Experimentally guided computational methods yield highly accurate insights into transmembrane interactions within the T cell receptor complex

Samyuktha Ramesh[1,2], Soohyung Park[3], Melissa J. Call[1,2], Wonpil Im[3,*] and Matthew E. Call[1,2,*]

1. The Walter and Eliza Hall Institute of Medical Research, Parkville, VIC 3052, Australia.
2. Department of Medical Biology, University of Melbourne, Parkville, VIC 3052, Australia.
3. Departments of Biological Sciences, Chemistry, Bioengineering, and Computer Science and Engineering, Lehigh University, Bethlehem, PA, USA

* Correspondence: wonpil@lehigh.edu, mecall@wehi.edu.au

ABSTRACT

Understanding how molecular interactions within the plasma membrane govern assembly, clustering and conformational changes in single-pass transmembrane (TM) receptors has long presented substantial experimental challenges. Our previous work on activating immune receptors has combined direct biochemical and biophysical characterizations with both independent and experimentally restrained computational methods to provide novel insights into the key TM interactions underpinning assembly and stability of complex, multi-subunit receptor systems. The recently published cryo-EM structure of the intact T cell receptor (TCR)-CD3 complex provides a unique opportunity to test the models and predictions arising from these studies and we find that they are accurate, which we attribute to robust simulation environments and careful consideration of limitations related to studying TM interactions in isolation from additional receptor domains. With this in mind, we revisit results in other immune receptors and look forward to how similar methods may be applied to understand receptors for which little or no structural information is currently available.

Introduction

Cell-surface receptors with a single α-helical transmembrane domain (TMD), known as single-pass membrane proteins, present special challenges for structural biologists, and a lack of methods to obtain simultaneous high-resolution views of both membrane-embedded and extracellular and/or intracellular domains (ECDs, ICDs) has long limited our understanding of their complete structures and signaling mechanisms. Interactions among TMDs play central roles in the assembly, structure and function of cytokine and growth factor receptors[1-4], death receptors[5, 6], adhesion receptors[7, 8] and activating immune receptors[9, 10]. These lipid-embedded domains are considerably more difficult to study in isolation than the water-soluble ECD and ICD fragments that have provided the vast majority of structural information available for single-pass receptors[11]. Biophysical and computational studies have nonetheless provided important insights into the types of interactions that are possible among isolated TMD fragments and identified key principles of helix-helix associations guided by small amino acid motifs and polar or aromatic sidechains (reviewed in[11-14]). The challenge in relating the structures obtained from these reductionist approaches to what happens within the intact receptors is that missing ECDs and ICDs or, in some cases, entire subunits, likely exert strong influences on the conformations of TMD interactions that have comparatively small binding interfaces.

Computational studies of single-pass receptor TM interactions

Predicting the interactions between two TM α-helices in a lipid bilayer is a problem that is inherently tractable using molecular dynamics (MD) simulations or other conformational space search methods, and significant insights into dimeric single-pass systems from computational studies have preceded and predicted experimentally determined structures. For example, early modeling of the glycophorin A (GpA) TM dimer[15] combined with parallel mutagenesis analysis[16] accurately predicted the right-handed coiled-coil, intimately packed around the now-iconic GxxxG motif, that featured in the later NMR and

crystal structures of TMD fragments in various membrane mimetics[17-20]. A purely computational study on the receptor tyrosine kinase (RTK) erbB2 (HER2/EGFR2) predicted that its TMD could stably interact through two different dimer interfaces based on N-terminal and C-terminal GxxxG-like motifs[21] and proposed that these correspond to active and inactive states of the receptor. Both conformations have now been observed in NMR structures of erbB2[22, 23] and the closely related EGFR[3, 24, 25] TM dimers under different sample conditions. MD simulations in the context of the entire EGFR protein in lipid bilayers[26] further suggested how switching between N-terminal (active) and C-terminal (inactive) packing conformations could regulate intracellular kinase activity via structured juxtamembrane domains. A model based on TMD cysteine crosslinking using full-length EFGR in live cells[27] supports the close N-terminal packing in the ligand-bound receptor *in situ*, but this study found little evidence from mutagenesis that it was required for receptor function and did not detect a C-terminal interface in ligand-free EGFR. Similar combinations of computational, biochemical, biophysical and cellular functional approaches have produced models of structural and functional roles for TMDs in, for example, other RTKs[28-32] and cytokine receptors[2, 4, 24, 33, 34].

Ultimately, testing the validity of these and other TMD-focused models and the strengths and accuracy of the methods used to derive them awaits the availability of high-resolution structures of full-length receptor complexes in defined activity states. Until recently no such structures had been reported for any single-pass receptor system. Similar to the above crosslinking study on EGFR in cells[27], our groups previously applied a disulfide-restrained MD simulation approach[35] to model TMD interactions at the core of one of the most complex single-pass receptor systems known: the eight-subunit T cell antigen receptor (TCR). The recent landmark report of a cryogenic electron microscopy (cryo-EM) structure of the intact TCR complex (**Figure 1A**)[10] now provides a completely unique opportunity to compare independent TMD modeling results to the interactions observed in the full receptor structure. Here we

describe our experimental approach, discuss the accuracy of the resulting model and its predictions in the context of the complete TCR cryo-EM structure, and compare this with other studies addressing the role of TMDs in immune receptor structure and function.

Experimental restraints guide MD simulations of TM interactions within the TCR complex

