EGFP). We transiently expressed DR5-EGFP, the corresponding TEV-cleavable version, and the dimer-breaking mutant (G217Y) separately in HEK293T cells, and used confocal microscopy to examine their surface distribution before and after treatment with ligand/enzyme. As shown in Figure S6B, treating the cells expressing WT DR5-EGFP with TRAIL clearly resulted in the appearance of several very bright and elongated puncta on the cell surface, suggesting receptor clustering. As expected, treating the cells expressing TEV-cleavable DR5-EGFP with TEV enzyme also induced many bright puncta on the cell surface. However, treating the cells expressing the dimer-breaking mutant, while slightly increased fluorescence on the cell surface, did not generate any sharp bright puncta.

Since the dimer-breaking mutation is in the TMH, the data suggest that the receptor clusters were mediated by TMH oligomerization.

TMH Higher-Order Clustering Alone Is Sufficient for DR5 Signaling, which is Inhibited by the Unliganded Extracellular Domain

The ability of DR5^{TMH} alone to mediate a dimer-trimer network formation prompted us to wonder why full-length DR5 does not signal in the absence of ligand binding, and whether the ECD physically hinders TMH from clustering and activation of the signaling pathway (Figure 6A). To test this hypothesis, we inserted a TEV cleavage sequence (ENLYFQGGGGGS) at residue 208 between the ECD and TMH for proteolytic removal of ECD and appended the receptor C-terminus with EGFP for facile separation of DR5⁺ cells using flow cytometry analysis. The cleavable DR5 was transiently expressed in DR5-deficient BJAB cells. The modified receptor was still active as addition of TRAIL induced strong Caspase-8 activity (Figure 6B). Remarkably, TEV protease treatment to remove the ECD activated DR5 in a dose-response manner (Figure 6C). The extent of Caspase-8 activation mimicked ligand-induced activation (Figure 6B). Additionally, increase in receptor activity correlated with ECD removal from the cell surface, which was assessed by anti-DR5 antibodies (Figure S7A, B). In parallel, WT DR5 without the TEV cleavage site

was not responsive to the protease except at a very high enzyme concentration (100 $\mu\text{g/ml}$) (Figure 6C). These results revealed that the pre-ligand state of ECD hinders the intrinsic ability of the TMH to assemble and activate the signaling pathway, and that the TMH, instead of the ECD, affords the driving force for assembly of the intracellular signaling complex.

Effect of TMH Oligomerization on the Pre-Ligand Form of DR5

To further examine the role of the TMH in pre-ligand and ligand-dependent DR5 associations, we implemented a fluorescence resonance energy transfer (FRET) assay used previously for investigating self-association of Fas and other receptors in the TNFRSF (Chan et al., 2000; Lee et al., 2009; Siegel et al., 2000a). In this experiment, the intracellular region of DR5 was replaced with either cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP). The fluorescent CFP- and YFP-fused WT DR5 constructs, or those harboring TMH mutations, were transiently co-expressed in HEK293T cells, and the FRET efficiency between CFP and YFP, which quantitates receptor association, were measured by acceptor photobleaching FRET (Chan et al., 2000). Before ligand stimulation, the FRET efficiencies of WT and the A222Y mutant that is defective for TMH trimerization did not show significant difference (Figure 6D, E). This is consistent with previous data on Fas that trimer-breaking mutations did not alter the Fas pre-ligand oligomerization (Fu et al., 2016). Upon ligand addition, the FRET efficiency of WT DR5 increased significantly (Figure 6D, E). Similarly, the G217Y mutant also showed a significant increase in FRET efficiency after TRAIL binding. However, for the A222Y mutant that is defective in TMH trimerization, the FRET increase upon ligand binding was not significant compared to the experimental uncertainty. Together these data seem to suggest DR5 TMH trimerization does not influence receptor pre-ligand association but is critical for ligand-induced receptor clustering.

Unliganded Extracellular Domain also Inhibits Receptor Activation for TNFR2 and OX40

We further investigated whether proteolytic removal of ECD can also activate TNFR2 or OX40, two other members of the TNFRSF. Since the TMHs of the two receptors, like that of DR5, are expected to cluster owing to the GXXXG motif (Figure 7A), removal of the ECD ought to free the TMHs, allowing them to cluster and activate downstream signaling. We inserted a TEV cleavage site between the ECD and TMH as was done for DR5 above and used an NF- κB luciferase reporter to read out receptor activation by the TEV protease in the absence of the native ligands. The results show that both TEV-cleavable TNFR2 and OX40 could be activated by the protease in a dose-dependent manner whereas the WT receptors were not responsive (Figure 7B,C).

DISCUSSION

Intrinsic Dimer-Trimer Network of DR5 TMH Is Sufficient for Signaling while a Role of the ECD Is Autoinhibition

In this study, we unexpectedly discovered that the single-pass TMH of DR5 has distinct trimerization and dimerization interfaces to allow the formation of higher-order structures in membrane, and that the TMH alone is sufficient to drive signaling in the absence of the ECD. While existing models of TNFRSF signaling rely on clustering by the ECD (Mukai et

al., 2010; Valley et al., 2012; Vanamee and Faustman, 2018; Yang et al., 2005), our results in contrast revealed the necessary and sufficient role of the TMH in DR5 clustering and signaling. First, both TMH interfaces are necessary as structure-based single-point mutations in the TMH that disrupt either the trimeric or dimeric interaction abolished TRAIL-induced cell death. Second, the TMH-mediated dimer-trimer network is sufficient as we demonstrated full DR5 activation by proteolytic removal of the ECD, in the absence of ligand engagement.

In this context, we hypothesize that the primary consequence of ligand binding is to overcome the inhibitory restraint that the ECD places on the TMH by altering the pre-ligand conformation (Figure 7D). This autoinhibition hypothesis provides an explanation for the long-standing puzzle on how dimeric agonistic antibodies or the dimeric ligand NGF activate TNFRSF family members as trimeric TNF family ligands do, because many different ECD interactions may overcome a specific autoinhibition state, while ECD clustering with an optimal geometry to position the TMH is unlikely to achieve in the different dimer-trimer, trimer-trimer or dimer-dimer networks that these different activating agents may assume with the receptors.

Disruption of the pre-ligand state by ligand binding is supported by the lack of crystal lattice-mediated dimeric associations of the unliganded TNFR1 ECD (Naismith et al., 1995) in the crystal structures of any ligand-receptor complexes in the TNFRSF (Vanamee and Faustman, 2018; Wu and Hymowitz, 2009). Additionally, mutations in the PLAD away from the ligand-binding site have been found to affect ligand binding, suggesting an allosteric coupling between ligand binding and PLAD association (Chan et al., 2000). There is also a tendency for agonistic antibodies to bind to the CRD1 and CRD2 regions of TNFRSF members that overlap with the ECD PLAD (Chodorge et al., 2012; Siegel et al., 2000b; Tamada et al., 2015), suggesting that breaking the PLAD interaction contributes to the agonistic phenotypes. Interestingly, O-linked glycosylation of DR5 ECD could augment ligand-induced receptor signaling (Wagner et al., 2007), which could also be explained by weakening of the PLAD interaction by O-Glycan modification of ECD. Collectively, our studies afford new opportunities and unique perspectives to modulate the signal transduction of these receptors for disease treatment including cancer immunotherapy.

The GXXXG Motif

The extraordinary function of a single TMH of DR5 to mediate both ligand-receptor complex assembly and higher-order receptor clustering may be attributed partly to the GXXXG motif. Previous studies of GXXXG-bearing receptor TMHs mainly reported the function of GXXXG in receptor assembly, e.g., EGFR (Endres et al., 2013) and ErbB2 (Bocharov et al., 2008). The current study uncovered an unrecognized function of GXXXG in receptor clustering. Notably, two other members of the TNFRSF, OX40 and TNFR2, also have TMHs that contain GXXXG, and they are expected to cluster similarly as that of DR5. Indeed, we found that both TNFR2 and OX40 could be efficiently activated by proteolytic removal of the receptor ECD in the absence of their respective ligands, suggesting that their TMHs drive downstream signaling in a manner similar to that of DR5. Furthermore, TMHs of some activating immunoreceptors such as the NKG2C-DAP12 complex also have

conserved GXXXG motif, which is curiously not involved in receptor assembly (Call et al., 2010). Therefore, the role of GXXXG in mediating receptor clustering may represent a more general concept in receptor biology.

Possible Determinants of Receptor Cluster Formation

While the presence of distinct dimerization and trimerization interfaces on opposite faces of DR5 TMH implies the ability of the TMH to form large clusters as modeled in Figure 4D, our data is insufficient to address the cluster size *in vivo*. In the native setting, the TMH aggregation number could depend on obvious factors including geometrical constraints posed by the extra- and intra-cellular domains of the receptor, the local receptor concentration, as well as the biophysical properties of the relevant membrane. The clusters, however, do not have to be rigidly and uniformly structured arrays if their purpose is simply to concentrate laterally the intracellular signaling components to a level that the signaling-competent DISC can form. As such, the reversible dimeric and trimeric interactions among the TMHs could suffice to concentrate the full-length receptors on the cell surface and form large, dynamic clusters. Indeed, our confocal microscopy data that TRAIL could induce large puncta formation by the WT DR5 but not the G217Y mutant is consistent with the ability of TMH to mediate large scale receptor clustering. Similar puncta have been observed previously for TNFR1 (Ko et al., 1999), Fas (Henkler et al., 2005), and 4-1BB (Gusti et al., 2014), although in those cases, factors other than the TMH could provide the dimeric interactions to achieve the dimer-trimer network.

