MSN (Moesin)

A Target Enabling Package (TEP)

Gene ID / UniProt ID / EC	4478 / P26038 / -
Target Nominator	AMP-AD (Allan Levey, Emory University)
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Target PI	Opher Gileadi
Therapeutic Area(s)	Alzheimer's disease
Disease Relevance	MSN is highly expressed in microglia and brain endothelium (as well as several non-brain tissues).
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SUMMARY OF PROJECT

Moesin (MSN; membrane-organizing extension spike protein) contains a FERM domain (Four-point-1, Ezrin, Radixin, Moesin) that links transmembrane receptors such as CD44 to the actin cytoskeleton, in a manner regulated by phosphorylation and PIP2. A proteomic, post-mortem analysis of >400 brains(1) identified a protein co-expression module that is highly correlated with pathological and cognitive measures of Alzheimer's disease (AD). Moesin and CD44 have emerged as key drivers in the module. MSN is highly expressed in microglia and brain endothelium (as well as several non-brain tissues)(2). This TEP targets the CD44-MSN interaction, to test the hypothesis that inhibiting the CD44-MSN interaction would reverse harmful activities of microglia and provide beneficial outcomes for AD patients.

SCIENTIFIC BACKGROUND

Moesin (MSN) is an eponymous member of the Ezrin-Radixin-Moesin (ERM) family of proteins that connect the actin cytoskeleton to the plasma membrane and thereby regulate the structure and function of specific domains of the cell cortex(3). Moesin tethers actin filaments by oscillating between a resting and an activated state providing transient interactions between moesin and the actin cytoskeleton(4,5). Once phosphorylated on its C-terminal threonine, moesin is activated leading to interaction with F-actin and cytoskeletal rearrangement. These rearrangements regulate many cellular processes, including cell shape determination, membrane transport, and signal transduction(6,7). The role of moesin is particularly important in immunity acting on both T and B-cells homeostasis and self-tolerance, regulating lymphocyte

egress from lymphoid organs (8,9). Moesin also participates in immunologic synapse formation(10). Mutations in MSN in mice and in humans are associated with defective immune responses (10-12).

RESULTS – THE TEP

Proteins purified

1. MSNA-c000: Full-length moesin (1-577) with N-terminal his6 tag and a TEV cleavage site.
2. MSNA-c001: FERM domain of moesin (1-346) with N-terminal his6 tag and a TEV cleavage site.

Both proteins expressed in recombinant *E. coli*. The shorter version has been used for crystallography and for binding assays.

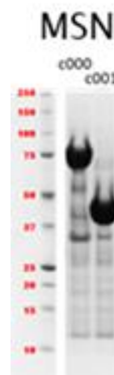

Figure 1. Purified full-length (c000) and FERM domain (c001) moesin proteins

Structural data

PDB: **6TXQ**, The high resolution structure of the FERM domain and helical linker of human moesin.
PDB: **6TXS**, The structure of the FERM domain and helical linker of human moesin bound to a CD44 peptide.

