The guidance and adhesion protein FLRT2 dimerizes *in cis* via dual Small-X₃-Small transmembrane motifs

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Abstract

Fibronectin Leucine-rich Repeat Transmembrane (FLRT 1-3) proteins are a family of broadly expressed single-spanning transmembrane receptors that play key roles in development. Their extracellular domains mediate homotypic cell-cell adhesion and heterotypic protein interactions with other receptors to regulate synapse development and cell guidance. These *in trans* FLRT interactions determine the formation of signaling complexes of varying complexity and function. Whether FLRTs also interact *in cis* remains unknown. Here, we reveal two dimerization motifs in the FLRT2 transmembrane helix. Molecular dynamics simulations and single particle tracking experiments show that these 'Small-X₃-Small' motifs synergize with a third dimerization motif encoded in the extracellular domain to permit the *cis* association and diffusion patterns of FLRT2 on cells. The results point to a competitive switching mechanism between *in cis* and *in trans* interactions which suggests that homotypic FLRT interaction mirrors the functionalities of classic adhesion molecules.

Introduction

Fibronectin Leucine-rich Repeat Transmembrane (FLRT) proteins are a family of cell adhesion molecules (CAMs) that are broadly expressed during vertebrate development^{1,2}. FLRTs are unusual CAMs as they perform both cell adhesive and repulsive functions, leading to their definition as Repellent CAMs (ReCAMs)^{3,4}. In neurons, FLRTs act as repulsive guidance cues during cortical cell migration^{3,5-7}, whereby they play key role in cortical folding⁸ and as adhesion molecules in synaptic complexes^{9,10}. Adhesive FLRT functions are elicited by homotypic binding^{1,2} or by binding to the G-protein coupled receptor Latrophilin (Lphn 1-3)^{6,9,11,12} on opposing cells while cell repulsion results from interaction with Uncoordinated-5 (Unc5A-D)^{4,11}. FLRT also interacts with Unc5 *in cis* to regulate Lphn-mediated adhesion, at least *in vitro*⁷. In migrating neurons, FLRT cooperates with the Lphn-binding receptor Teneurin to form a ternary trans-synaptic complex that mediates cell repulsion¹³, while the three proteins also function in promoting synapsing¹⁴. Thus, FLRT acts in a context-dependent manner to determine the formation of different higher order cell-guidance signalling complexes and regulate brain development¹⁵. Here we ask whether FLRT forms homotypic *cis* complexes and how this may modulate *cis* and *trans* interactions with other partners.

FLRTs share a common architecture¹⁶ (**Fig. 1A**) beginning with an N-terminal Leucine-Rich Repeat (LRR) extracellular domain, which contains a concave surface on which both FLRT and Lphn bind^{3,6}. Unc5 binds to an adjacent surface on the LRR domain, which is compatible at least with Lphn-binding⁷. The LRR domain is linked to a type III fibronectin (FN) domain which then leads into the single-spanning transmembrane (TM) domain and a ~100 amino acid long intracellular domain (ICD) of unknown structure. FLRT2 TM domains contained two consecutives "Small-X₃-Small" motifs¹⁷⁻¹⁹ (**Fig. 1B**) which may promote FLRT interactions *in cis*. Indeed, this motif play fundamental roles in other single-spanning transmembrane receptors, including epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR), integrins, and EphA receptors²⁰⁻²⁶.

Characterizing dynamic membrane protein structures is challenging^{18,27,28}. However, the recent developments in molecular dynamics (MD) simulations²⁹ have provided a powerful tool to study membrane receptors and their interactions³⁰⁻³⁵. Coarse-grained molecular dynamics (CG-MD) simulations have emerged as the method of choice to explore the self-assembly of TMs³⁶⁻³⁸ and have been exhaustively validated³⁹⁻⁴¹. Combining MD simulations with well-established experimental assays such as Single Molecule Tracking (SMT) microscopy^{42,43}, which tracks the clustering of receptors⁴⁴⁻⁴⁹ on live cells, is a powerful approach to provide new insights into the dynamic assemblies of cell receptors^{21,33,50}.