STAR METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact James Chou (james_chou@hms.harvard.edu)

EXPERIMENTAL MODEL AND SUBJECT DETAILS

DR5-deficient BJAB cell line—Human Burkitt lymphoma B cell line -DR5-deficient BJAB was maintained in RPMI 1640 (Gibco, ThermoFisher Scientific) plus 10% fetal bovine serum (Gibco, ThermoFisher Scientific), and 100U/ml Pen-Strep (Gibco, ThermoFisher Scientific), at 37° C, 5% CO₂.

HEK293T cell line—Human kidney epithelial cell line HEK293T and related DR5-expressing stable cell lines were maintained in DMEM (Gibco, ThermoFisher Scientific) plus 10% fetal bovine serum (Gibco, ThermoFisher Scientific), and 100U/ml Pen-Strep (Gibco, ThermoFisher Scientific), at 37° C, 5% CO₂.

METHOD DETAILS

Protein Expression and Purification—The human DR5 (isoform 1) fragment, residues 208 – 242, corresponding to the TMH was synthesized by GenScript (Piscataway, NJ) (Figure S1A). Amino acid C209 within stalk region was mutated to glycine, as DR5S (short form) with no stalk region is functional as DR5L (long form). This fragment is designated DR5^{TMH}. Expression constructs were created by fusing the DR5^{TMH} fragment to the C-

terminus of the His9-TrpLE expression sequence in pMM-LR6 vector (a gift from S.C. Blacklow, Harvard Medical School), with an added methionine in-between for cleavage by cyanogen bromide. Mutant constructs were generated by standard PCR protocols and confirmed by DNA sequencing. For NMR sample preparation, transformed *E. coli* strain BL21 (DE3) cells were grown in M9 minimal media supplemented with Centrum multivitamins and stable isotopes. Cultures were grown at 37° C to an absorbance of ~0.6 at 600 nm, and cooled to 28° C before induction with 600 μ M isopropyl β -D-thiogalactopyranoside at 28° C for overnight. For fully deuterated proteins, bacterial cultures were grown in 99.8% D₂O (Sigma Aldrich, St. Louis, MO) with deuterated glucose (Cambridge Isotope Laboratories, Tewksbury, MA). The DR5^{TMH} protein was extracted, cleaved by cyanogen bromide, purified and lyophilized as described (Fu et al., 2016). Some of the differences are stated below. To keep Cys232 reduced, 20 mM BME (2-Mercaptoethanol) was used throughout the Ni-NTA purification step. Because DR5^{TMH} is very hydrophobic, it was still bound to the column after the elution gradient from 50% (v/v) isopropanol with 0.1% (v/v) trifluoroacetic acid (Buffer A) to 100% (v/v) acetonitrile with 5% (v/v) isopropanol and 0.1% (v/v) trifluoroacetic acid (Buffer B) (Figure S1B). A second elution gradient from 0% to 100% Buffer B was needed to elute DR5^{TMH} (Figure S1B).

Reconstitution of DR5^{TMH} in Bicelles—To reconstitute the DR5^{TMH} variants in bicelles, 1~2 mg of purified and lyophilized protein was mixed with 9 mg 1,2-Dimyristoyl-*sn*-Glycero-3-Phosphocholine (DMPC; protonated or deuterated from Avanti Polar Lipids, Alabaster, AL) and dissolved in hexafluoro-isopropanol. The mixture was slowly dried to a thin film under nitrogen stream, followed by overnight lyophilization. The dried thin film was redissolved in 3 ml of 8 M Urea containing ~27 mg 1,2-Dihexanoyl-*sn*-Glycero-3-Phosphocholine (DHPC, D6PC; protonated or deuterated from Avanti Polar Lipids) and 20 mM 2-Mercaptoethanol (BME). The mixture was dialyzed twice against a 20 mM phosphate buffer (pH 7) (1 L each time) to remove the denaturant, and 10 mg D6PC was added to the sample before the second dialysis to compensate its loss. The DMPC:DHPC ratio was monitored by 1D NMR throughout the reconstitution process. If needed, additional D6PC was added to make the final DMPC:DHPC ratio between 0.5 and 0.6 (Figure S1C). The sample was concentrated using Centricon (EMD Millipore, Billerica, MA) to ~350 ml. The final NMR sample contained ~0.8 mM DR5^{TMH} (monomer), ~50 mM DMPC, ~100 mM DHPC, 20 mM phosphate buffer (pH 7), 0.02% NaN₃ and 5% D₂O. For all NOE experiments, the protein was reconstituted using DMPC and DHPC with deuterated acyl chains (Avanti Polar Lipids).

Analysis of the Oligomeric State of the WT and Mutant DR5^{TMH} in Bicelles by SDS-PAGE and OG-Label—Standard SDS-PAGE was first used to examine the oligomeric state of the bicelle-reconstituted DR5^{TMH}. The WT or mutant DR5^{TMH} was reconstituted in bicelles ($q \sim 0.5$), and then mixed with a SDS sample buffer (Invitrogen) without boiling, followed by SDS-PAGE at 200 volts for 30 minutes and Commassie blue staining. In the cases of the WT DR5^{TMH} and the G217Y and V227Y mutants, the unreconstituted protein migrated at apparent MW of ~5 kDa (theoretical MW of DR5^{TMH} is 3.6 kDa), and the bicelle-reconstituted protein migrated at ~16 kDa, which corresponds to a trimer. Although the trimeric interaction of DR5^{TMH} resisted SDS, the dimeric interaction

did not. Therefore, the oligomeric state was further examined using the non-denaturing method known as OG-label.

In the OG-label method, each of the protomers in the membrane protein oligomer was labeled, non-covalently, with a soluble crosslinkable protein (SCP) protein. Then the SCPs were crosslinked with Lomant's reagents to read out the oligomeric state (schematically illustrated in Figure 2E). The SCP used was a small Ig-fold protein named GB1 (MW = 8.4 kDa), and its N-terminus was linked to a TriNTA molecule via a PEG-2-SMCC ((succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate)) to form the TriNTA-GB1 conjugate as previously described (Chen et al., 2018). The membrane protein to be examined has a His₆-tag. TriNTA has high binding affinity to His₆-tag (20 ± 10 nM) (Lata et al., 2005), which can strongly attach GB1 to the individual protomers of the membrane protein oligomer in bicelles. Then, the concentration of stoichiometric number of GB1 to the membrane protein oligomer allows for more efficient crosslinking than the free GB1 in solution. The crosslinked GB1 can be released from the oligomer by addition of EDTA and analyzed by SDS-PAGE.

To implement the OG-label method for DR5^{TMH}, the protein was modified by a C-terminus addition of a His₆-tag. The His₆-tagged DR5^{TMH} was expressed, purified, and reconstituted in bicelles ($q \sim 0.5$) in the same way as described above for other NMR samples except HEPES (pH 7.2) was used as buffer for better crosslinking efficiency. To prevent undesirable crosslinking between DR5^{TMH} and GB1, we first blocked all the active amine groups with the addition of 100-fold molar excess of Sulfo-NHS Acetate (Thermo Fisher Scientific) to the bicelle sample in 25 mM HEPES buffer (pH 7.2) for 1 hour at room temperature. Excessive Sulfo-NHS Acetate was removed by dialysis while tightly controlling the bicelle q . After dialysis, 20 mM DR5^{TMH} (monomer concentration) was mixed with 30 mM TriNTA-GB1 to ensure the His₆-tagged DR5^{TMH} are saturated with TriNTA-GB1. The mixture was then treated with 0.6 mM of DTSSP for 30 min, followed by incubation with various concentration (0.1, 0.5, 1.0, and 2.5 mM) of glutaraldehyde for 5 min. The crosslinking reaction was quenched with a 20 mM Tris buffer (pH 7.5). As a negative control, 0.6 mM of DTSSP and 0.5 mM of glutaraldehyde were sequentially added to 30 mM TriNTA-GB1 in the absence of the His₆-tagged DR5^{TMH}. The crosslinked species were boiled and examined by SDS-PAGE using the 4–12% Bis-Tris protein gels (Thermo Fisher Scientific).

Assignment of NMR Resonances—All NMR data was collected at 30°C (303k) on Bruker spectrometers operating at ¹H frequency of 900 MHz, 800 MHz, 750 MHz, or 600 MHz and equipped with cryogenic probes. NMR data was processed using NMRPipe (Delaglio et al., 1995) and spectra analysis was performed in NMRPipe (Delaglio et al., 1995), Sparky (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco), and XEASY (Bartels et al., 1995). Sequence specific assignment of backbone chemical shifts was accomplished using two pairs of TROSY-enhanced triple resonance experiments (Salzmann et al., 1999), recorded using a (¹⁵N, ¹³C, 85% ²H)-labeled sample. The triple resonance experiments included HNCA, HN(CO)CA, HN(CA)CO, and HNCO. Backbone amide resonance assignments were confirmed using a 3D ¹⁵N-edited NOESY-TROSY-HSQC spectrum ($\tau_{\text{NOE}} = 200$ ms), which was recorded on a 750 MHz spectrometer

using (^{15}N , ^2H)-labeled sample. Protein aliphatic and aromatic resonances were assigned using a combination of 2D ^{13}C HSQC, 3D ^{15}N -edited NOESY-TROSY ($\tau_{\text{NOE}} = 120$ ms) and ^{13}C -edited NOESY-HSQC ($\tau_{\text{NOE}} = 150$ ms) recorded on a 900 MHz spectrometer. These experiments were performed using a (^{15}N , ^{13}C)-labeled protein samples in deuterated bicelles, i.e., the acyl chains of DMPC and DHPC were deuterated (Anatrace). Specific stereo assignment of the methyl groups of valines and leucines were obtained from a 28 ms constant-time ^1H - ^{13}C HSQC spectrum recorded using a 15% ^{13}C -labeled sample (Szyperski et al., 1992). Assignments of the backbone NH and sidechain methyl groups are shown in Figure 1B, 1D, S2A&B.