The TCR is organized into four dimeric modules – one responsible for extracellular ligand binding (TCR$\alpha\beta$) and the other three responsible for intracellular signal initiation (CD3$\delta\varepsilon$, CD3$\gamma\varepsilon$ and $\zeta\zeta$)[36, 37]. TCR and CD3 dimers each assemble through ECD interfaces[38-42], while the $\zeta\zeta$ dimer forms via a disulfide-stabilized TMD interface[43, 44]. To assemble a complete TCR complex, the three signaling modules are recruited to the TCR via complementary charged/polar residues between the TCR$\alpha\beta$ TMDs (arginine/lysine) and the CD3$\delta\varepsilon$, CD3$\gamma\varepsilon$ and $\zeta\zeta$ TMDs (aspartic/glutamic acids)[36, 45, 46]. We hypothesized that TCR$\alpha\beta$ could form a structured 'hub' that organizes the complex within the membrane and set out to determine whether there was a specific interface between these two TMDs. Stable interactions between TCRα and β TMD fragments could not be identified by solution NMR or in unrestrained MD simulations (reference[35] and our unpublished observations), but a TMD cysteine-scanning analysis carried out in full-length, membrane-embedded TCR complexes identified positions that were close enough to form intermolecular disulfide crosslinks in the native receptor[35]. These displayed a clear helical periodicity, indicating that a specific helix-helix interface exists within the assembled TCR complex. We converted these crosslinks to distance restraints and combined them with solution NMR-derived secondary structure analysis of TMD peptides to guide assembly of a TCR$\alpha\beta$ TMD heterodimer using replica-exchange MD simulations[14, 47]. Since these simulations were carried out in an implicit membrane model[48], we mutated the basic TMD residues that would normally be shielded from the membrane interior by their CD3 assembly partners. The resulting structure (**Figure 1B**) was stable in longer, unrestrained MD simulations carried out in an explicit palmitoyl-oleoyl-phosphatidylcholine

(POPC) bilayer, and the model showed highly specific packing around an inter-helical hydrogen-bond network (dotted lines) involving conserved polar residues that had not previously been implicated in TCR assembly. The biochemical stability of the octameric complex was surprisingly sensitive to mutations in this interface[35] and this was strongly correlated with increased dynamics and substantial departures from the native structure in MD simulations of the same mutations[49]. Further study of this network in two additional types of TCR heterodimers ($\gamma\delta$- and pre-TCR) by sequence alignment and MD simulation indicated that a similar structural motif exists in both, with the stability of the interface modulated by small sequence differences in each TCR type[49] that may have functional relevance. It is noteworthy that the MD simulations alone brought to light the key polar interactions at the TCR$\alpha\beta$ interface, providing completely new structural observations in a field that is heavily reliant on identifying known sequence motifs to predict TMD interactions. This convergence of biochemical and simulation data indicated that the core TCR$\alpha\beta$ TMD structure is a linchpin for formation of compact and highly cooperative eight-TMD assemblies in all TCR types throughout vertebrate evolution.

An alternative model for TM roles in the TCR complex

This model was challenged by a subsequent NMR study of the isolated TCRα TMD in lipid micelles[50] that reported a bent helix structure where the basic arginine and lysine residues (TCRα R253 and K258 in **Figure 1B**) were solvent exposed and the region around the key asparagine (TCRα N263 in **Figure 1B**) was non-helical. This distortion is generally consistent with our own unpublished observations that without their natural assembly partners, receptor TMD peptides containing central basic residues do not adopt stable trans-bilayer orientations in NMR samples or in MD simulations. Based on this structure of an isolated TMD fragment, the authors proposed a TCR activation model in which the basic residues in a bent TCRα TMD interact primarily with lipid headgroups rather than CD3 modules, and that force applied through ligand binding causes a partial straightening of the helix[50, 51]. This was predicted to

dislodge the signaling modules in a 'dissociative activation' mechanism. This model proposes an alternative role for the conserved asparagine residue that we identified at the TCR$\alpha\beta$ TMD interface[35, 49] (as a 'hinge' in an unstructured region) and it rejects the biochemical evidence that the basic and acidic residues drive receptor assembly (9, 45, 52 and references therein).

Evaluation of experimental and computational TM models against the intact TCR complex structure

As a major milestone for T cell biology and receptor structural biology, the structure of the intact TCR complex was recently determined using cryo-EM (**Figure 1A**)[10]. This is the first structure of any full-length single-pass receptor complex where the TMDs were well resolved (3.8 Å local resolution). The structure in this region revealed a compact bundle of eight undistorted TMD helices and provided atomic-resolution evidence confirming the roles of basic and acidic residues in assembly (more on this below). The receptor is organized around a specific TCR$\alpha\beta$ TMD coiled-coil structure that is nearly identical to the model derived from our experimentally guided restrained simulations[35] (**Figure 1B**), with 0.63 Å Cα RMSD between the model and the corresponding region of the cryo-EM structure. The authors independently noted the stabilizing hydrogen bond between the TCRα N263 carboxamide sidechain and the backbone carbonyl oxygen of TCRβ A289, but interestingly, could not identify the second one between TCRα T267 and TCRβ Y292 (see **Figure 1B** inset). This is likely because the quality of the electron density maps did not allow accurate identification of the TCRα T267 sidechain rotamer. The only pre-existing experimental structure of a subcomplex from the membrane-embedded regions, the solution NMR structure of the $\zeta\zeta$ dimer in detergent micelles[43], also aligns well to the cryo-EM structure (Cα RMSD 0.91 Å; **Figure 1C**), though it is not clear whether the central Y42-T47 interhelical hydrogen bonds identified in the NMR structure (dotted lines) are present in the context of the fully assembled receptor (see **Figure 1C** inset). This is potentially of functional relevance, since a separate study using biochemical and fluorescence-based proximity measurements[53] reported that the $\zeta\zeta$ TMDs separate like

chopsticks when incorporated in the TCR and snap back together as part of a conformational activation mechanism. The cryo-EM structure does not show evidence of such a significant departure from the NMR structure, suggesting that either this model is incorrect or the complex captured by cryo-EM represents an activated state. Notwithstanding these structural features, the aspartic acid pair in the ζζ TMDs directly contacts TCRα R253 in the cryo-EM structure, and the authors independently noted the potential for hydrogen bonding with TCRβ Y282 (**Figure 1D**), both interactions predicted by our model[35] and prior biochemical data[43, 54].

Several key considerations in our restrained simulation approach contributed to the accuracy of the TCRαβ TMD model and its associated predictions. NMR analysis defined the stable helical regions, allowing us to eliminate crosslink restraints derived from unstructured areas. With the application of carefully curated disulfide restraints collected on intact receptor complexes, the replica-exchange MD assembly in an implicit membrane model was highly efficient, converging on the final structure in just a few nanoseconds of simulation time. Mutation of the basic residues in the TCRαβ TMDs for MD and NMR analysis of the isolated fragments used our knowledge of existing biochemical data to prevent the artificial structural distortion that was evident in the later TCRα-only NMR study[50]. Finally, the all-atom simulations in explicit POPC bilayers enabled a careful analysis of hydrogen bond occupancy in both WT and interface-mutant complexes[35, 49] to understand the important contributions of these interactions to stabilizing the native structure.