Here, we use molecular dynamics simulations and live cell SMT experiments to reveal how FLRT2 dimerizes *in cis* via two Small-X₃-Small motifs. Unexpectedly, these motifs work synergistically with the extracellular dimerization motif in the ligand-

82 binding domain³ to produce FLRT-FLRT association. The results suggest a bipartite
83 structural mechanism that underlies the diverse functions of FLRT, and a competitive
84 mechanism for *in cis* versus *in trans* binding via the extracellular domain.
85

Figure 1: Dimerization of FLRT2 TM domains. **A-** Schematic of FLRT proteins engaging *in trans* and potentially also *in cis* interactions. **B-** Sequence of the FLRT2 TM helix. The two Small-X₃-Small motifs, key residues for the formation of the helix/helix interface highlighted by the CG-MD simulations, are colored in green and red. Two constructs were used as inputs for MD simulations: the core TM helix of 24 residues (TM₂₄) and an extended version with the four most N- and C-terminal residues (TM₃₂). **C-** The CG-MD protocol to assess TM helix interactions. The two helices are positioned 60 Å apart and diffuse freely in the membrane. The colored bars show, for each simulation, the distance in between the two TM helices as a function of the time. **D-** The TM contact bars of the 30 simulations for the TM₃₂ helices in a membrane of 80% POPC and 20% cholesterol. Details for all the simulations can be found in Sup. Fig. 1 and Sup. Fig. 2.

86 Results

87 FLRT2 TM dimerization involves two Small-X₃-Small motifs

89 As no structural information exists for the FLRT2 TM domain, we have used
90 secondary structure prediction tools (see Methods) to predict the membrane-
91 embedded helical region of FLRT2 (Fig. 1B). We obtained 24 residues as the core TM
92 helix (denoted TM₂₄). This length is consistent with the average length for a plasma
93 membrane-spanning TM helix⁵¹. We also extended the helical segment with four N-
94 and C-terminal residues, which were modelled as coils (denoted TM₃₂).

95 We then performed multiple runs of coarse-grained molecular dynamics (CG-
96 MD) to model the associations of both TM₂₄ and TM₃₂ in three different types of
97 membrane: POPC, DPPC, and a lipid mixture of 80% POPC and 20% cholesterol
98 (Sup. Table 1). For each of these three lipid compositions, we positioned the two
99 helices 60 Å apart allowing them to diffuse freely until there is an encounter which
100 predominantly leads to the formation of a stable helix dimer (Fig. 1D and Sup. Fig. 1

and 2). The TM helices interacted through a network of residues distributed along each peptide. Among these residues, we identified two consecutive Small-X₃-Small motifs known to favor TM interactions^{52,53}: A₅₄₄-X₃-G₅₄₈ and G₅₄₅-X₃-G₅₄₉ (Sup. Fig. 3).

Figure 2: CG-MD simulations uncover two Small-X₃-Small motifs as key areas for the dimerization of FLRT2 TM domains. **A-** TM₃₂ helix dimer structural populations for different membrane compositions (see Sup. Fig. 5 to 7 for individual simulations). A positive value for the crossing angle corresponds to a left-handed (LH) dimer, and a negative value to a right-handed (RH) dimer (details for TM₂₄ systems are presented in Sup. Fig. 4 and Sup. Fig. 8 to 10). The simulations have highlighted two Right Handed conformations (RH1 and RH2) and one Left Handed (LH). RH1 is defined with a crossing angle between -18° and 0°, RH2 between -36° and -18°, and LH between 0° and 18°. **B-** The three main representative structures for each crossing angle peak with A₅₄₄-G₅₄₈ dimerization motif in green, and A₅₄₅-G₅₄₉ dimerization motif in red. For each conformation (RH1, RH2 and LH), averaged TM contact matrix extracted from simulations of TM₃₂ in 80% DPPC and 20% of cholesterol highlight the TM dimerization via the A₅₄₄-G₅₄₈ motif (RH2), G₅₄₅-G₅₄₉ motif (RH1) or a combination of both (LH).