Backbone Chemical Shift and TALOS Analyses—The assigned chemical shift values of backbone ^{15}N , $^{13}\text{C}\alpha$, and $^{13}\text{C}'$ were used as input for the TALOS+ program (Shen et al., 2009) for predicting backbone dihedral angles. Out of 32 residues with assignments, the dihedral angles of 28 residues were considered by TALOS+ as ‘GOOD’. Furthermore, the $^{13}\text{C}\alpha$ secondary shifts of DR5^{TMH} and the G217Y mutant are shown in Figure S2C, providing a secondary structure mapping of the TM fragment.

Assignment of NOE Restraints—Intramolecular distance restraints derived from nuclear Overhauser enhancements (NOEs) were obtained from the above-mentioned ^{15}N -edited and ^{13}C -edited NOESY spectra recorded on a 900 MHz spectrometer. Determining the inter-protomer distance restraints faced the challenge of measuring NOEs between structurally equivalent protomers having the same chemical shifts. To solve this problem, we prepared a mixed sample in which half of the protomers were ^{15}N , ^2H -labeled (0.4 mM) and the other half 15% ^{13}C -labeled (0.4 mM). Recording a 3D ^{15}N -edited NOESY-TROSY-HSQC ($\tau_{\text{NOE}} = 200$ ms) on this sample allowed measurement of exclusive NOEs between the ^{15}N -attached protons of one subunit and aliphatic protons of the neighboring subunits. The non-deuterated protein was 15% ^{13}C -labeled for recording the ^1H - ^{13}C HSQC spectrum as internal aliphatic proton chemical shift reference. Inter-protomer NOE strips of the WT DR5^{TMH} is shown in Figure 2A.

To distinguish trimer-specific NOEs from the dimer-specific NOEs, the same mixed NOE experiment above was performed for the G217Y mutant. As shown in Figure 2B, the dimer-specific NOEs have vanished. In parallel, a control sample of the G217Y mutant with only (^{15}N , ^2H)-labeled protein (0.4 mM) was used to record an identical 3D ^{15}N -edited NOESY-TROSY-HSQC (Figure 2C). Comparison of the mixed and control spectra allows rigorous confirmation that a particular inter-protomer NOE was due solely to the mixing of protomers and NOT to incomplete deuteration of the protein.

Structure Calculation—Structure was calculated using the program XPLOR-NIH (Schwieters et al., 2003). We first determined the trimer structure of the G217Y mutant. The monomer structure was derived using intramonomer restraints and backbone dihedral restraints, determined from chemical shifts using the TALOS+ program (Shen et al., 2009). Using the monomer structure and inter-protomer NOEs from the mutant spectrum, the trimer assembly solution was derived using the ExSSO program (Yang et al., 2017). The unique trimer solution was then fed to XPLOR-NIH for further refinement against all NOE restraints (intra- and inter- protomer NOEs) and dihedral restraints. For each inter-protomer

restraint between two adjacent protomers, three identical distance restraints were assigned respectively to all pairs of neighboring protomers to satisfy the condition of C3 rotational symmetry. The XPLOR refinement used a simulated annealing (SA) protocol in which the temperature in the bath was cooled from 1000 to 200 K with steps of 20 K. The NOE restraints were enforced by flat-well harmonic potentials, with the force constant ramped from 2 to 30 kcal/mol Å⁻² during annealing. Backbone dihedral angle restraints were taken from the ‘GOOD’ dihedral angles from TALOS+, all with a flat -well (\pm the corresponding uncertainties from TALOS+) harmonic potential with force constant ramped from 5 to 1000 kcal/mol rad⁻². A total of 100 structures were calculated and 15 lowest energy structures were selected as the final structural ensemble (Figure S3 and Table S2).

To calculate the dimer-of-trimer structure of the WT DR5^{TMH}, the trimer-specific inter-protomer NOEs, which are almost the same as those of the G217Y mutant, were first used to generate a trimer structure using the ExSSO program (Yang et al., 2017). Then, two copies of the trimer structure, or the hexamer, were used as input to XPLOR-NIH for further calculation to satisfy both trimer- and dimer-specific inter-protomer NOEs restraints. The XPLOR protocol used was the same as above. A total of 100 structures were calculated and 15 lowest energy structures were selected as the final structural ensemble of the dimer-of-trimer (Figure S4A&S4B and Table S2).

Analysis of Transmembrane Partition of the Trimeric Mutant of DR5^{TMH}—A

previously published Paramagnetic Probe Titration (PPT) method (Piai et al., 2017a; Piai et al., 2017b) was used to determine the transmembrane partition of the trimeric DR5^{TMH} mutant in bicelles. This method is based on the notion that if the bicelle is sufficiently wide ($q > 0.5$), the lateral solvent paramagnetic relaxation enhancement (PRE) becomes negligible, thus allowing the use of measurable solvent PRE to probe residue-specific depth immersion of the protein in the bilayer region of the bicelle. We reconstituted the G217Y mutant in bicelles with $q = 0.6$ (Figure 4C) to perform the PPT as described previously (Piai et al., 2017b). The water-soluble and membrane-inaccessible paramagnetic agent, Gd-DOTA (Sigma), was used to generate solvent PREs. Gd-DOTA (in 200 mM stock solution) was titrated into the bicelle sample to reach final concentrations of 0.0, 1.0, 2.0, 4.0, 8.0, 10.0, 15 and 20 mM. At each concentration, a 2D ¹H-¹⁵N TROSY-HSQC spectrum was recorded at 600 MHz to measure residue-specific PRE, defined here as the ratio of peak intensity in the presence (I) and absence (I₀) of the paramagnetic agent. For each of the residues, we used *Origin* (OriginLab, Northampton, MA) to fit the PRE titration curve to exponential decay

$$\frac{I}{I_0} = 1 - \text{PRE}_{\text{amp}}(1 - e^{-[\text{Gd-DOTA}]/\tau}) \quad (1)$$

to derive the residue-specific PRE amplitude (PRE_{amp}) (Figure S4D). To determine the position of the trimer relative to the bilayer center, we calculated, for each residue i , the distance (r_z) along the protein symmetry axis, which is parallel to the bilayer normal, from the amide proton to an arbitrary reference point based on the structure of the DR5^{TMH} mutant trimer. This calculation converted PRE_{amp} vs. (residue number) to PRE_{amp} vs. r_z

(Figure S4E), which was then analyzed using *the sigmoidal fitting* method. Briefly, the trimer structure was moved along the 3-fold axis in increment of 0.3 Å (Figure S4F) to achieve the best fit to the symmetric sigmoid equation

$$\text{PRE}_{\text{amp}} = \text{PRE}_{\text{amp}}^{\text{min}} + \frac{(\text{PRE}_{\text{amp}}^{\text{max}} - \text{PRE}_{\text{amp}}^{\text{min}})}{1 + e^{(r_z^I - |r_z|)/\text{SLOPE}}}, \quad (2)$$

where $\text{PRE}_{\text{amp}}^{\text{min}}$ and $\text{PRE}_{\text{amp}}^{\text{max}}$ are the limits within which PRE_{amp} can vary, r_z^I is the inflection point (the distance from the bilayer center at which PRE_{amp} is halfway between $\text{PRE}_{\text{amp}}^{\text{min}}$ and $\text{PRE}_{\text{amp}}^{\text{max}}$), and SLOPE is a parameter which reports the steepness of the curve at the inflection point. The best fit gave an adjusted coefficient of determination (R^2_{adj}) of ~0.9 (Figure S4F), and was used to determine the position of the trimer structure with respect to the bilayer center ($r_z = 0$).

TRAIL Preparation—TRAIL was prepared as described (Ashkenazi et al., 1999). DNA encoding the human TRAIL (residues 114–281) was synthesized by Genescript and subcloned into the pET28a (+) vector. The plasmid was transformed into *E. coli* strain BL21 (DE3) cells for protein expression. The cells were grown in LB medium supplemented with 50 mg/ml kanamycin. Cultures were grown at 37°C to an absorbance of ~0.6 at 600 nm, and cooled to 28°C, followed by overnight induction with 100 μM isopropyl β-D-thiogalactopyranoside (IPTG) at 28°C. The cells were lysed in the *Lysis Buffer* (100 mM NaH₂PO₄, 350 mM NaCl, pH 7.4). TRAIL was purified from the soluble lysate by Ni-NTA affinity chromatography in the *Lysis Buffer*, supplemented with 150 mM imidazole for elution. Ammonium Sulfate was added to TRAIL elution from Ni-NTA to a final concentration of 0.4 M. The elution was loaded to Hitrap phenyl FF column (GE, hydrophobic interaction chromatography (HIC) column) pre-equilibrated in the *FPLC Buffer* (100 mM NaH₂PO₄, 350 mM NaCl, 0.4 M (NH₄)₂SO₄, pH 7.4). The elution fractions containing pure TRAIL were collected, pooled and exchanged into the PBS buffer (pH 7.4) using PD10 column (GE). Purified TRAIL solution was concentrated by centricon (10 kDa cut-off, Millipore), filtered (0.22 μm) and stored at –80°C before use.