Apart from confirmation of these previously predicted TMD interactions, the TCR cryo-EM structure also revealed some surprising features in and near the membrane. Most striking were the very long helices of the TCRαβ TMDs (best seen in **Figure 1E**) and the very small interaction surfaces between the CD3 TMDs within each dimer (**Figure 1A, D, E**) in comparison to the functionally homologous ζζ dimer that

has extensive contacts along its entire length. Finally, what were thought to be long and unstructured connecting peptide regions between the TCRα ECDs and TMDs are in fact intimately associated with one another and with CD3/ζ components in the extracellular juxtamembrane space. Their potential role in any activation-induced conformational transitions can now be investigated with a precise structural rationale. The interfaces among TCR and CD3 components in the ECDs, while not the focus of this perspective, also provide structural context for the re-examination of an enormous number of prior biochemical, biophysical and modeling studies that are just beginning to be addressed in very recent reviews and commentaries[55-57].

Insights from related immune receptor systems

Other prior experimental and computational studies contributed significantly to our understanding of the extensive intramembrane polar networks that can now be seen holding the receptor together in the cryo-EM structure[10]. Association of TCR and CD3 subunits via basic-acidic TMD interactions was first proposed three decades ago based on biochemical data[46, 58, 59], but the current model in which one basic residue in a TCR TMD directly contacts two acidic residues in a signaling dimer TMD stems from later *in vitro* assembly and stoichiometry experiments[45, 60]. Structural studies of a different lymphocyte receptor complex (NKG2C-DAP12) with a similar assembly mechanism[61] showed that, as in $\zeta\zeta$, the two aspartic acid residues in the DAP12 signaling dimer TMDs were close together in the helix-helix interface. This study also confirmed that the lysine residue in the NKG2C receptor TMD was the direct binding site for the aspartic acid pair and revealed an additional contributor to the polar network: a threonine residue in DAP12, located one helical turn below the key aspartic acid (making two DxxxT motifs in the dimer). Similarly located serine/threonine residues are present in the CD3γ, CD3δ and CD3ε TMDs, and mutations at these positions disrupted TCR-CD3 assembly[61]. Despite the biochemical evidence that all five of these polar residues were required for assembly, the NKG2C-DAP12 NMR structure showed that

the DxxxT motif in one chain of the DAP12 dimer faced away from the receptor, which would point into the nonpolar hydrocarbon core of a lipid bilayer and is therefore energetically unlikely. An independent all-atom MD refinement of the three-helix NKG2C-DAP12 complex[62] using NMR-derived inter-proton distance restraints[61] showed that, in a lipid bilayer environment, the second aspartic acid rotated inward to face the lysine more directly. A similar orientation is seen in the ζζ aspartic acids contacting TCR chains in the cryo-EM structure (**Figure 1D**), a rotation and break in symmetry that accounts for most of the small differences between the cryo-EM and NMR structures of this module (**Figure 1C**). Three subsequent unrestrained simulation studies by others[63-65] showed similar findings for DAP12-NKG2C and for TCRα-CD3δε and TCRβ-CD3γε trimeric interactions, indicating that all four polar residues in the DxxxT motifs could directly contact the basic residue.

Direct experimental support for this arrangement came from structures of DAP12 trimers and tetramers crystallized in lipid cubic phase bilayers[66]. Without a basic residue from an assembly partner, the DAP12 TMD complexes scavenged metal cations from crystallization solutions to stabilize their DxxxT motifs, and we noted at the time that two chains from the trimer structure with their coordinated K^+ ion were likely to reflect the structure of a CD3 dimer with the (+1 charged) lysine from a single TCR chain (**Figure 1E**). As shown in **Figure 1F**, this arrangement indeed compares very favorably to the TCRα-CD3δε interaction site from the cryo-EM structure. As noted above in reference to other regions in this structure, the threonine sidechain rotamers in CD3δε TMDs are likely incorrect due to lack of sufficient resolution to assign them based on electron density alone, and thus the appearance that CD3δ T115 and CD3ε T141 do not directly contribute polar contacts in the cryo-EM structure is likely inaccurate. We therefore conclude that the arrangement in **Figure 1F** is likely to accurately represent the structures of TMD complexes of many activating immune receptors that rely on very similar assembly mechanisms[9, 52].

Concluding remarks

The validation of combined experimental and computational methods to elucidate TMD interactions provided by these TCR studies has now set a strong precedent for similar approaches to be applied to other multi-component single-pass receptor systems. A subset of activating immune receptors, including the B cell antigen receptor and some antibody Fc receptors, do not contain the canonical assembly-mediating basic residues and have little to no experimentally derived structural information available for the membrane-embedded regions[9]. Our own work on the FcγRIIIA, FcεR1α and FcγRI receptor TMD associations with FcεR1γ or ζζ signaling dimers shows that *de-novo* assembly simulations reach a consensus structural arrangement that is very well supported by mutagenesis data from intact receptors[67]. These results suggest a motif of interacting polar and aromatic TMD residues that has not yet been validated by experimental structures in these or any other receptor systems to our knowledge. Cytokine receptors, with TMDs whose roles in assembly and function are less defined but can harbor key disease-causing mutations, could similarly benefit from studies using experimentally restrained simulation techniques. While rapid advances in cryo-EM promise more high-resolution single-pass receptor structures to come, great technical challenges remain for this class of proteins. As such, experimentally guided MD simulations can provide great value in understanding how membrane-embedded protein interactions contribute to the structures and functions of these deceptively simple proteins in their physiological environments.

BIOGRAPHIES

Samyuktha Ramesh is a Ph.D. candidate at the Walter and Eliza Hall Institute of Medical Research and the University of Melbourne, with a focus on mapping transmembrane interactions in immune receptors. With experience in engineering structure in intrinsically disordered proteins, understanding sequence-structure and -function relationships is her keen interest.

Soohyung Park received his Ph.D. in Chemistry from Seoul National University in 2006. After postdoctoral studies in diffusion-influenced reversible reactions at the Hebrew University of Jerusalem and polyelectrolyte solutions at the University of Wisconsin-Madison, he joined Dr. Wonpil Im's laboratory. His research focuses on modeling of membrane protein and biophysics of membrane and membrane proteins.