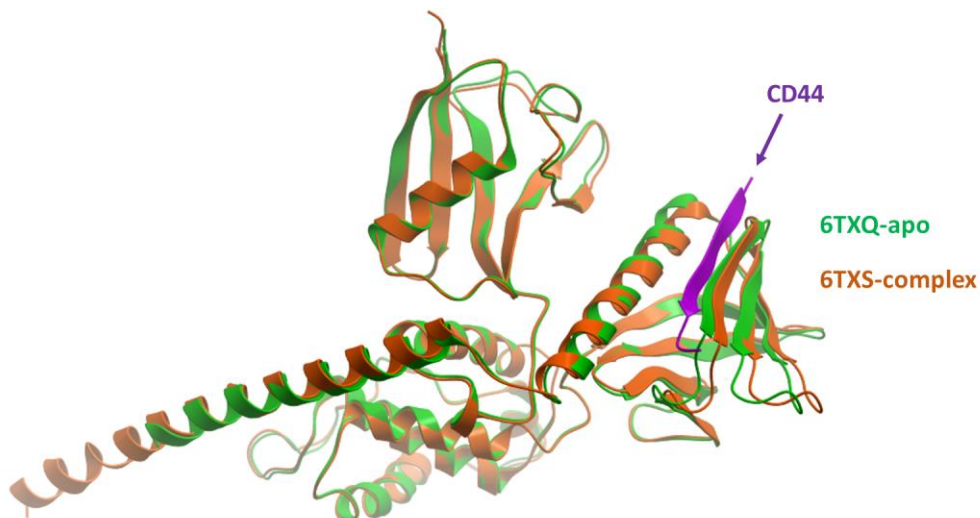

Figure 2. Ribbon representation of Moesin FERM domain structures. 6TXQ (green) is the apo structure, 6TXS (orange) is the structure of moesin in complex with a CD44-derived peptide (purple). The two protein structures closely align, with some movement of the β-sheet to accommodate CD44 as an additional strand.

Assays

This TEP focusses on a specific interaction of MSN with the cytoplasmic tail of the cell-surface receptor CD44.

TR-FRET assay

This assay is based on Time-resolved Fluorescence energy transfer. An anti-His antibody conjugated to europium serves as the fluorescence donor, bound to the His_6 tag of recombinant MSN. A peptide derived from CD44 (672-691), biotinylated at the N-terminal, is bound to streptavidin conjugated to XL665, a phycobiliprotein pigment. When the peptide is bound to MSN, excitation of the donor (ex 340 nm, em 620 nm) results in energy transfer to the acceptor dye (em. 664 nM). As a control, we performed a competition of the biotinylated peptide with increasing concentrations of an unmodified peptide; this results in a dose-dependent decrease of energy transfer. The assay has been put to use in a High-throughput screen performed at Emory University (Prof. Haian Fu) (**Fig. 3**).

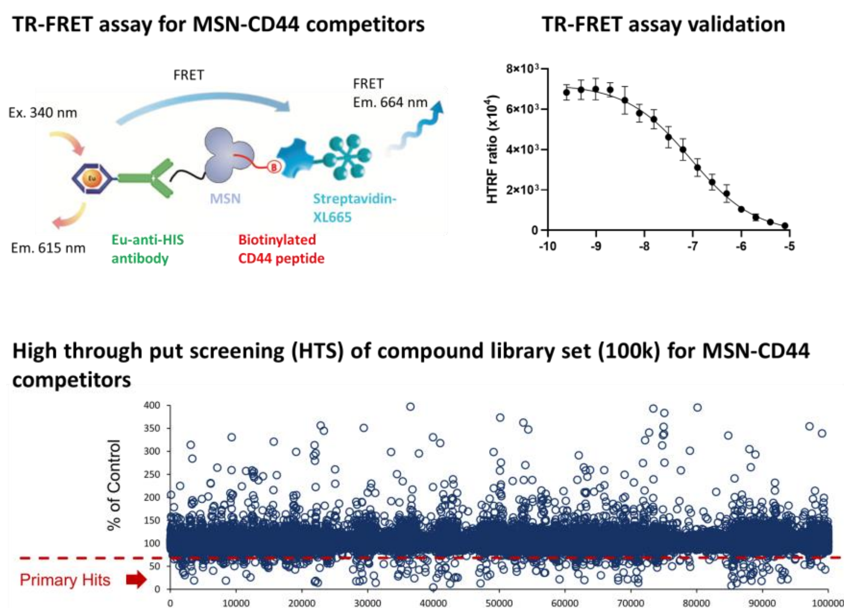

Figure 3. TR-FRET Binding assay of biotinylated CD44 peptide to His6-tagged MSN.

SPR

We have used Surface Plasmon Resonance (SPR) with IMAC sensors to measure the binding of the unlabelled peptide (and, potentially, small molecule inhibitors) to immobilized MSN protein. A trace of the peptide binding experiment is shown in **Fig 4**.