We next performed crossing angle analysis to analyze the geometry of the TM helices^{54,55}. For both the TM₂₄ and TM₃₂ models, this revealed three dimer populations (Fig. 2A and Sup. Fig. 4): two main right-handed populations with average crossing angles of approximately -27° (RH1) and -9° (RH2) and one minor left-handed population with an average crossing angle of around +9° (LH). For each crossing angle population, a clear interface was identified (Fig. 2B). RH1 conformations were driven

by interactions around the G₅₄₅-X₃-G₅₄₉ motif while RH2 conformations were centered on the A₅₄₄-X₃-G₅₄₈ motif. The LH population was constituted by peptides interacting via both motifs.

To obtain a more detailed view of the dynamical TM dimer association, we plotted the helix crossing angle against the distance between the two Small-X₃-Small motifs. This revealed several sub-populations associated with each crossing angle peak (**Fig. 2A**). Notably, membrane lipid composition appeared to modulate this dynamical equilibrium (**Fig. 2A and Sup. Fig. 4**) as seen in other studies⁵⁶⁻⁶⁰. Furthermore, for each membrane composition, the juxtamembrane regions appeared to modulate TM dimer formation (**Fig. 2A and Sup. Fig. 4**) as previously reported⁶¹. Plotting individual simulations (**Sup. Fig. 5 to 10**) suggested that the RH1 conformation can exchange with the LH conformation. RH2 seemed to also exchange with RH1 conformation but to a lesser extent. Thus, RH1 may be seen as a transition state in between RH1 and LH conformations.

We then refined the three main TM dimer structures (RH1, RH2 and LH) by performing 400 ns of atomistic MD simulations (see Methods) (**Sup. Fig. 11-A**). The RH1 and RH2 dimers remained in a right-handed conformation though we observed a slight increase of the crossing angle for the RH2 structure (with a final crossing angle of -37°). Interestingly, the LH structure switched to a right-handed conformation at the beginning of the simulation and then returned to a stable left-handed conformation. This confirmed the exchanges between RH1 and LH configurations seen in coarse-grain simulations (**Sup. Fig. 5 to 10**). For all three structures, the interactions between the Small-X₃-Small motifs were stable throughout the simulation (**Sup. Fig. 11-B**).

Thus, MD simulations revealed a dynamic equilibrium of dimer structures involving the two consecutive Small-X₃-Small motifs: A₅₄₄-X₃-G₅₄₈ and G₅₄₅-X₃-G₅₄₉.

Distinctive mutations in the Small-X₃-Small motifs selectively modulate FLRT2 TM dimerization

To assess the individual contributions of the two Small-X₃-Small motifs to the dimerization, we performed CG-MD simulations with several mutants replacing glycine residues with isoleucine or valine residues, the larger hydrophobic side chains of which are expected to disturb the TM dimerization⁶²⁻⁶⁶ (**Fig. 3A and Sup. Table 1**). For each mutant, we evaluated both the crossing angle populations and the spatial distributions for the TM₃₂ construct in a DPPC bilayer.

Figure 3: *In silico* mutations in the two Small-X₃-Small motifs affect the TM dimerization and dynamical equilibrium. **A-** Table of mutations for *in silico* and SMT experiments. The LRR/TM₁₊₂ mutant was only used for the SMT experiments. **B-** Spatial distribution profiles of one TM₃₂ helix relative to the other for the CG simulations of both WT and mutants. The diagram shows the probability density of finding the backbone particles of one TM₃₂ helix at a given point in the bilayer plane around the other helix. Diagrams are colored such that white represents low probability up to blue for higher probabilities. Green (respectively red) circles depict averaged positions of A₅₄₄ and G₅₄₈ (respectively G₅₄₅ and G₅₄₉) residues. **C-** Helix crossing angle distributions for both TM₃₂ WT and mutants. **D-** Averaged TM contact matrices of the different mutants based on different representative TM conformations.

