Characterisation of the FAM171 neuronal receptors

Tom Bird, Sepideh Valimehr, David Wood, Zac Tillett, Gerd Mittelstädt, Renwick Dobson, Eric Hanssen, **Davide Comoletti**

Victoria University of Wellington, Ian Holmes Imaging Centre, ARC Centre for Cryo-electron Microscopy of Membrane Proteins, University of Canterbury, Ferrier Research Institute

Introduction

- The **FAM171 proteins are a three-member family (A1, A2, B)** of understudied neuronal receptors.
- Only 22 publications on PubMed (as of September 2024).
- Genetic implications in cancers (bile duct[1], bladder[2], breast[3], colon[4], colorectal[5], tongue[6]) and neurodegenerative diseases[7] (Alzheimer's and Parkinson's).
- High expression in microglia and cerebral vascular endothelium.
  - Each FAM171 protein localises to a different area of the filopodia of neurons (unpublished data).
- Extracellular domains are 36-40 kDa in size, have high sequence similarity and AlphaFold predicted structure similarity (Figure 1).
- Currently there is no biophysical characterisation of interactions or experimental structural data available for any FAM171 protein.

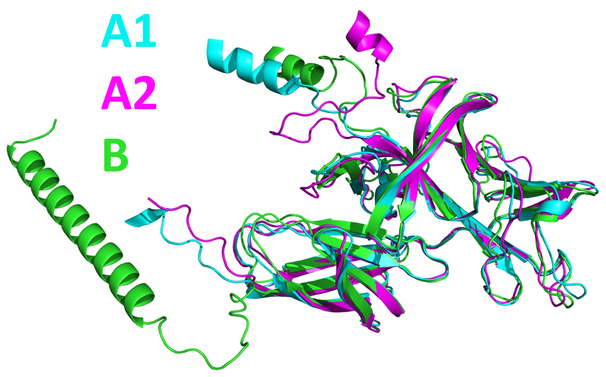


Figure 1. AlphaFold predicted structures of FAM171 extracellular domains.

Our aim is to characterise the structure and behaviour of the FAM171 family of proteins to further understand the role they play in human disease.

Methods

ELISA-based Proteomic Screen

Protein Expression

- Expi293 cells were used for expression of AP- and Fc-fused proteins constructs.
- Fc-protein expression was confirmed by dot blot and AP-protein expression confirmed by reaction with PnPP.

Proteomic Screen

- 384-well ELISA plates were coated with an anti-AP antibody overnight.
- Conditioned media containing AP-tagged FAM171 constructs and Fc-tagged neuronal proteins were added separately with an anti-IgG-HRP antibody. Interactions were detected using TMB substrate. Hits are defined as > 3-fold over background and unique to a plate.

Protein Expression and Purification

Stable Cell Line Generation

- Stable HEK293S GnTI- cell lines were generated to express Fc-fused or FLAG-tagged constructs.
- Protein production utilised triple-layer flasks or a BelloCell bioreactor.

Protein Purification

- Proteins were purified by affinity chromatography using anti-FLAG resin (where there the Fc-tag was to remain intact) or Protein A resin (where the Fc tag was cleaved via a 3C protease site engineered between the C-terminal of the protein and the start of the Fc region).
- Where required, proteins were further purified by size exclusion chromatography (SEC) (Superdex 200 10/300 GL or Superose 6 Increase 10/300 GL).

ELISA Binding Affinity Assay

- 96 well plates were coated with 3 µg/mL anti-AP antibody and used to capture AP-tagged FAM171 protein from conditioned media
- Serial dilutions of purified Fc-FAM171 receptor were added in triplicate and their interactions with the AP-protein detected using an anti-IgG-HRP antibody.

Analytical Ultracentrifugation (AUC)

- FAM171 proteins at concentrations of 2 and 20 µM were run in an An-50 Ti rotor at 50,000 rpm using a Beckman Optima XL-I analytical ultracentrifuge and data processed using SedFit.

Cryo-EM

- Cryo-electron microscopy data was collected using a Krios G4 running at 300 kV, equipped with a K3 detector and a BioQuantum imaging filter at the Ian Holmes Imaging Centre. Data was processed using CryoSPARC and initial models fit using Model Angelo. Phenix and Coot were used for structure refinement.