Melissa Call received her Ph.D. in Molecular Medicine from the University of Auckland in 2002 and during her post-doctoral work at the Dana-Farber Cancer Institute in Boston went on to study how peptides are loaded into MHC molecules for presentation to T cells. She became an Instructor at Harvard Medical School in 2008 and in 2010 she joined the Walter and Eliza Hall Institute of Medical Research as a laboratory head. Her work focuses on how natural and engineered immune receptors can be manipulated to treat disease using a combination of structure determination by crystallography, deep mutational scanning, and functional assays in cells.

Wonpil Im received in bachelor's and master's degrees from Hanyang University in Seoul. He then earned his Ph.D. in Biochemistry from Cornell University. He did his post-doctoral research at the Scripps Research Institute in La Jolla, California. In 2005, he was hired as an assistant professor in the Center for Computational Biology and Department of Molecular Biosciences at the University of Kansas,

Lawrence. In 2011, he was promoted to associate professor and then professor in 2015. In 2016, he joined the Faculty in Departments of Biological Sciences and Bioengineering at Lehigh University, and he has been named the Presidential Endowed Chair in Health - Science and Engineering. Wonpil was awarded the Alfred P. Sloan Research Fellowship (2007), ACS HP Outstanding Junior Faculty Award (2011), Meredith Docking Scholar (2013), University Scholarly Achievement Award (2015), the Friedrich Wilhelm Bessel Research Award from the Humboldt Foundation (2017), and was named a KIAS Scholar from the Korea Institute for Advanced Study (2016). Research in his lab is focused on the applications of theoretical/computational methods to chemical and physical problems in biology and material sciences. In particular, he is interested in modeling and simulations of biological membranes and associated proteins, glycoconjugates, and protein-ligand (drug) interactions. In addition, his lab has been developing CHARMM-GUI for the molecular modeling and simulation community.

Matthew Call studied Biology at Trinity University (San Antonio, TX) and received his Ph.D. in Immunology from Harvard University in 2007. After postdoctoral studies in solution NMR at Harvard Medical School, he joined the Walter and Eliza Hall Institute of Medical Research as a laboratory head in 2010. Since 2017 he has been Division Head (Structural Biology) at the Walter and Eliza Hall Institute and Associate Professor of Medical Biology at the University of Melbourne. His research focuses on the structure and function of natural and engineered immune receptors in health and disease.

ACKNOWLEDGMENTS

This work was supported in part by grants from NSF MCB-1810695 (WI) and NHMRC GNT1011352 (MEC, MJC). The authors thank the many researchers who contributed to the experimental and computational work described herein.

REFERENCES

1. Bridgford, J. L.; Lee, S. M.; Lee, C. M. M.; Guglielmelli, P.; Rumi, E.; Pietra, D.; Wilcox, S.; Chhabra, Y.; Rubin, A. F.; Cazzola, M.; Vannucchi, A. M.; Brooks, A. J.; Call, M. E.; Call, M. J., Novel drivers and modifiers of MPL-dependent oncogenic transformation identified by deep mutational scanning. *Blood* **2020,** *135* (4), 287-292.
2. Brooks, A. J.; Dai, W.; O'Mara, M. L.; Abankwa, D.; Chhabra, Y.; Pelekanos, R. A.; Gardon, O.; Tunny, K. A.; Blucher, K. M.; Morton, C. J.; Parker, M. W.; Sierecki, E.; Gambin, Y.; Gomez, G. A.; Alexandrov, K.; Wilson, I. A.; Doxastakis, M.; Mark, A. E.; Waters, M. J., Mechanism of activation of protein kinase JAK2 by the growth hormone receptor. *Science* **2014,** *344* (6185), 1249783.
3. Endres, N. F.; Das, R.; Smith, A. W.; Arkhipov, A.; Kovacs, E.; Huang, Y.; Pelton, J. G.; Shan, Y.; Shaw, D. E.; Wemmer, D. E.; Groves, J. T.; Kuriyan, J., Conformational Coupling across the Plasma Membrane in Activation of the EGF Receptor. *Cell* **2013,** *152* (3), 543-56.
4. Staerk, J.; Defour, J. P.; Pecquet, C.; Leroy, E.; Antoine-Poirel, H.; Brett, I.; Itaya, M.; Smith, S. O.; Vainchenker, W.; Constantinescu, S. N., Orientation-specific signalling by thrombopoietin receptor dimers. *EMBO J* **2011,** *30* (21), 4398-413.
5. Fu, Q.; Fu, T. M.; Cruz, A. C.; Sengupta, P.; Thomas, S. K.; Wang, S.; Siegel, R. M.; Wu, H.; Chou, J. J., Structural Basis and Functional Role of Intramembrane Trimerization of the Fas/CD95 Death Receptor. *Mol Cell* **2016,** *61* (4), 602-613.
6. Pan, L.; Fu, T. M.; Zhao, W.; Zhao, L.; Chen, W.; Qiu, C.; Liu, W.; Liu, Z.; Piai, A.; Fu, Q.; Chen, S.; Wu, H.; Chou, J. J., Higher-Order Clustering of the Transmembrane Anchor of DR5 Drives Signaling. *Cell* **2019,** *176* (6), 1477-1489 e14.
7. Lau, T. L.; Kim, C.; Ginsberg, M. H.; Ulmer, T. S., The structure of the integrin alphaIIbbeta3 transmembrane complex explains integrin transmembrane signalling. *Embo J* **2009,** *28* (9), 1351-61.
8. Yang, J.; Ma, Y. Q.; Page, R. C.; Misra, S.; Plow, E. F.; Qin, J., Structure of an integrin alphaIIb beta3 transmembrane-cytoplasmic heterocomplex provides insight into integrin activation. *Proc Natl Acad Sci U S A* **2009,** *106* (42), 17729-34.
9. Berry, R.; Call, M. E., Modular Activating Receptors in Innate and Adaptive Immunity. *Biochemistry* **2017,** *56* (10), 1383-1402.
10. Dong; Zheng, L.; Lin, J.; Zhang, B.; Zhu, Y.; Li, N.; Xie, S.; Wang, Y.; Gao, N.; Huang, Z., Structural basis of assembly of the human T cell receptor-CD3 complex. *Nature* **2019,** *573* (7775), 546-552.
11. Trenker, R.; Call, M. J.; Call, M. E., Progress and prospects for structural studies of transmembrane interactions in single-spanning receptors. *Curr Opin Struct Biol* **2016,** *39*, 115-123.
12. Bocharov, E. V.; Mineev, K. S.; Pavlov, K. V.; Akimov, S. A.; Kuznetsov, A. S.; Efremov, R. G.; Arseniev, A. S., Helix-helix interactions in membrane domains of bitopic proteins: Specificity and role of lipid environment. *Biochim Biophys Acta Biomembr* **2017,** *1859* (4), 561-576.
13. Li, E.; Wimley, W. C.; Hristova, K., Transmembrane helix dimerization: beyond the search for sequence motifs. *Biochim Biophys Acta* **2012,** *1818* (2), 183-93.
14. Mori, T.; Miyashita, N.; Im, W.; Feig, M.; Sugita, Y., Molecular dynamics simulations of biological membranes and membrane proteins using enhanced conformational sampling algorithms. *Biochim Biophys Acta* **2016,** *1858* (7 Pt B), 1635-51.
15. Treutlein, H. R.; Lemmon, M. A.; Engelman, D. M.; Brünger, A. T., The glycophorin A transmembrane domain dimer: sequence-specific propensity for a right-handed supercoil of helices. *Biochemistry* **1992,** *31* (51), 12726-32.
16. Lemmon, M. A.; Flanagan, J. M.; Treutlein, H. R.; Zhang, J.; Engelman, D. M., Sequence specificity in the dimerization of transmembrane alpha-helices. *Biochemistry* **1992,** *31* (51), 12719-25.
17. MacKenzie, K. R.; Prestegard, J. H.; Engelman, D. M., A Transmembrane Helix Dimer: Structure and Implications. *Science* **1997,** *276* (5309), 131-133.