In contrast with the wild type, mutations in the A₅₄₄-X₃-G₅₄₈ motif (mutants TM₀ and TM₁) favored formation of a dimer with a spatial distribution focused on the G₅₄₅-X₃-G₅₄₉ motif (Fig. 3B) and switched of crossing angle populations towards RH1 conformations (Fig. 3C). For these conformations, TM helices are packed around the G₅₄₅-X₃-G₅₄₉ motif (Fig. 3D). Mutations in the G₅₄₅-X₃-G₅₄₉ motif (mutants TM₂ and TM₃) drove interactions through the G₅₄₄-X₃-G₅₄₈ motif (Fig. 3B,D) favoring RH2 crossing angle populations (Fig. 3C) and allowing TM domains to explore a wider area around the G₅₄₄-X₃-G₅₄₈ motif compared to the WT (Fig. 3B). Mutations of both motifs (mutant TM₁₊₂) enabled one TM domain to explore the entire bilayer plane surrounding its TM partner (Fig. 3B), thereby abolishing the specificity of the TM helix interactions. For this double mutant, the crossing angle density was clearly more diffuse than for the WT or the other mutants, further highlighting a loss of specificity (Fig. 3C). Mutating both motifs also limited the interactions in between the TM helices for the two main conformations RH2 and LH (Fig. 3D).

Thus, these mutations highlighted two distinct dynamical behaviors of the TMD dimer specific to each motif. Only mutation of both motifs resulted in an almost complete loss of interactions between the two monomers.

Mutations in the Small-X₃-Small motifs affect FLRT2 co-localization in cells

To support the *in silico* results, we performed SMT experiments to assess the contribution of the predicted key residues in the Small-X₃-Small motifs to dimer formation by mutating glycine residues to isoleucine or valine residues (Fig. 3A). We tracked FLRT2 receptors on live cells with a sub-pixel accuracy by SMT in two different channels using the dyes Alexa549 and CF640R (Fig. 4A-C). Based on receptor frame to frame proximity in each channel (Fig. 4C), we then built a distribution of the durations of co-localization events (Fig. 4D), referred to as τ_{on} . The duration of co-localization events is a characteristic of the stability of any interaction or association between the tracked receptors, and is independent of expressed receptor concentration⁴⁷. Comparing the τ_{on} distributions of WT and mutants (Fig. 4E) revealed that mutations in only one of the two motifs (either TM₁, TM₂ or TM₃ alone) was insufficient to reduce the baseline average τ_{on} of wild-type FLRT2. However, mutation of both Small-X₃-Small motifs (TM₁₊₂ mutant) resulted in a significant shift in the τ_{on} distribution towards lower values (Fig. 4E and Sup. Fig. 12B). These results are in agreement with a previous study showing that mutation of both Small-X₃-Small transmembrane motifs is necessary to disrupt the EGFR TM dimer and affect receptor function⁶⁷. The significant shift in τ_{on} was also seen for the mutation in the LRR ectodomain, known to abolish FLRT-FLRT trans-interactions³, and for the triple mutation LRR+TM₁₊₂. This provides experimental support for the *in silico* results demonstrating that the two Small-X₃-Small motifs are required for FLRT interactions *in cis*.