Ligand-Induced DR5 Activation Assay Using DR5-Expressing Stable Cell Line—Full-length human DR5 cDNA encoding isoform 1 (NCBI Ref. NP_003833.4) was cloned into the pVCR8400-Puro vector (gift from the Springer Lab) to generate the plasmid for expressing the WT DR5. Plasmids with mutations (G217Y, A222Y, A222Y/G217Y or V227Y) were prepared by site-directed mutagenesis based on the WT DR5 plasmid. These plasmids were used with the HEK293T cells to establish DR5-expressing stable cell lines. Briefly, 48 h after transfection, cells were treated with 2 μg/mL puromycin. Clonal populations were produced by limited dilution, and individual clones were screened for DR5 surface expression. FITC-labeled anti-DR5 antibody was used to evaluate cell surface expression level of DR5 by flow cytometry (ACEA NovoCyte™, ACEA Biosciences) (Figure S5B).

In vitro efficacy study was performed on the monoclonal stable HEK293T cells with similar DR5 (or mutant) expression level. Cells were seeded at 10^4 per well in 96-well plates. On the morrow, adherent cells were incubated with serial concentrations of TRAIL (DR5 ligand) for 12 h to allow full cytotoxic effect of TRAIL. Ultimately, cells were incubated with 10% (v/v) Cell count kit (CCK-8, Dojindo) agent for about 1 h. Absorbance at 450 nm was measured by using the BioRad Model 680 Microplate Reader. IC_{50} , defined as the concentration of TRAIL that reduced the response of untreated control group by half, was calculated using GraphPad Prism 6.01.

Cell Imaging—WT or mutant DR5-EGFP constructs were transfected into DR5-deficient BJAB cells by electroporation as described above. Twelve hours post-transfection, cells were transferred to glass-bottomed dishes pretreated by poly-glycine and then stained by Hoechst for 1 hour for nucleus staining. All the images were taken under Olympus Fluoview FV1000 confocal microscope (Figure S6A).

DR5-EGFP, TEV-cleavable DR5-EGFP, and the mutant DR5-EGFP (G217Y) constructs (0.6 μ g) were transfected into 2.0×10^6 HEK293T cells in a 35 mm glass bottomed dishes (MatTek corporation) by lipofectamine 2000 (Invitrogen). Eight hours post-transfection, cells were stained by Hoechst for 1 hour for nucleus staining and imaging. For TEV cleavage assay and TRAIL stimulation assay, cells were treated by 100 μ g/ml TEV enzyme or 250 ng/ml TRAIL for another 2 hours before imaging. All the images were taken under Olympus Fluoview FV1000 confocal microscope (Figure S6B).

Ligand-Induced DR5 Activation Assay Using Transiently Transfected Cells—The same full-length human DR5 was cloned into the pcDNA3-GFP-LIC vector (a gift from Scott Gradia (Addgene plasmid # 30127)) using ligation independent cloning for expression of C-terminal GFP-tagged DR5. All DR5 mutants were generated by site-directed mutagenesis. 2 μ g plasmids of the WT, DR5-TEV and mutant DR5 were transfected into 2.0×10^6 DR5-deficient BJAB cells (gift from Dr. Thorburn) by electroporation using cell line nucleofector Kit V (Lonza). 14 h post-transfection, cells were treated with TRAIL ligand for 5 h and then analyzed for active Caspase-8 using the CaspGLOW red Caspase-8 activity kit (Biovision) and flow cytometry (BD FACSAria II) (Figures 5B and S5A). Caspase-8 inhibitor IETD-FMK was conjugated to sulfo-rhodamine (Red-IETD-FMK, or DsRed2) as the fluorescent in situ marker to label apoptotic cells. The 561 nm laser with 585/42 band pass (BP) emission filter was used for DsRed2, and the 488 nm laser was used to excite the GFP and measured with a 520/20 BP filter.

Fluorescence Resonance Energy Transfer—In the Fluorescence Resonance Energy Transfer (FRET) assay, the intracellular fragment of DR5 was replaced by ECFP or EYFP. Truncated DR5 cDNAs encoding residues 1 – 245 (WT and mutants) was cloned into the pECFP-N1 or pEYFP-N1 vector (Clontech Laboratories). After confirmation of DR5 sequences and expression, the YFP and CFP fused DR5 variants were co-transfected into the HEK293T cells. Empty pECFP-N1 and pEYFP-N1 vectors were also co-transfected into the HEK293T cells for FRET negative control. Then, 48 h after transfection, cells were treated with 10 μ g/mL TRAIL or PBS for 4 h, followed by fixation with 4% paraformaldehyde before the FRET assay. Acceptor photobleaching experiments were performed using the

Nikon A1 confocal microscope (FV3000-fluoview) with 40 mW argon lasers. CFP (donor) and YFP (acceptor) were excited with 405 nm and 515 nm lasers, respectively. Before YFP channel bleaching, both CFP and YFP channels were excited to obtain pre-bleaching FRET signal. Cells were examined with a 60× oil-immersion objective lens and bleached in the YFP channel to analyze region of interest (ROI, e.g., plasma membrane bound DR5) using the 515 nm laser at 100% intensity (95 mW laser power). Bleaching lasted 2 to 4 seconds, depending on the locations of bleached ROIs. CFP images were acquired to calculate FRET efficiency. Fifteen-eighteen images in the CFP channel were taken per ROI after photobleaching. At least 10 ROIs were used for each sample ($n > 10$). FRET efficiency ($FRET_{eff}$) was quantified as $FRET_{eff} = (D_{post} - D_{pre}) / D_{post}$, where D_{post} is the fluorescence intensity of the Donor (DR5-CFP) after acceptor (DR5-YFP) photobleaching, and D_{pre} is the fluorescence intensity of the Donor before acceptor photobleaching.

Apoptosis Induced by TEV Cleavage-Mediated DR5 ECD Removal—TEV

cleavage sequence (ENLYFQGGGGGS) was introduced into the linker region between the ECD and TMH of human DR5 isoform 1 after residue 208. The TEV cleavage construct (DR5-TEV) was transfected into DR5-deficient BJAB cells by electroporation. The DR5 with TEV cleavage sequence can also be activated by TRAIL and showed similar sensitivity to TRAIL (Figure 6B). 14 h after transfection, cells were treated with 10, 20, 40, or 100 $\mu\text{g}/\text{ml}$ TEV enzyme for 5 h at 37°C. The TEV-treated cells were analyzed for active Caspase-8 using the CaspGLOW red Caspase-8 activity kit and flow cytometry (BD FACSAria II). The 561 nm laser with 585/42 band pass (BP) emission filter was used for DsRed2, and the 488 nm laser was used to excite the GFP and measured with a 520/20 BP filter.

For activation of TNFR2 (Figure 7B) and OX40 (Figure 7C) by proteolytic removal of the ECD in the absence of ligands, TEV cleavage sequence (ENLYFQGGGGGS) was introduced into the linker region between the ECD and TMH for human TNFR2/OX40. In 24-well plates, 20 mg of NF- κ B plasmid (pHAGE-NF- κ B, a gift from Darrell Kotton; Addgene plasmid # 49343) and 50 mg of plasmid of the TEV-cleavable TNFR2/OX40 or the WT TNFR2/OX40 were co-transfected into HEK293T cells using Lipofectamine 3000 (Invitrogen). 14 h after transfection, cells were treated with 10, 40, or 100 $\mu\text{g}/\text{ml}$ TEV enzyme for 5 h at 37°C. The cell lysates of TEV-treated cells were analyzed for luminescence using the Dual-Luciferase Reporter Assay kit (Promega). Normalized relative luminescence is calculated as the difference between luminescence of the TEV-treated and non-TEV treated cell lysate divided by the cell lysate input (mg/ml). Results were from 3 independent experiments ($n=3$), and expressed as mean \pm SEM.

TEV Cleavage Validation by Flow Cytometry—TEV-treated cells were washed with PBS buffer 3 times and incubated with primary anti-DR5 monoclonal antibodies (R&D systems, catalog number: mab631) for 30 min on ice. Then the cells were washed 3 times with cold PBS, followed by incubation with Alexa Fluor-647 conjugated secondary antibodies (Abcam) for 30 min on ice. The cells were washed again with cold PBS 3 times and analyzed by flow cytometry (BD FACSAria II) (Figure S7A, B). The 633 nm laser with

670/20 band pass (BP) emission filter was used for Alexa fluor 647, and the 488 nm laser was used to excite the GFP and measured with a 520/20 BP filter.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical significance was calculated by using GraphPad Prism 6.01 (unpaired Student's test). The number of independent experiment of duplicates, the statistical significance, and the statistical test used to determine the significance are indicated in each figure or figure legend or method section where quantification is reported.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Transmembrane helix of death receptor 5 oligomerizes to drive downstream signaling
2. The transmembrane helix in lipid bilayer forms dimer-trimer interaction network
3. Receptor ectodomain in pre-ligand state inhibits receptor clustering and activation
4. Ligand binding overcomes the pre-ligand autoinhibition

terminus. See Figure S2C for Comparison of the $^{13}\text{C}\alpha$ secondary chemical shifts between the WT and the G217Y mutant.