18. Mineev, K. S.; Bocharov, E. V.; Volynsky, P. E.; Goncharuk, M. V.; Tkach, E. N.; Ermolyuk, Y. S.; Schulga, A. A.; Chupin, V. V.; Maslennikov, I. V.; Efremov, R. G.; Arseniev, A. S., Dimeric structure of the transmembrane domain of glycophorin a in lipidic and detergent environments. *Acta Naturae* **2011,** *3* (2), 90-8.
19. Smith, S. O.; Jonas, R.; Braiman, M.; Bormann, B. J., Structure and orientation of the transmembrane domain of glycophorin A in lipid bilayers. *Biochemistry* **1994,** *33* (20), 6334-41.
20. Trenker, R.; Call, M. E.; Call, M. J., Crystal Structure of the Glycophorin A Transmembrane Dimer in Lipidic Cubic Phase. *J Am Chem Soc* **2015,** *137* (50), 15676-9.
21. Fleishman, S. J.; Schlessinger, J.; Ben-Tal, N., A putative molecular-activation switch in the transmembrane domain of erbB2. *Proceedings of the National Academy of Sciences* **2002,** *99* (25), 15937-15940.
22. Bocharov, E. V.; Mineev, K. S.; Volynsky, P. E.; Ermolyuk, Y. S.; Tkach, E. N.; Sobol, A. G.; Chupin, V. V.; Kirpichnikov, M. P.; Efremov, R. G.; Arseniev, A. S., Spatial structure of the dimeric transmembrane domain of the growth factor receptor ErbB2 presumably corresponding to the receptor active state. *J Biol Chem* **2008,** *283* (11), 6950-6.
23. Bragin, P. E.; Mineev, K. S.; Bocharova, O. V.; Volynsky, P. E.; Bocharov, E. V.; Arseniev, A. S., HER2 Transmembrane Domain Dimerization Coupled with Self-Association of Membrane-Embedded Cytoplasmic Juxtamembrane Regions. *J Mol Biol* **2016,** *428* (1), 52-61.
24. Bocharov, E. V.; Bragin, P. E.; Pavlov, K. V.; Bocharova, O. V.; Mineev, K. S.; Polyansky, A. A.; Volynsky, P. E.; Efremov, R. G.; Arseniev, A. S., The Conformation of the Epidermal Growth Factor Receptor Transmembrane Domain Dimer Dynamically Adapts to the Local Membrane Environment. *Biochemistry* **2017,** *56* (12), 1697-1705.
25. Bocharov, E. V.; Lesovoy, D. M.; Pavlov, K. V.; Pustovalova, Y. E.; Bocharova, O. V.; Arseniev, A. S., Alternative packing of EGFR transmembrane domain suggests that protein-lipid interactions underlie signal conduction across membrane. *Biochim Biophys Acta* **2016,** *1858* (6), 1254-61.
26. Arkhipov, A.; Shan, Y.; Das, R.; Endres, N. F.; Eastwood, M. P.; Wemmer, D. E.; Kuriyan, J.; Shaw, D. E., Architecture and membrane interactions of the EGF receptor. *Cell* **2013,** *152* (3), 557-69.
27. Lu, C.; Mi, L. Z.; Grey, M. J.; Zhu, J.; Graef, E.; Yokoyama, S.; Springer, T. A., Structural evidence for loose linkage between ligand binding and kinase activation in the epidermal growth factor receptor. *Mol Cell Biol* **2010,** *30* (22), 5432-43.
28. Li, P. C.; Miyashita, N.; Im, W.; Ishido, S.; Sugita, Y., Multidimensional umbrella sampling and replica-exchange molecular dynamics simulations for structure prediction of transmembrane helix dimers. *J Comput Chem* **2014,** *35* (4), 300-8.
29. Manni, S.; Mineev, K. S.; Usmanova, D.; Lyukmanova, E. N.; Shulepko, M. A.; Kirpichnikov, M. P.; Winter, J.; Matkovic, M.; Deupi, X.; Arseniev, A. S.; Ballmer-Hofer, K., Structural and functional characterization of alternative transmembrane domain conformations in VEGF receptor 2 activation. *Structure* **2014,** *22* (8), 1077-1089.
30. Matsuoka, D.; Kamiya, M.; Sato, T.; Sugita, Y., Role of the N-Terminal Transmembrane Helix Contacts in the Activation of FGFR3. *J Comput Chem* **2020,** *41* (6), 561-572.
31. Sarabipour, S.; Ballmer-Hofer, K.; Hristova, K., VEGFR-2 conformational switch in response to ligand binding. *Elife* **2016,** *5*, e13876.
32. Tamagaki, H.; Furukawa, Y.; Yamaguchi, R.; Hojo, H.; Aimoto, S.; Smith, S. O.; Sato, T., Coupling of transmembrane helix orientation to membrane release of the juxtamembrane region in FGFR3. *Biochemistry* **2014,** *53* (30), 5000-7.
33. Defour, J. P.; Itaya, M.; Gryshkova, V.; Brett, I. C.; Pecquet, C.; Sato, T.; Smith, S. O.; Constantinescu, S. N., Tryptophan at the transmembrane-cytosolic junction modulates thrombopoietin receptor dimerization and activation. *Proc Natl Acad Sci U S A* **2013,** *110* (7), 2540-5.