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Figure 4: Mutations in the TM domains affect colocalization of FLRT2 monomers at the cell surface. **A-** Single molecule TIRF image of HeLa cells expressing wtFLRT2 labelled with both SNAP-549 and BG-CF640R. **B-** Single molecule tracks are generated from time series of the molecules under observation. **C-** An example pair of colocalized tracks where the tracks are separated by less than 1 pixel (160 nm) during at least 5 frames (250 ms). **D-** Example τ_{on} distribution for the WT (n represents the number of tracks analyzed). **E-** Distributions of τ_{on} for wtFLRT2 and each of the six FLRT2 mutants tested. Significance analysis of these distributions available in Sup. Fig 12B. **F-** Distributions of diffusion values for wtFLRT2 and each of the six FLRT2 mutants tested. Significance analysis of these distributions available in Sup. Fig 12C.

187 We next analyzed the diffusion coefficients for the WT and mutants as an
 188 alternative approach to provide further insight into the role of these motifs in dimer
 189 formation (Fig. 4F). In line with the τ_{on} results, only diffusion values for the mutants
 190 TM₁₊₂, LRR, and LRR+TM₁₊₂, increased significantly from the WT (Sup. Fig. 12C,D).
 191 Notably, diffusion calculations in MD simulations also indicated a significant difference
 192 in between the double mutant TM₁₊₂ and the WT (Sup. Fig. 12E). Although the spatial
 193 resolution of single molecule tracking is insufficient to discriminate between direct
 194 pairwise interactions and co-confinement or joint interactions with the same larger
 195 protein complex, this correlation between the decreased duration of co-localization
 196 events and the increased diffusion constant of the tracked receptors is consistent with
 197 the mutations disrupting interactions that usually occur between WT FLRT2 TM
 198 domains.

199 Taken together, these data indicate that the extracellular motif and both G₅₄₄-
 200 X₃-G₅₄₈ and G₅₄₅-X₃-G₅₄₉ motifs in the transmembrane region are required for wild-type
 201 FLRT2 homotypic interactions *in cis*.

Discussion

TM dimer association is a highly dynamic process involving multiple states and weak interactions hence challenging structural studies. As a consequence, only a limited number of TM dimer structures are available and these are often restricted to one state of the TM dimer⁶⁸. To complement structural studies, MD simulations can provide details of these dynamic assemblies. Here, we have used MD simulations to gain structural insights into the formation of FLRT2 TM dimers. Our models revealed a dynamical equilibrium between conformations involving two successive Small-X₃-Small motifs: G₅₄₄-X₃-G₅₄₈ and G₅₄₅-X₃-G₅₄₉ motifs. To further assess these models and the conformational changes governing the FLRT2 dimer association, one could use

Figure 5: Model of the FLRT cis-interaction. A- Sequence alignment of the TM domain for FLRT1-3 in human and for FLRT2 in other species (human:h, chicken:g, frog:x, fish:dr). **B-** Model of FLRT2 cis-interactions that may compete with different FLRT2 trans interactions. The interconversion in between RH1, involving the G₅₄₅-G₅₄₉ motif (in red), and RH2 interactions, driven by the A₅₄₄-G₅₄₈ motif (in green), may be modulated by mutations in the TM domain or environmental conditions such as changes in the lipid composition of the membrane.

accelerated MD simulations combined with free energy calculations^{69,70}. To enable this, we are releasing the three main conformations (RH1, RH2 and LH) both in coarse

grained and atomistic representations (<https://github.com/MChavent/FLRT>).

The TM helices of other receptors such as EGFRs and EPHAs dimerize via Small-X₃-Small motifs to transduce extracellular signals into intracellular activity^{21,22,24}. Unlike these receptors, there is no enzymatic activity associated with FLRT. Instead, FLRTs act as crucial adaptor proteins that define the architecture of cell surface signalling hubs^{3,7,13}, and also regulate the trafficking of diverse receptors. For example, FGF receptor traffics with FLRT1-3^{71,72}, while FLRT3 regulates the cell surface levels of the Deleted in Colorectal Carcinoma (DCC) receptor in thalamic axons⁷³. Dimerization of the EGFR Small-X₃-Small motif also regulates EGFR trafficking⁶⁵. Thus, *in cis* dimerization via the Small-X₃-Small motifs in the TM helix of FLRTs may act similarly and play a central role in receptors trafficking and thereby signal transduction.