(E) Same as in B except G217 is mutated to tyrosine (see Figures 2B and 2C for NOE data), showing disappearance of about half of the inter-protomer NOEs.

See also Figure S2.

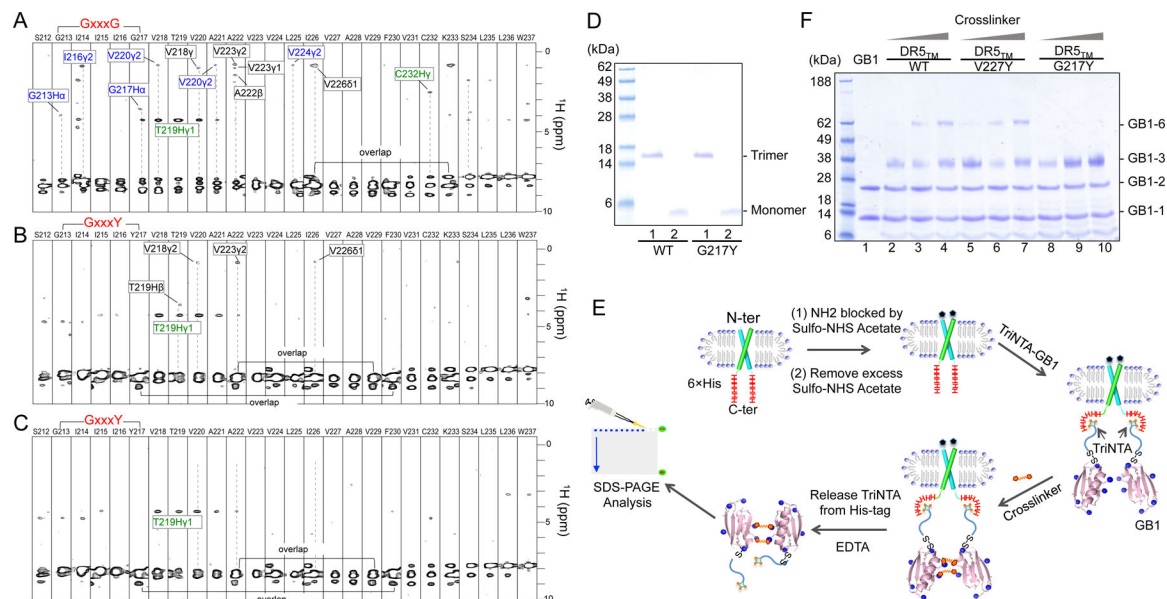


Figure 2. GXXXG Motif Mediated Minimal Dimer-Trimer Assembly of DR5^{TMH}

(A) Inter-protomer NOEs from the WT DR5^{TMH}. Strips from 3D ¹⁵N-edited NOESY-TROSY-HSQC ($\tau_{\text{NOE}} = 200$ ms) spectra recorded using an isotopically mixed sample, consisting of 0.4 mM (¹⁵N,²H)-labeled DR5^{TMH} and 0.4 mM (15% ¹³C)-labeled DR5^{TMH}, premixed at the cell level before cell lysis and protein purification. The spectrum was recorded at ¹H frequency of 900 MHz. The assignment of NOE cross peaks from the dimer interface (blue) and from the trimer interface (black) were possible using the NOE data of the G217Y mutant that eliminated the dimer interface (see B and C). Peaks labeled in green are due to labile (or exchangeable) protons.

(B) Inter-protomer NOEs from the G217Y mutant. The NOE data were collected and assigned as in (A) except the spectrum was recorded at ¹H frequency of 800 MHz.

(C) The negative control sample for (B) containing only 0.4 mM (¹⁵N,²H)-labeled G217Y mutant. The spectrum was recorded exactly the same way as in (B). TMH

(D) Oligomerization of DR5^{TMH} in bicelles analyzed by standard SDS-PAGE. The gel lanes from left to right are: (1) purified WT DR5^{TMH} or the G217Y mutant reconstituted in bicelles; powder dissolved in gel loading buffer; (2) pure WT DR5^{TMH} or the G217Y mutant powder dissolved in gel loading buffer. Samples were run under non-denaturing conditions (mixed with loading buffer and loaded onto the gel without boiling).

(E) Schematic illustration of the OG-label method for determining the oligomeric state of DR5^{TMH} in bicelles (Chen et al., 2018).

(F) Oligomerization of DR5^{TMH} analyzed by the OG-label method, which preserves the native state of the TMD assembly in bicelles (see Method).

See also Figure S2.

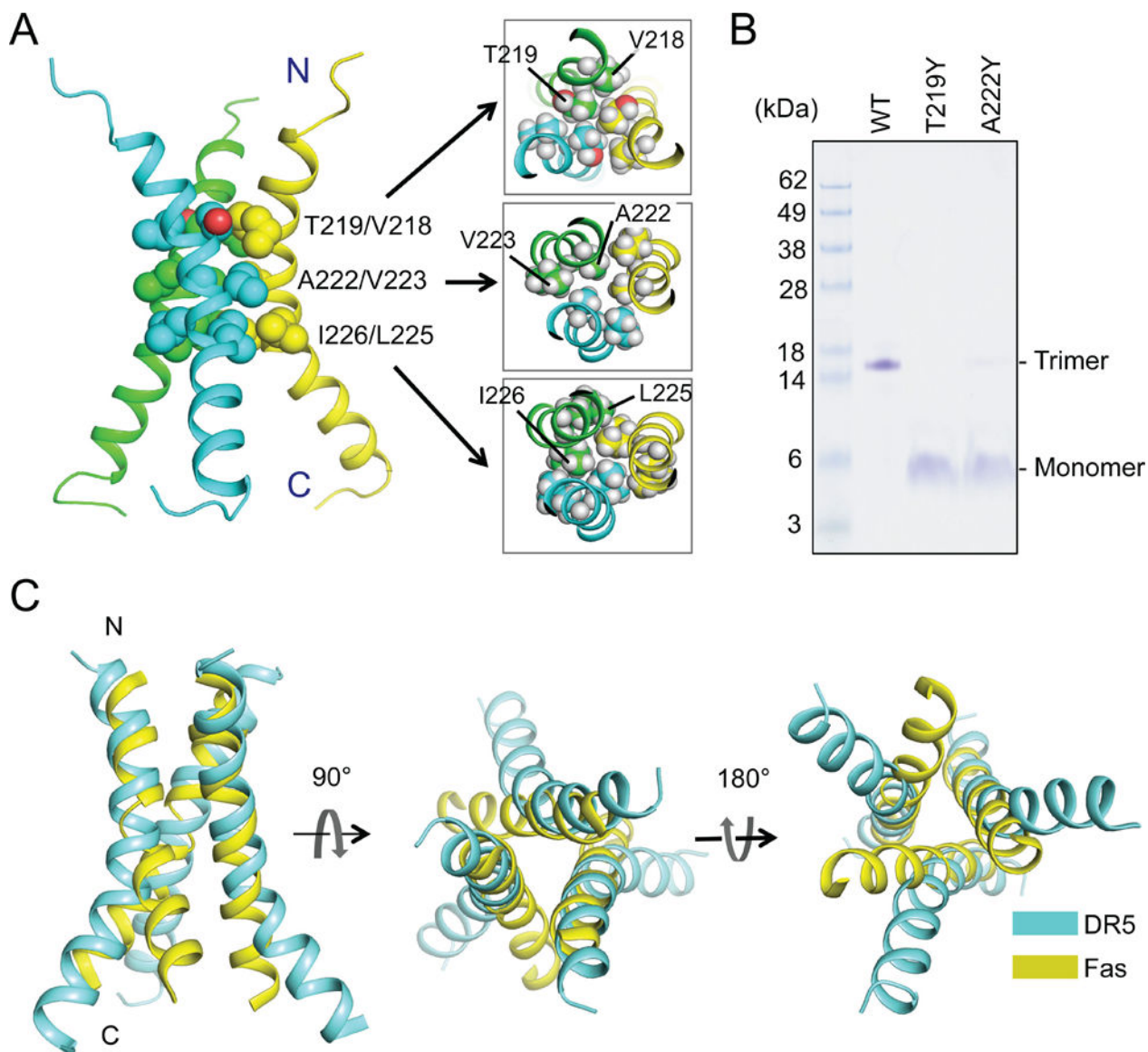


Figure 3. Structure of the Trimeric Interfaces of Human DR5^{TMH} in Bicelles

(A) Ribbon representation (left) of the trimeric G217Y mutant TMD with core residues highlighted (sidechain heavy atoms shown as spheres). The sidechain packing at three different levels along the TM helices are further illustrated with sectional top views of the trimer (right) (sidechain heavy atoms and protons included).

(B) SDS-PAGE analysis of several trimer-breaking mutations, introduced on top of the dimer-breaking G217Y mutation. Samples were run under non-denaturing condition (DR5^{TMH} variants were reconstituted into bicelles, mixed with loading buffer and loaded to the gel without boiling).