Results

Interactions

ELISA-Based Proteomic Screen

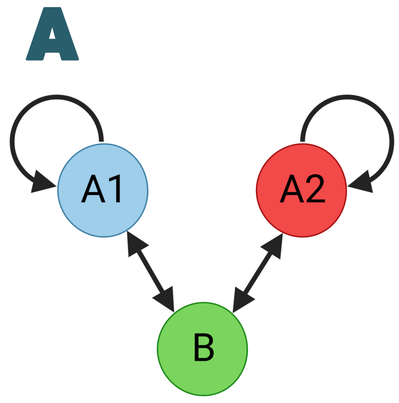
- **965** proteins screened against **3** FAM171 constructs using an ELISA-based proteomic screen.
- **2,895** total interactions.
- FAM171 interaction network identified (Figure 2A).

Binding Affinities

- A binding affinity ELISA using purified protein was used to validate hits found in the proteomic screen.
- FAM171 extracellular domains interact with each other with **nanomolar affinities** (Figure 2B).

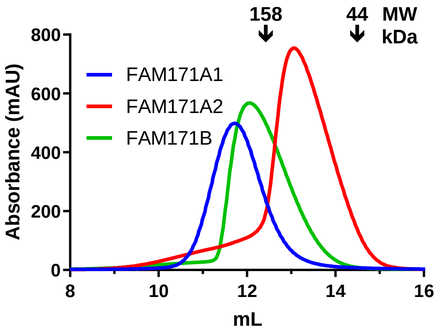
Oligomeric States

- SEC suggested that despite similar expected masses (36 - 40 kDa), each FAM171 extracellular domain may exist in a different oligomeric state (Figure 2C).
- AUC experiments show (Figure 2D):
  - **FAM171A1** is a stable trimer.
  - **FAM171A2** and **FAM171B** both exist in a concentration-dependent monomer-trimer equilibrium.
  - **FAM171A2** and **FAM171B** interact as a 1:1 complex.



B

Binding (nM)	A1-AP	A2-AP	B-AP
A1-Fc	**8.92** ± 4.9	No binding	**14.5** ± 10
A2-Fc	**515** ± 156	**389** ± 170	**27.2** ± 5.6
B-Fc	**157** ± 36	**195** ± 12	No binding



D

	Mass (kDa)	Monomers
A1	103	**2.6**
A2	39	**1**
	111	**2.9**
B	33	**0.9**
	102	**2.7**
A2+B	80	**1:1**

Figure 2. (A) FAM171 interaction network. (**B**) Binding affinities between each FAM171 extracellular domain. (**C**) SEC trace of purified FAM171 extracellular domains. (**D**) Masses determined by AUC.

Cryo-EM Structures

FAM171A1

- Data collection and 3D refinement of 231,630 particles gave a **2.3 Å resolution map** (Figure 3A).
- Formed a 60-mer, an icosahedron made up of 20 trimers (High symmetry helped achieve better resolution).
  - Individual trimers were also found at a lower resolution.
- Unlikely to be a 60-mer in a biological context (the intracellular domain would be inside the 60-mer)
- Possible the 60-mer could be flattened on a cell surface, making a receptor 'carpet' for cell - cell interactions.

FAM171A2

- **Trimeric structure** (Figure 3B) was found to be similar to FAM171A1 (RMSD = 1.49 Å).
- Differences between key trimer interactions in **FAM171A1** and **FAM171A2** may explain the stability of the **FAM171A1** trimer in contrast to the concentration dependent oligomerisation of **FAM171A2** (Figure 3C).