A functional role for the Small-X₃-Small motifs is supported by their conservation in all three FLRT human homologues (FLRT 1-3) as well as for FLRT2 in different species (Fig. 5A). In addition, the COSMIC database⁷⁴ lists a number of cancer-related mutations targeting the TM domain of FLRT2. Two such mutations (A544V and G545V) map to the Small-X₃-Small motifs described here, and may affect FLRT2 function and dynamics as seen in MD simulations (Fig. 3). Furthermore, the lipid environment can modulate WT TM association⁷⁵⁻⁷⁷ (Fig. 2A, Sup. Fig. 4) and may accentuate deleterious effects of these mutations.

Unexpectedly, our results show that the same mutation in the LRR domain that disrupts FLRT-FLRT interactions *in trans*³ also disrupts FLRT-FLRT interaction *in cis*, posing the question whether FLRT *cis* and *trans* interactions are competitive (Fig. 5B). Adding complexity to this issue is the observation that the same mutation also abolishes *trans* FLRT-Lphn interactions^{3,6}. These findings suggest that Lphn may also compete with *in cis* FLRT-FLRT dimerization, leading to a mechanism in which FLRTs switch between *in cis* dimerization and *in trans* interactions (Fig. 5B). Interplay between *cis* and *trans* interactions are essential for the formation of clusters of adhesion proteins leading to cell-cell recognition⁷⁸. One canonical example is the formation of adherens junction by the clustering of cadherins for which both *cis* and *trans* interactions are required^{79,80}. In addition, a competitive switch between *in trans* and *in cis* interactions occurs for EphA receptors with their ephrin-A ligands^{81,82}. Our results describe the structural basis and nature of *in cis* dimerization of FLRT2 receptors and suggest how these interactions may compete with interactions *in trans* to determine the formation of specific signalling complexes and regulate fundamental developmental processes, such as cortical folding and synapsing.

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Competing interests

No competing interests declared

Methods

Modeling Transmembrane domain and Molecular Dynamics Simulations

Results from the PSIPred⁸³, PRED-TMR2⁸⁴, and HMMTOP⁸⁵ servers were combined to predict the membrane embedded helical region of FLRT2. Twenty-four residues of human FLRT2 (residues 541 – 564) were selected to form the core of the TM helix (TM₂₄). The transmembrane domain was created using the Pymol secondary structure creation script: build_seq.py (<http://pldserver1.biochem.queensu.ca/~rlc/work/pymol/>) and then converted into coarse-grained model. For TM₃₂, the four residues both N- and C-terminal of TM24 were modelled as random coils using Modeller 9v9⁸⁶.

Coarse-grained MD (CG-MD) simulations were performed using GROMACS 4.6 (www.gromacs.org)^{87,88} with the MARTINI 2.1 forcefield^{89,90}. The temperature was 323K. Electrostatic interactions were shifted to zero between 0 and 1.2 nm and the Lenard-Jones interactions between 0.9 and 1.2 nm. A Berendsen thermostat in combination with a Berendsen barostat⁹¹ with a coupling constant of 1.0 ps, a compressibility of $5.0 \times 10^{-6} \text{ bar}^{-1}$, and a reference pressure of 1 bar were used. The integration timestep was 20 fs. Simulations were run for either 1 or 2 μs (Sup. Table 1) over twenty to thirty replicates to ensure exhaustive sampling of TM helix dimer structures.