34. Staerk, J.; Lacout, C.; Sato, T.; Smith, S. O.; Vainchenker, W.; Constantinescu, S. N., An amphipathic motif at the transmembrane-cytoplasmic junction prevents autonomous activation of the thrombopoietin receptor. *Blood* **2006,** *107* (5), 1864-71.
35. Krshnan, L.; Park, S.; Im, W.; Call, M. J.; Call, M. E., A conserved alphabeta transmembrane interface forms the core of a compact T-cell receptor-CD3 structure within the membrane. *Proc Natl Acad Sci U S A* **2016,** *113* (43), E6649-E6658.
36. Call, M. E.; Wucherpfennig, K. W., The T cell receptor: critical role of the membrane environment in receptor assembly and function. *Annu Rev Immunol* **2005,** *23*, 101-25.
37. Kuhns, M. S.; Davis, M. M.; Garcia, K. C., Deconstructing the form and function of the TCR/CD3 complex. *Immunity* **2006,** *24* (2), 133-9.
38. Arnett, K. L.; Harrison, S. C.; Wiley, D. C., Crystal structure of a human CD3-epsilon/delta dimer in complex with a UCHT1 single-chain antibody fragment. *Proc Natl Acad Sci U S A* **2004,** *101* (46), 16268-73.
39. Kjer-Nielsen, L.; Dunstone, M. A.; Kostenko, L.; Ely, L. K.; Beddoe, T.; Mifsud, N. A.; Purcell, A. W.; Brooks, A. G.; McCluskey, J.; Rossjohn, J., Crystal structure of the human T cell receptor CD3 epsilon gamma heterodimer complexed to the therapeutic mAb OKT3. *Proc Natl Acad Sci U S A* **2004,** *101* (20), 7675-80.
40. Rossjohn, J.; Gras, S.; Miles, J. J.; Turner, S. J.; Godfrey, D. I.; McCluskey, J., T cell antigen receptor recognition of antigen-presenting molecules. *Annu Rev Immunol* **2015,** *33*, 169-200.
41. Sun, Z. J.; Kim, K. S.; Wagner, G.; Reinherz, E. L., Mechanisms contributing to T cell receptor signaling and assembly revealed by the solution structure of an ectodomain fragment of the CD3 epsilon gamma heterodimer. *Cell* **2001,** *105* (7), 913-23.
42. Sun, Z. Y.; Kim, S. T.; Kim, I. C.; Fahmy, A.; Reinherz, E. L.; Wagner, G., Solution structure of the CD3epsilondelta ectodomain and comparison with CD3epsilongamma as a basis for modeling T cell receptor topology and signaling. *Proc Natl Acad Sci U S A* **2004,** *101* (48), 16867-72.
43. Call, M. E.; Schnell, J. R.; Xu, C.; Lutz, R. A.; Chou, J. J.; Wucherpfennig, K. W., The structure of the zetazeta transmembrane dimer reveals features essential for its assembly with the T cell receptor. *Cell* **2006,** *127* (2), 355-68.
44. Rutledge, T.; Cosson, P.; Manolios, N.; Bonifacino, J. S.; Klausner, R. D., Transmembrane helical interactions: zeta chain dimerization and functional association with the T cell antigen receptor. *Embo J* **1992,** *11* (9), 3245-54.
45. Call, M. E.; Pyrdol, J.; Wiedmann, M.; Wucherpfennig, K. W., The organizing principle in the formation of the T cell receptor-CD3 complex. *Cell* **2002,** *111* (7), 967-79.
46. Cosson, P.; Lankford, S. P.; Bonifacino, J. S.; Klausner, R. D., Membrane protein association by potential intramembrane charge pairs. *Nature* **1991,** *351* (6325), 414-6.
47. Sugita, Y.; Okamoto, Y., Replica-exchange molecular dynamics method for protein folding. *Chem Phys Lett* **1999,** *314* (1-2), 141-151.
48. Im, W.; Feig, M.; Brooks, C. L., 3rd, An implicit membrane generalized born theory for the study of structure, stability, and interactions of membrane proteins. *Biophys J* **2003,** *85* (5), 2900-18.
49. Park, S.; Krshnan, L.; Call, M. J.; Call, M. E.; Im, W., Structural Conservation and Effects of Alterations in T Cell Receptor Transmembrane Interfaces. *Biophys J* **2018,** *114* (5), 1030-1035.
50. Brazin, K. N.; Mallis, R. J.; Boeszoermenyi, A.; Feng, Y.; Yoshizawa, A.; Reche, P. A.; Kaur, P.; Bi, K.; Hussey, R. E.; Duke-Cohan, J. S.; Song, L.; Wagner, G.; Arthanari, H.; Lang, M. J.; Reinherz, E. L., The T Cell Antigen Receptor alpha Transmembrane Domain Coordinates Triggering through Regulation of Bilayer Immersion and CD3 Subunit Associations. *Immunity* **2018,** *49* (5), 829-841 e6.
51. Mallis, R. J.; Brazin, K. N.; Duke-Cohan, J. S.; Hwang, W.; Wang, J. H.; Wagner, G.; Arthanari, H.; Lang, M. J.; Reinherz, E. L., NMR: an essential structural tool for integrative studies of T cell