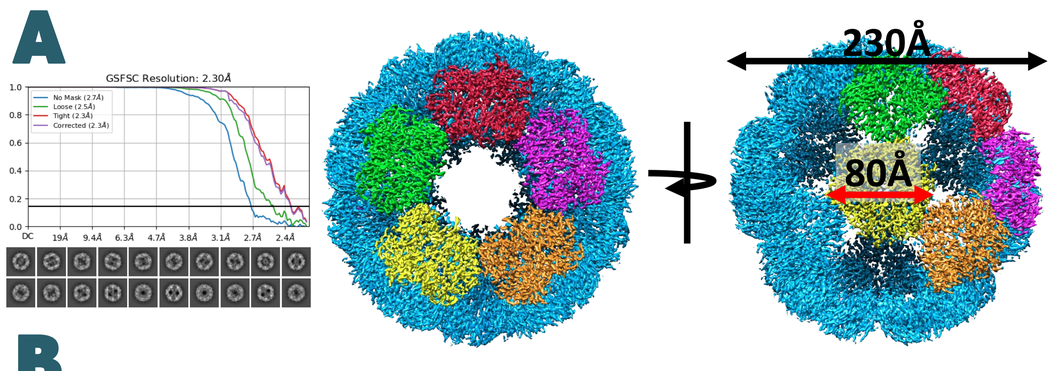


Figure 3. (**A**) **FAM171A1** cryo-EM FSC curve and 20 representative 2D classes (left), 3D model of the **FAM171A1** 60-mer showing five trimers in different colours (right).

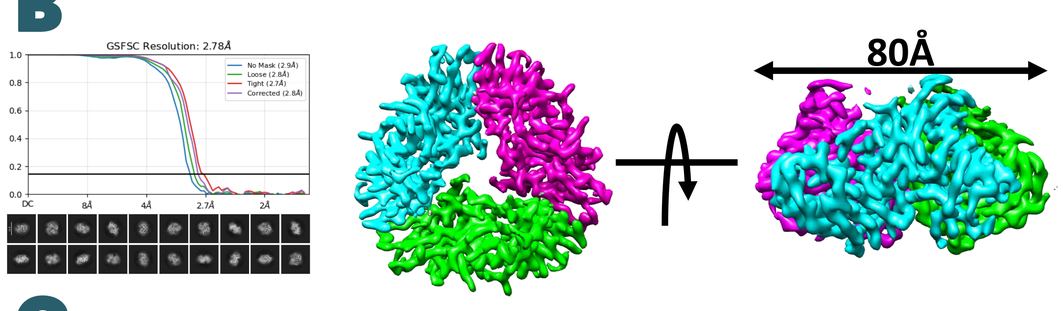


Figure 3. (**B**) **FAM171A2** cryo-EM FSC curve and 20 representative 2D classes (left), 3D model of the **FAM171A2** trimer showing each monomer in a different colour.

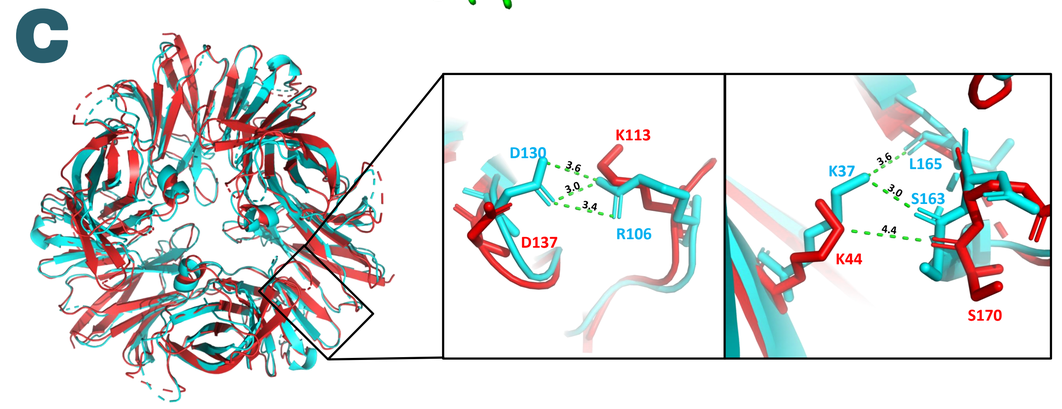


Figure 3. (**C**) Overlayed structures of **FAM171A1** (cyan), **FAM171A2** (red) (left) and key trimer stabilising interactions (right), H-bond and salt bridges distances highlighted by green dashed lines.

Conclusions/Future Work

- FAM171 extracellular domains interact with each other at nanomolar affinities and form trimers of varying stabilities.
- We still want to determine the FAM171A2/B complex structure (X-ray crystallography or cryo-EM) and compare interfaces with homotrimeric structures. Cell aggregation assays can be used to determine if interactions are cis- or trans-cellular.

References

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