We then converted the three main representative (RH1, RH2, and LH) coarse grained structures into atomistic models using the CHARMM-GUI MARTINI to All-atom converter (<http://www.charmm-gui.org/?doc=input/converter.martini2all>). Atomistic simulations were performed with GROMACS 2018 in combination with the CHARMM36 forcefield⁹²⁻⁹⁴ and TIP3P water model. The temperature was held at 310K. A first step of energy minimization was performed using the steepest descent algorithm and was equilibrated with a constant temperature (canonical ensemble, NVT, 310 K) ensemble for 100 picoseconds, followed by a 100 picoseconds equilibration at constant pressure (isothermal-isobaric, NPT, 1 bar). We then ran 100 ns of equilibration by keeping the protein backbone constrained followed by 400 ns of unrestrained production run. We applied a Nosé-Hoover thermostat on the system, coupled with the Parrinello–Rahman barostat, with a compressibility of $4.5 \times 10^{-5} \text{ bar}^{-1}$. Long-range electrostatics were modeled using the Particle-Mesh Ewald method⁹⁵. All bonds were treated using the LINCS algorithm⁹⁶. The integration time step was 1 femtosecond.

Simulation analysis:

Protein and lipid structures were rendered using VMD⁹⁷. Simulations trajectories were analyzed using a combination of Tcl/VMD and Python scripts. Matplotlib was used to create graphs and images of TMD monomer distances, contact matrices, TMD density rendering, and crossing angles analysis. All the scripts used to

perform these analyses are available at: <https://github.com/MChavent/FLRT>. Distances were calculated in between the two centers of mass of each TM helix. Density, TM contacts and crossing angles calculations were performed every nanosecond for the part of the trajectory where a dimer was formed. In Figure 2-A (resp. 3-B,C), the values were renormalized to take into account of both the maximum values and time of interactions to properly compare the different membrane (resp. Wild Type and mutants) systems.

Diffusion analyses of dimers were carried out by extracting time-averaged mean squared displacement (MSD) for all simulation replicates, for the part of the trajectory where a dimer was formed. For each replicate, a MSD vs. Δt was plotted for Δt in the range 0.2 ns – 200 ns. The diffusion coefficient (D) for each trajectory was calculated by fitting the equation $MSD = 4D\Delta t$ to the first two points in the plot, in order to obtain an instantaneous diffusion coefficient. The diffusion analysis script used to perform the analysis can be found at:

https://github.com/tylerjerredy/diffusion_analysis_MD_simulations.

Cloning

SNAP-FLRT2 was cloned into the EcoRI/XhoI restriction sites of the pHSec vector⁹⁸. In SNAP-FLRT2 an N-terminal SNAP tag (containing the RPTP σ signal sequence) was fused to murine FLRT2 (residues A35 – T660) via an HA-tag. Mutations were introduced using molecular cloning.

Cell Culture and Transfection

HeLa cells were seeded onto uncoated 4-well μ -Slides, #1.5 polymer coverslips (Ibidi) at a density of 1.1×10^5 cells/well in 600 μ L phenol red-free DMEM + 10% FBS + 1% L-Gln + 1% NEAA (complete medium). After 24 h, each well was transfected with 2.0 μ g plasmid DNA using FuGENE6, according to the manufacturer's instructions. Cells were maintained at 37 °C, 5% CO₂ and were prepared for experiments 12-18 hours post-transfection.

BG-CF640R Conjugation

CF640R succinimidyl ester (Biotium) was reacted with BG-NH₂ (New England Biolabs) to produce the benzylguanine functionalised dye BG-CF640R. 1 μ mol of CF640R succinimide ester was reconstituted in DMSO and dissolved in 10 ml 0.1 M sodium bicarbonate buffer at pH 8.4. 1.5 μ mol BG-NH₂ in DMSO was added to the dye mixture and vortexed well. The reaction was shaken at room temperature overnight before dilution with deionised water. For all subsequent dilutions the conjugation efficiency was assumed to be 100%.