development, pMHC ligand recognition and TCR mechanobiology. *J Biomol NMR* **2019,** *73* (6-7), 319-332.
52. Call, M. E.; Wucherpfennig, K. W., Common themes in the assembly and architecture of activating immune receptors. *Nat Rev Immunol* **2007,** *7* (11), 841-50.
53. Lee, M. S.; Glassman, C. R.; Deshpande, N. R.; Badgandi, H. B.; Parrish, H. L.; Uttamapinant, C.; Stawski, P. S.; Ting, A. Y.; Kuhns, M. S., A Mechanical Switch Couples T Cell Receptor Triggering to the Cytoplasmic Juxtamembrane Regions of CD3zetazeta. *Immunity* **2015,** *43* (2), 227-39.
54. Fuller-Espie, S.; Hoffman Towler, P.; Wiest, D. L.; Tietjen, I.; Spain, L. M., Transmembrane polar residues of TCR beta chain are required for signal transduction. *Int Immunol* **1998,** *10* (7), 923-33.
55. Chai, J., Atomic-resolution view of complete TCR-CD3 revealed. *Protein Cell* **2020,** *11* (3), 158-160.
56. Mariuzza, R. A.; Agnihotri, P.; Orban, J., The structural basis of T-cell receptor (TCR) activation: An enduring enigma. *Journal of Biological Chemistry* **2020,** *295* (4), 914-925.
57. Xu, X.; Li, H.; Xu, C., Structural understanding of T cell receptor triggering. *Cellular & Molecular Immunology* **2020,** *17* (3), 193-202.
58. Blumberg, R. S.; Alarcon, B.; Sancho, J.; McDermott, F. V.; Lopez, P.; Breitmeyer, J.; Terhorst, C., Assembly and function of the T cell antigen receptor. Requirement of either the lysine or arginine residues in the transmembrane region of the alpha chain. *J Biol Chem* **1990,** *265* (23), 14036-43.
59. Bonifacino, J. S.; Cosson, P.; Shah, N.; Klausner, R. D., Role of potentially charged transmembrane residues in targeting proteins for retention and degradation within the endoplasmic reticulum. *Embo J* **1991,** *10* (10), 2783-93.
60. Call, M. E.; Pyrdol, J.; Wucherpfennig, K. W., Stoichiometry of the T-cell receptor-CD3 complex and key intermediates assembled in the endoplasmic reticulum. *Embo J* **2004**.
61. Call, M. E.; Wucherpfennig, K. W.; Chou, J. J., The structural basis for intramembrane assembly of an activating immunoreceptor complex. *Nat Immunol* **2010,** *11* (11), 1023-9.
62. Cheng, X.; Im, W., NMR observable-based structure refinement of DAP12-NKG2C activating immunoreceptor complex in explicit membranes. *Biophys J* **2012,** *102* (7), L27-9.
63. Dube, N.; Marzinek, J. K.; Glen, R. C.; Bond, P. J., The structural basis for membrane assembly of immunoreceptor signalling complexes. *J Mol Model* **2019,** *25* (9), 277.
64. Sharma, S.; Juffer, A. H., An atomistic model for assembly of transmembrane domain of T cell receptor complex. *J Am Chem Soc* **2013,** *135* (6), 2188-97.
65. Wei, P.; Xu, L.; Li, C. D.; Sun, F. D.; Chen, L.; Tan, T.; Luo, S. Z., Molecular dynamic simulation of the self-assembly of DAP12-NKG2C activating immunoreceptor complex. *PLoS One* **2014,** *9* (8), e105560.
66. Knoblich, K.; Park, S.; Lutfi, M.; van 't Hag, L.; Conn, C. E.; Seabrook, S. A.; Newman, J.; Czabotar, P. E.; Im, W.; Call, M. E.; Call, M. J., Transmembrane Complexes of DAP12 Crystallized in Lipid Membranes Provide Insights into Control of Oligomerization in Immunoreceptor Assembly. *Cell Rep* **2015,** *11* (8), 1184-92.
67. Blazquez-Moreno, A.; Park, S.; Im, W.; Call, M. J.; Call, M. E.; Reyburn, H. T., Transmembrane features governing Fc receptor CD16A assembly with CD16A signaling adaptor molecules. *Proc Natl Acad Sci U S A* **2017,** *114* (28), E5645-e5654.