(C) Comparison between the TMH structures of human DR5 and Fas. The ribbon representations of DR5 and Fas TMHs are shown in cyan and yellow, respectively. Structural overlay shows that the Fas TMH trimer is overall more intimate than the DR5 TMH trimer. See also Figure S3.

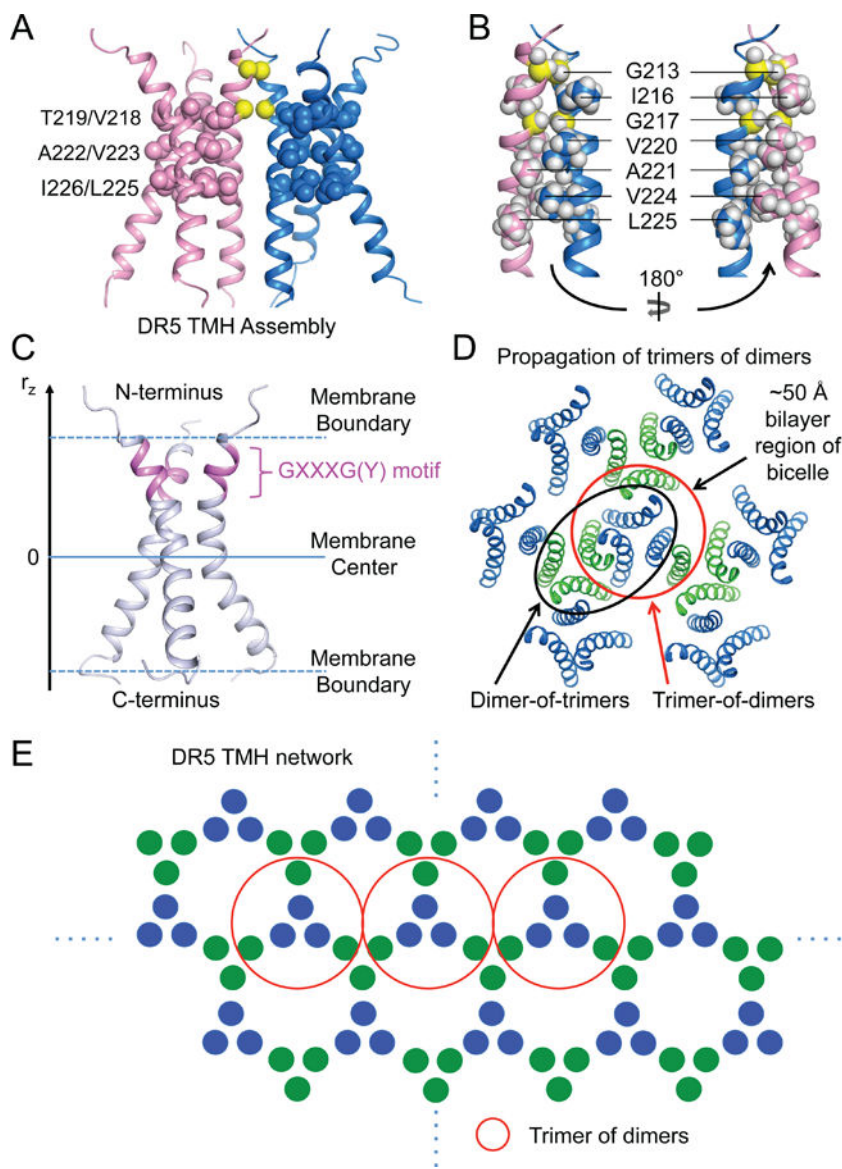


Figure 4. Propagation of Dimerization and Trimerization Mediates Higher-Order Assembly of DR5

(A) Ribbon representation of the dimer-of-trimer structure of the WT DR5^{TMH}. Residues involved in the trimer-specific inter-protomer contacts are highlighted (sidechain heavy atoms shown as spheres). In addition, the Ca atoms of G213 and G217 are shown as yellow spheres. Although the dimer-of-trimer model is calculated using the inter-molecular NOE restraints, a trimer-of-dimer in the bicelle is also possible (see Figure S4C).

(B) Close up view of the residues involved in the dimer-specific inter-protomer contacts (sidechain heavy atoms and protons shown as spheres).

(C) Transmembrane partitioning of the G217Y mutant trimer determined by the PPT method (Figure S4, D–F), showing the positions of the trimerization core and the GXXXG motif relative to the lipid bilayer.

(D) Higher-order assembly model of DR5 mediated by propagation of dimerization and trimerization. Fitting multiple dimer-of-trimer structures from Figure 4A into the network,

demonstrating the possibility of such network without steric clash. Red and Black circles represent trimer-of-dimer and dimer-of-trimer unit respectively in the clustering network. Both of them fits in the lipid bilayer region of bicelle ($q=0.55$, ~ 50 Å diameter).

(E) Schematic illustration of potential clustering network mediated by DR5 TMH, which contains both dimer and trimer interfaces. Representative trimer-of-dimer cluster units are labeled with red circle.

See also Figure S4.

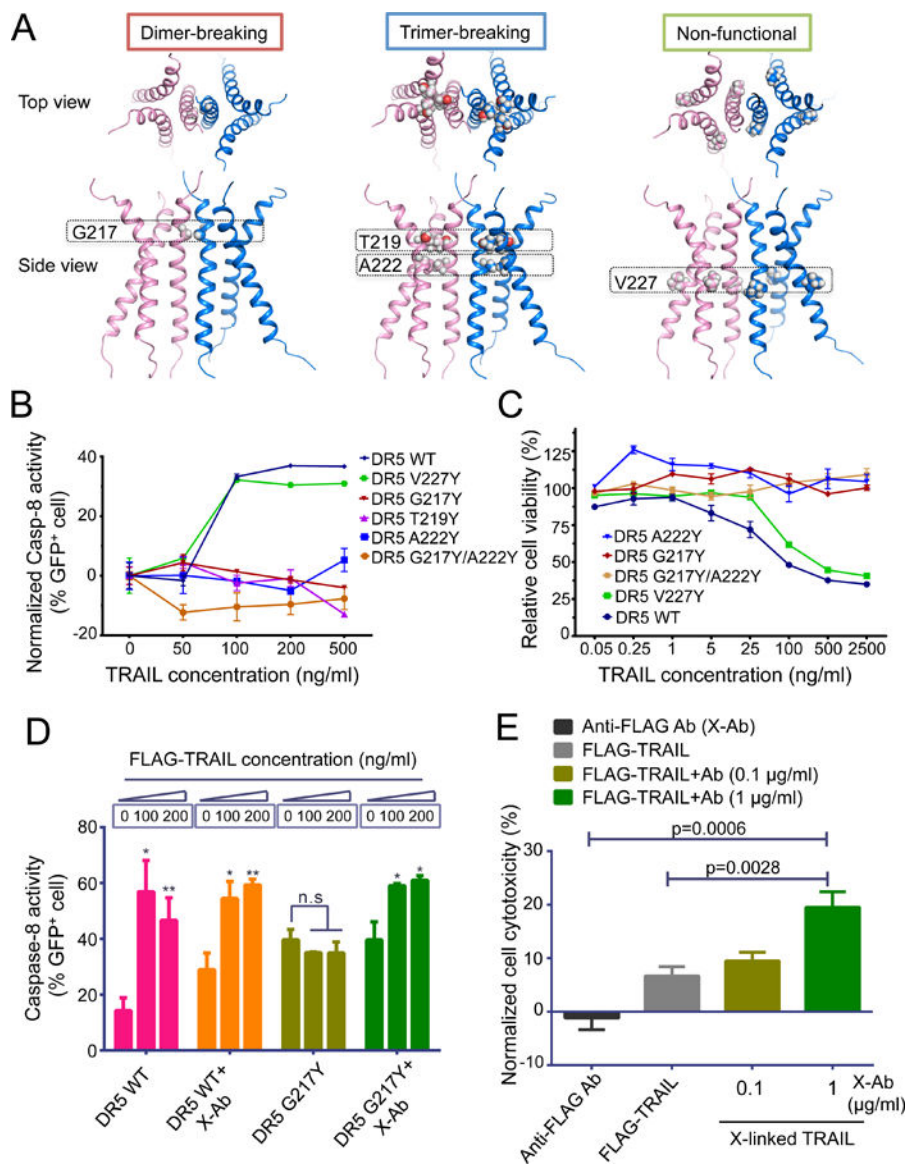


Figure 5. Essential Role of TMH Clustering in DR5 Signaling

(A) Positions of mutations in the DR5^{TMH} hexamer. Cartoon representation of the side- and over-views of the dimer-of-trimers structure of DR5^{TMH}, showing the positions of dimer-breaking mutation (G217Y), trimer-breaking mutations (T219Y and A222Y) and non-functional mutation (V227) (spheres) in the structure. The structure shows that V227 is not involved in either dimeric or trimeric interactions.

(B) TRAIL-induced apoptosis in DR5-deficient BJAB cells after transient transfection of the WT DR5 or mutants. After transient transfection, cells were treated with TRAIL for 5 h. Caspase-8 activity was measured using the CaspGLOW red Caspase-8 activity kit and flow cytometry, and calculated as percentage of DsRed2+/GFP+ cells relative to GFP+ cells (see Figure S5A). Results were obtained from 3 independent experiments (n=3) and expressed as mean ± SEM.