Two-Colour Fluorescent Labelling

To achieve an approximately equal ratio of single molecules labelled with SNAP Dy549 and BG-CF640R a two-step staining procedure was used. Firstly, the medium was removed from each well of the 4-well μ -Slides and the cells washed twice with 300 μ L complete medium. BG-CF640R was diluted in complete medium to a final concentration of 10 nM and applied to each well of the μ -Slide for 5 min. The medium was then exchanged for 150 μ L 10 nM SNAP-Dy549 (SNAP-Surface 549, New England Biolabs) in complete medium and incubated for a further 5 min. All labelling steps were performed at 37 °C, 5% CO₂. Labelled cells were then washed three times with complete medium and the final wash replaced with Live Cell Imaging Solution plus 1:50 ProLong Antifade reagent (both ThermoFisher) and incubated for at least 15 min, at 37 °C, 5% CO₂ before beginning experiments.

Single molecule image acquisition and feature tracking

Single-molecule images were acquired using an Axiovert 200M microscope with an iLas2 TIRF illuminator (Cairn, UK), with a $\times 100$ oil-immersion objective (α -Plan-Fluar, NA = 1.46; Zeiss, UK) and an EMCCD (iXon X3; Andor, UK). The microscope is also equipped with a wrap-around incubator (Pecan XL S1). The 561 and 642 nm lines of a LightHub laser combiner (Omicron-laserage Laserprodukte GmbH) were used to illuminate the sample and an Optosplit Image Splitter (Cairn Research) was used to separate the image into its spectral components as described previously⁹⁹. The field of view of each channel for single-molecule imaging was 80 $\times$ 30 μ m. Typically, for each condition at least 50 fields of view comprising one or more cells were acquired from a total of 4 independent biological replicates. Single molecules were tracked in each field of view for 30s, by which time the majority of molecules had undergone photobleaching. All single-molecule time series data were analyzed using the multidimensional analysis software described previously¹⁰⁰. Briefly, this software performs frame-by-frame Bayesian segmentation to detect and measure features to sub-pixel precision, then links these features through time to create tracks using a simple proximity-based algorithm. The software determines cubic polynomial registration transformations between wavelength channels from images of fluorescent beads. Feature detection and tracking was performed independently in each channel.

Calculation of colocalisation and τ_{ON}

Two-colour TIRF images of the basolateral surfaces of cells were chromatically separated by a beam splitter and registered using custom-made software to map the relative positions of the probes over the time course of data acquisition¹⁰⁰ and extract single molecule tracks. A colocalisation event was defined as one in which a track in one channel moves within one pixel of a track in the other channel before they move apart again (Sup. Fig 12A). The duration of each such event is one measurement of τ_{ON} . This parameter indicates the stability of presumptive receptor interactions while

beingin sensitive to variation in expression of the receptors between cells or different levels of labelling with the two probes within cells⁴⁷. The track positions were registered between channels prior to this analysis. To reduce the impact of localisation error on these results a temporal Gaussian smoothing filter of FWHM 4 frames (200 ms) was applied to the position traces before the colocalisation analyses. τ_{ON} distributions were compared between conditions using the two sample Kolmogorov-Smirnov test to decide which were significantly different (**Sup. Fig 12B**).

Mean squared displacement and diffusion calculation

From single particle tracks, mean squared displacement (MSD) curves were calculated as $MSD(\Delta T) = \langle |\mathbf{r}_i(T+\Delta T) - \mathbf{r}_i(T)|^2 \rangle$ where $|\mathbf{r}_i(T+\Delta T) - \mathbf{r}_i(T)|$ is the displacement between position of track i at time T and time $T+\Delta T$ and the average is over all pairs of points separated by ΔT in each track. The average instantaneous diffusion coefficient (D) for these tracks was calculated by fitting a straight line to the first two points of the MSD curve then calculating D directly from the gradient m of the fit, $D = m/4$. The tracks for each single molecule field of view (FOV) were pooled into one MSD curve per FOV to produce a sample of D values, one value per FOV per condition. These D distributions were compared between conditions using the Kolmogorov-Smirnov test to decide which were significantly different.

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