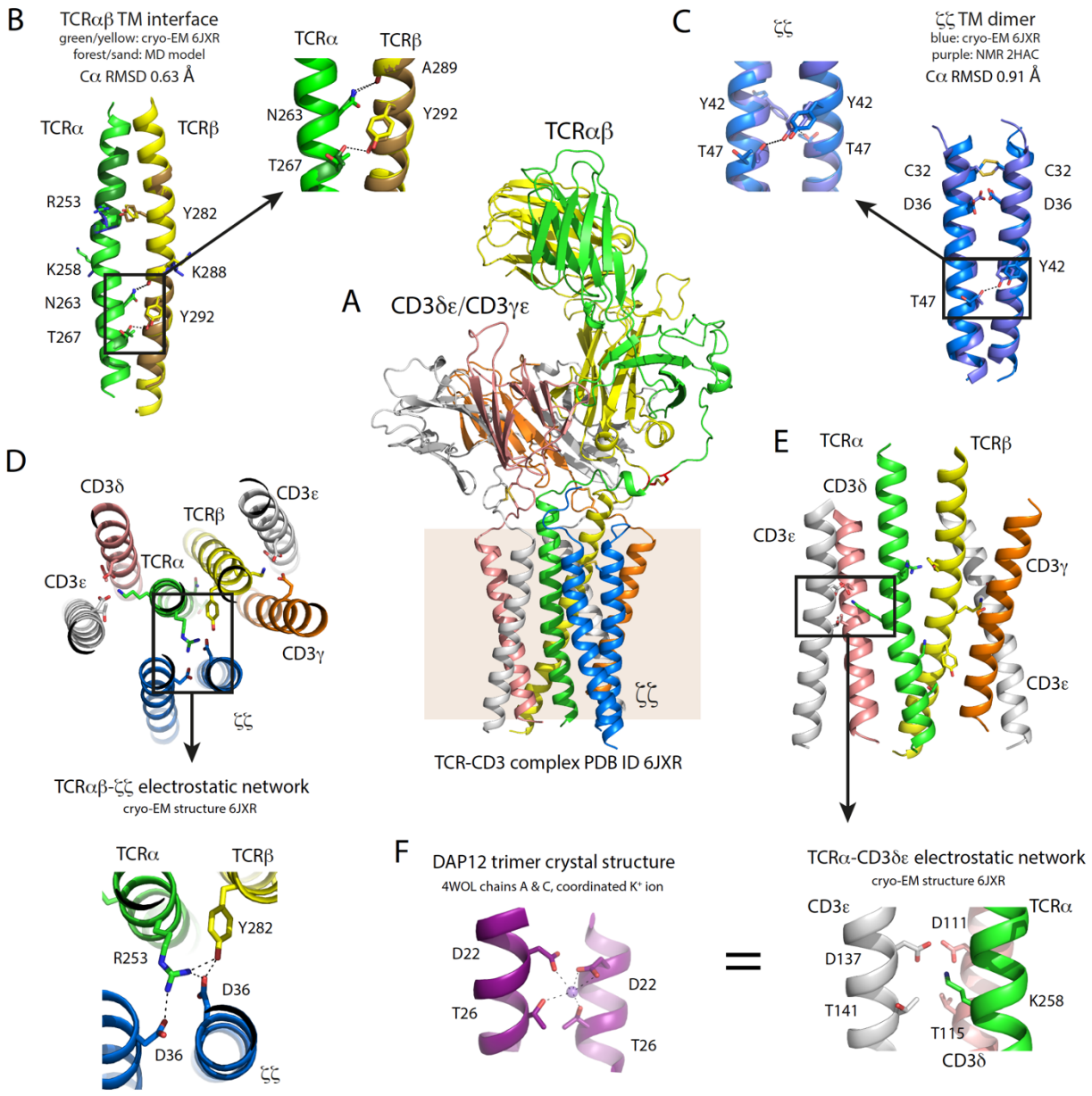
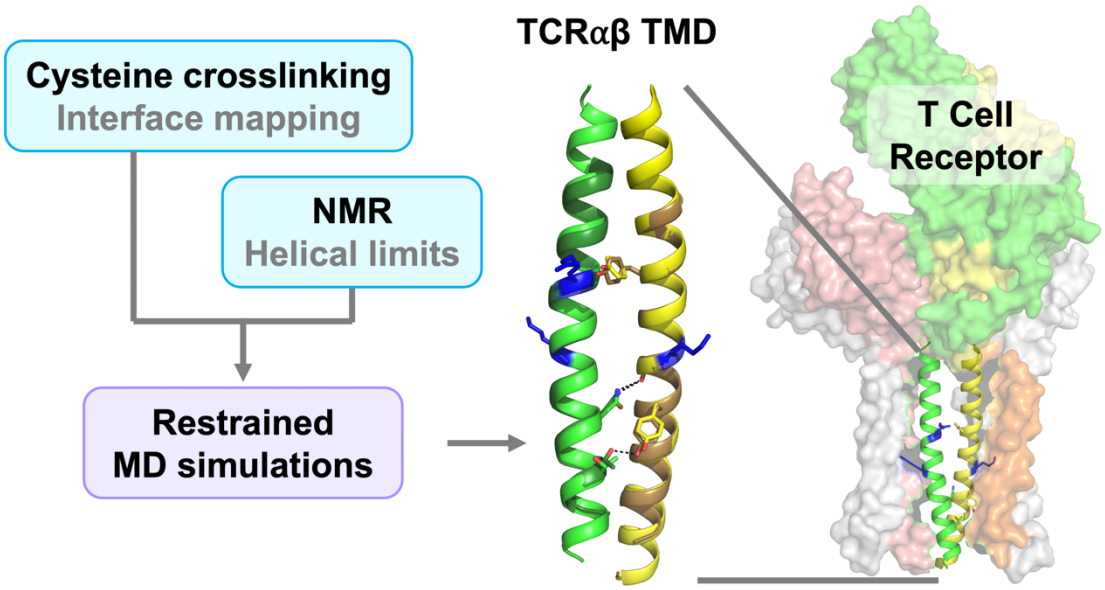


Figure 1: Models and predictions about receptor TMD organization that are borne out by the cryo-EM structure of an intact T cell receptor (TCR)-CD3 complex. The center panel **(A)** shows a profile view of the TCR-CD3 complex structure (PDB ID 6JXR) determined by cryo-EM using digitonin-extracted and lightly glutaraldehyde-crosslinked receptors. Beige box approximates where the lipid bilayer would be located. All subunits were well resolved in the ECD and TMD (their intracellular tails were not resolved, consistent with a largely unstructured state) and are colored here as follows: TCRα (green), TCRβ

(yellow), CD3γ (orange), CD3δ (salmon), CD3ε (white), and ζ (blue). **(B)** Overlay of TCRαβ TMD coiled coil structures from restrained simulations (forest and sand)[35] and cryo-EM structure 6JXR (green and yellow)[10]. Root mean squared deviation (RMSD) is calculated from Cα alignment of the MD model to residues TCRα L247-L270 and TCRβ L270-A303 in 6JXR. Boxed region is expanded to show a closer view of key interface hydrogen bonds. **(C)** Overlay of the ζζ TMD dimer NMR structure (2HAC; purple)[43] and the corresponding region from the cryo-EM structure 6JXR (blue). RMSD is calculated from Cα alignment of the lowest-energy NMR model to ζζ L31-F55 in 6JXR. Boxed region is expanded to show a closer view of key interface hydrogen bonds. **(D)** A view down the long axis of the eight-helix TMD assembly in 6JXR from the extracellular side. Boxed region is expanded to show the electrostatic network stabilizing the interaction between TCRαβ and ζζ dimers. **(E)** Side view of the six-helix TCR-CD3 TMD sub-assembly in 6JXR (with ζζ removed for clarity). Boxed region is expanded to show the electrostatic network stabilizing the interaction between TCRα and CD3δε. **(F)** Close-up view of an electrostatic network analogous to that from TCRα and CD3δε shown in (E) taken from DAP12 TMD trimer crystal structure 4WOL[66]. Purple sphere represents coordinated K^+ cation, sphere size does not represent the ionic radius of K^+ but is small so that D22 and T26 sidechains can be seen. All residue numbering in TCR-CD3 components is taken from cryo-EM structure 6JXR for ease of comparison.

TOC Graphic


Cysteine crosslinking
Interface mapping
NMR
Helical limits
Restrained
MD simulations
TCRαβ TMD
T Cell
Receptor