(C)TRAIL-induced apoptosis in DR5-expressing HEK293T stable cell lines. Monoclonal HEK293T stable cell lines expressing the WT DR5 or mutants were treated with TRAIL for 12 h. Cell viability was determined by a cell count kit (CCK-8) through measurement of dehydrogenase activities in live cells. Results were from 4 duplicates (n=4), repeated two times in independent experiments, and expressed as mean \pm SD.

(D)Rescue of the dimerization-defective mutant (G217Y) with crosslinked TRAIL in the DR5-deficient BJAB cells. After transient expression of WT or G217Y DR5, cells were treated with two different concentrations of FLAG-TRAIL (100, 200 ng/ml), each in the absence and presence of 500 ng/ml anti-FLAG IgG (designated X-Ab) for 5 h. Caspase-8 activity was measured as in (B) except the data was not normalized. Results were obtained from 3 independent experiments (n=3) and expressed as mean \pm SEM. Data was analyzed by unpaired Student's test, * for $p<0.05$ and ** for $p<0.005$.

(E)The same study as in (D) performed using the HEK293T stable cells. Monoclonal HEK293T stable cell line expressing DR5 WT or G217Y mutant was treated with FLAG-TRAIL (100 ng/ml) in the absence and presence of 0.1 and 1 mg/ml anti-FLAG IgG (X-Ab) for 12 h. Cell cytotoxicity (100% – cell viability) was determined using cell counting kit-8 (CCK-8) as described in (C). Results were from 3 duplicates (n=3), and expressed as mean \pm SD.

See also Figures S5 and S6.

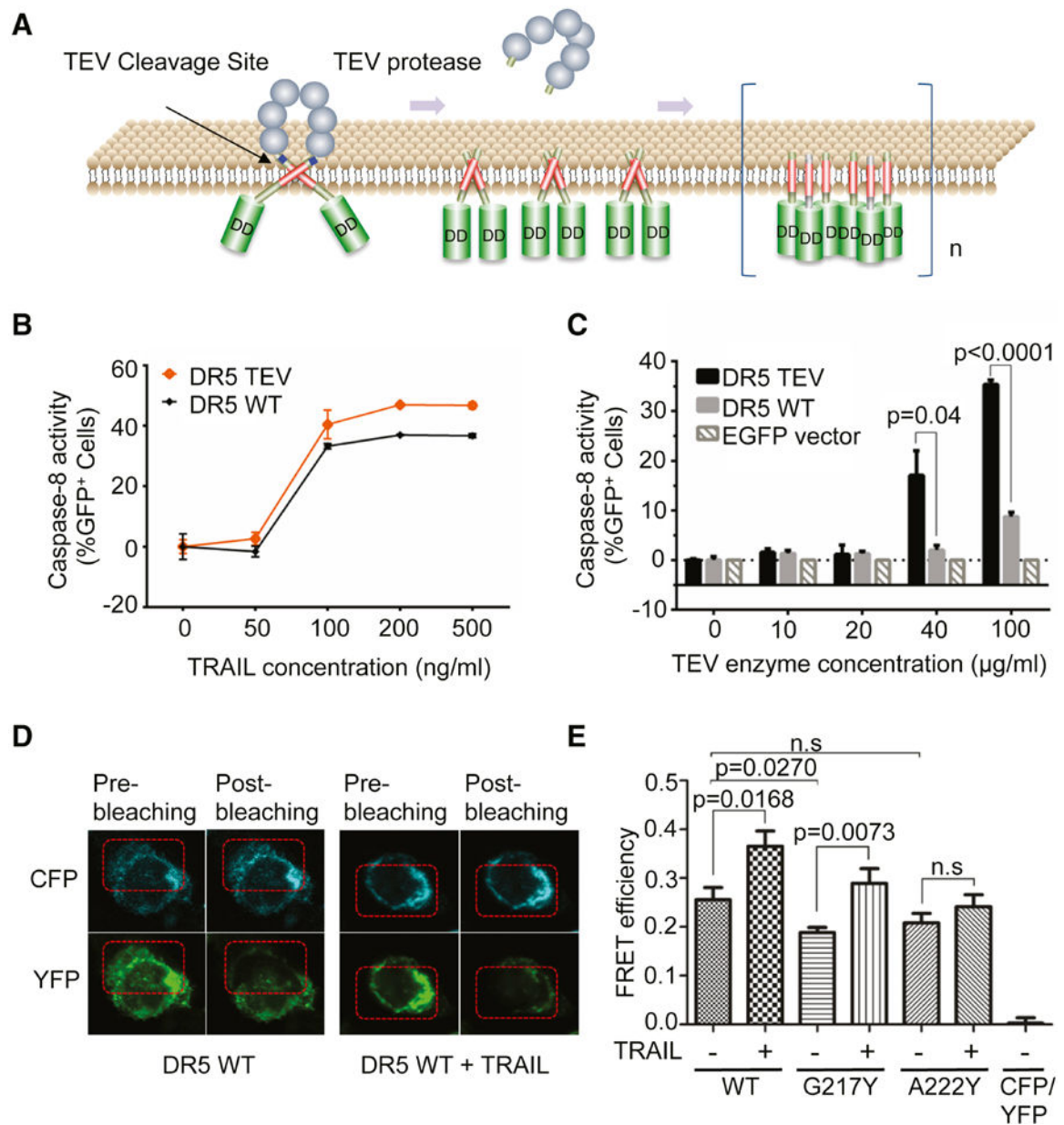


Figure 6. Higher-Order Clustering is Inhibited by the Unliganded Extracellular Domain
(A) Schematic illustration of possible mechanism underlying proteolytic activation of DR5.
(B) Comparing TRAIL sensitivity of the DR5-TEV-expressing BJAB cells to that of the DR5-WT-expressing BJAB cells. Caspase-8 activity was measured using the CaspGLOW red Caspase-8 activity kit (Biovision) and flow cytometry (BD FACSARIA II) and was calculated as percentage of DsRed2+/GFP+ cells divided by percentage of GFP+ cells. Results were from 3 independent experiments (n=3), and expressed as mean \pm SEM.
(C) TEV-induced apoptosis. DR5-deficient BJAB cells were transiently transfected with WT DR5-EGFP with (DR5-TEV) or without (DR5-WT) TEV cleavage site between the ECD and TMD. After transfection, BJAB cells were treated with TEV for 5 h before analysis.

(D)Examples of CFP and YFP photobleaching for WT DR5 without (left) and with (right) TRAIL.

(E)Receptor self-association of the WT DR5 and mutants. The effects of dimer-breaking mutation (G217Y) and trimer-breaking mutation (A222Y) with or without TRAIL pre-treatment are quantitated in the form of FRET efficiency. CFP/YFP co-transfected cells were used as FRET negative control. N.S indicates not significant. Twelve regions of interest (n=12) (ROI, e.g., plasma membrane bound DR5) from different cells were examined by FRET study.

See also Figure S6 and S7.

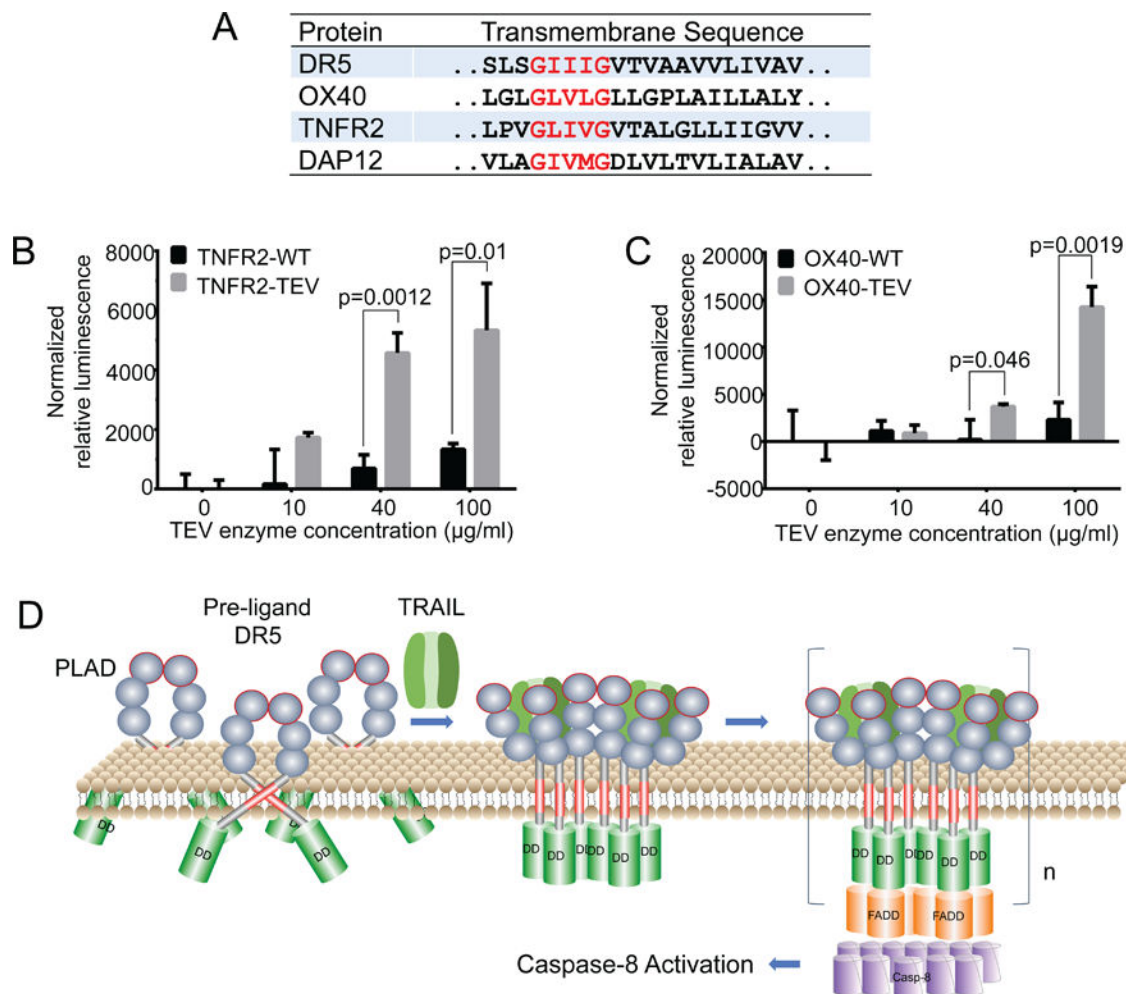


Figure 7. Proposed Roles of TMH in Ligand-Induced Receptor Assembly and Receptor Clustering

(A) Examples of other human receptor TMDs bearing the GXXXG motif that may mediate receptor clustering.

(B, C) Activation of TNFR2 and OX40 by proteolytic removal of the ECD in the absence of ligands. TEV cleavage site was inserted between the ECD and TMH for human TNFR2 and OX40. In 24-well plates, 20 mg of NF- κ B plasmid and 50 mg of plasmid of the TEV-cleavable TNFR2/OX40 or the WT TNFR2/OX40 were co-transfected into HEK293T cells using. 14 h after transfection, cells were treated with 10, 40, or 100 μ g/ml TEV enzyme for 5 h at 37°C. The cell lysates were analyzed for luminescence. Normalized relative luminescence is calculated as the difference between luminescence of the TEV-treated and non-TEV treated cell lysate divided by the cell lysate input (mg/ml). Results were from 3 independent experiments (n=3), and expressed as mean \pm SEM.

(D) A model of DR5 activation by TRAIL, highlighting how TRAIL binding to the ECD unleashes the TMH to trimerize and to form higher order cluster to activate downstream signaling.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-DR5 mAbs-FITC	Abcam	Cat# ab53319
Anti-FLAG mAbs, M2	Sigma-Aldrich	Cat# F1804
Primary anti-DR5 mAbs	R&D systems	Cat# mab631
Goat Anti-Mouse IgG H&L (Alexa Fluor® 647)	Abcam	Cat# ab150115
Bacterial Strains and mammalian cells		
E. coli BL21(DE3)	New England Biolabs	Cat# C2527
E. coli DH5-alpha	New England Biolabs	Cat# C2987
HEK293T	ATCC	CRL-3216
DR5-deficient BJAB cell	Andrew Thorburn	N/A
Chemicals, Peptides, and Recombinant Proteins		
Cyanogen Bromide	Sigma-Aldrich	Cat# C91492
Isotopes	Cambridge Isotope Laboratories, Inc	N/A
Kanamycin monosulfate	Sigma-Aldrich	Cat# BP861
Ampicillin, Sodium Salt	Sigma-Aldrich	Cat# 171254
Puromycin dihydrochloride	Sigma-Aldrich	Cat# P8833
isopropyl β -D-thiogalactopyranoside (IPTG)	Sigma-Aldrich	Cat# I5502
Imidazole	Sigma-Aldrich	Cat# I5513
Ammonium sulfate	Sigma-Aldrich	Cat# A4418
DTSSP (3,3'-dithiobis (sulfosuccinimidyl propionate))	ThermoFisher Scientific	Cat# 21578
Sulfo-NHS Acetate	Thermo Scientific	Cat# 26777
DMPC Lipid	Avanti Polar Lipids	Cat# 850345
DH ⁶ PC Detergent	Avanti Polar Lipids	Cat# 850305
Deuterated DMPC lipid	Avanti Polar Lipids	Cat# 860345C
Deuterated DH ⁶ PC Detergent	Avanti Polar Lipids	Cat# 790427C
TriNTA	Medicilon Inc.	N/A
SMCC Crosslinker	Life Technology	Cat# 22103
Triethylammonium Acetate	Calbiochem	Cat# 625718
Gd-DOTA	Macrocyclics, Inc.	Cat# M-147
16-DSA	Sigma-Aldrich	Cat# 253596
TRAIL ₁₁₄₋₂₈₁	This paper	N/A
FLAG-TRAIL	Enzo Life Sciences	Cat# ALX-522-003-C010
TEV enzyme	James Chou lab	N/A
Software and Algorithms		
Sparky	Goddard, UCSF	www.cgl.ucsf.edu/home/sparky

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Nmrpipe	Delaglio, 1995	www.ibbr.umd.edu/nmrpipe
Ccpnmr	Vranken, 2005	http://www.ccpn.ac.uk
Origin	OriginLab	www.originlab.com
Pymol	Schrodinger, LLC	www.pymol.org
Talos+	Shen, 2009	www.ibbr.umd.edu/nmrpipe
Graphpad prism 6.01	Graphpad	https://www.graphpad.com/scientific-software/prism/
Vectors and plasmids		
pVRC8400-puro	Tim Springer lab	N/A
pVRC-DR5 WT, for stable cell line	This paper	N/A
pVRC-DR5 G217Y, for stable cell line	This paper	N/A
pVRC-DR5 T219Y, for stable cell line	This paper	N/A
pVRC-DR5 A222Y, for stable cell line	This paper	N/A
pVRC-DR5 G217Y/A222Y, for stable cell line	This paper	N/A
pVRC-DR5 A227Y, for stable cell line	This paper	N/A
pEYFP-N1 vector	Clontech	Cat# 6006-1
pECFP-N1 vector	Clontech	Cat# 6900-1
pEYFP-N1-DR5 WT CT	This paper	N/A
pECFP-N1-DR5 WT CT	This paper	N/A
pEYFP-N1-DR5 G217Y CT	This paper	N/A
pECFP-N1-DR5 G217Y CT	This paper	N/A
pEYFP-N1-DR5 A222Y CT	This paper	N/A
pECFP-N1-DR5 A222Y CT	This paper	N/A
pMM-LR6 vector	Stephen C. Blacklow	N/A
pMM-DR5 _{TM} WT	This paper	N/A
pMM-DR5 _{TM} G217Y	This paper	N/A
pMM-DR5 _{TM} T219Y	This paper	N/A
pMM-DR5 _{TM} A222Y	This paper	N/A
pMM-DR5 _{TM} WT-6HIS, for OG-Label	This paper	N/A
pMM-DR5 _{TM} G217Y-6HIS, for OG-Label	This paper	N/A
pMM-DR5 _{TM} A227Y-6HIS, for OG-Label	This paper	N/A
pcDNA3-GFP-LIC vector	Scott Gradia	Addgene plasmid # 30127
pcDNA3-GFP-DR5 WT	This paper	N/A
pcDNA3-GFP-DR5 G217Y	This paper	N/A
pcDNA3-GFP-DR5 T219Y	This paper	N/A
pcDNA3-GFP-DR5 A222Y	This paper	N/A
pcDNA3-GFP-DR5 G217Y/A222Y	This paper	N/A
pcDNA3-GFP-DR5 V227Y	This paper	N/A
pcDNA3-GFP-DR5 TEV	This paper	N/A
pHAGE-NF-kappa B	Darrell Kotton	Addgene plasmid # 49343

REAGENT or RESOURCE	SOURCE	IDENTIFIER
pcDNA3.1(+)-OX40	Genscript	N/A
pcDNA3.1(+)-TNFR2	Genscript	N/A
pET28a(+)-TRAIL ₁₁₄₋₂₈₁	Genscript	N/A
Other		
Hispur Ni-NTA Resin	ThermoFisher Scientific	Cat# 88223
Zorbax SB-C18 Column	Agilent	Cat# 880995-202
Centricon Concentrator	EMD Millipore	Cat# UFC901024
StrepTrap HP Column	GE Healthcare	Cat# 28907547
Superdex S75 26/60	GE Healthcare	Cat# 28989334
NHS-Activated Agrose Resin	Thermo Fisher	Cat# 26196
Hitrap phenyl FF column	GE Healthcare	Cat # 45-000-249
PD-10 column	GE Healthcare	Cat # 17085101
CaspGLOW Red Active Caspase-8 Staining Kit	BioVision	Cat # K198
Cell Counting Kit-8	Dojindo	Cat # CK04
Hoechst 33342 Solution	ThermoFisher Scientific	Cat # 62249
Lipofectamine 2000 Transfection Reagent	ThermoFisher Scientific	Cat # 11668019
Lipofectamine 3000 Transfection Reagent	ThermoFisher Scientific	Cat # L3000008
Cell Line Nucleofector Kit V	Lonza	Cat# VVCA-1003
Dual Luciferase Reporter Assay kit	Promega	Cat# E1910
NuPAGE 10% Bis-Tris Protein Gels	ThermoFisher Scientific	Cat# NP0302BOX
NuPAGE 4-12% Bis-Tris Protein Gels	ThermoFisher Scientific	Cat# NP0322BOX