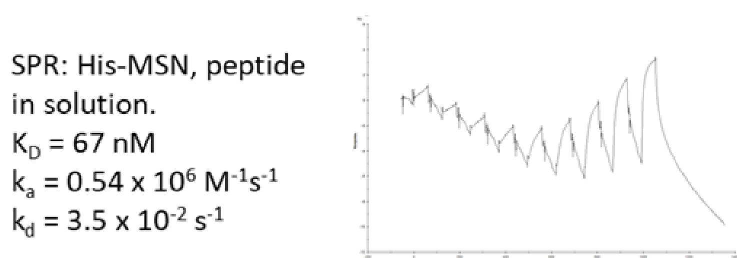

Figure 4. Surface Plasmon Resonance (Biacore) assay: His6-tagged moesin immobilized on chip, binding to increasing concentrations of CD44 peptide.

Chemical Matter

In this TEP, we present the CD44-derived peptide as chemical matter. The peptide binds at 67 nM (by SPR). Fig 5 shows the binding of an 8-residue CD44 peptide, QKKKLVIN; the leucine and isoleucine residues are buried

in a hydrophobic pocket (**Fig 5A**) which is conserved (**Fig 5B**) with another FERM protein, EPB41L3, which is the subject of a separate TEP.

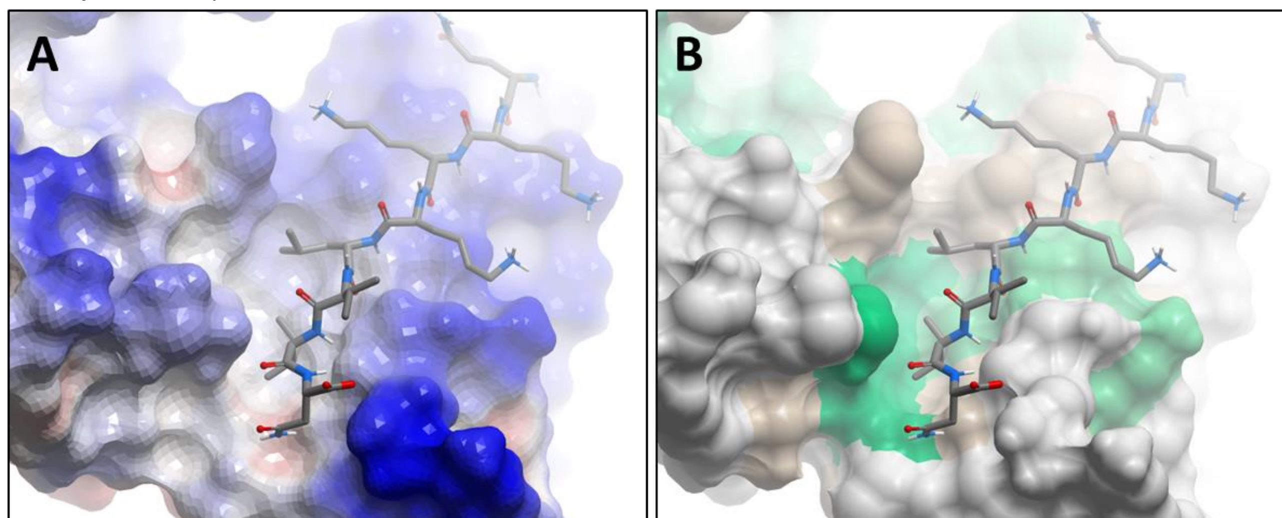

Figure 5. Close-up view of the peptide-binding pocket. A. surface potential representation. B. surface coloured by the degree of conservation with EPB41L3 (green-more conserved).

IMPORTANT: Please note that the existence of small molecules within this TEP indicates only that chemical matter might bind to the protein in potentially functionally relevant locations. The small molecule ligands are intended to be used as the basis for future chemistry optimisation to increase potency and selectivity and yield a chemical probe or lead series. As such, the molecules within this TEP should not be used as tools for functional studies of the protein, unless otherwise stated, as they are not sufficiently potent or well-characterised to be used in cellular studies.

Selectivity panel

A set of FERM domain proteins, Ezrin, Radixin, EPB41L3 and the distantly-related FERMT2, have been purified and will serve to test and improve the selectivity of compounds during probe development.

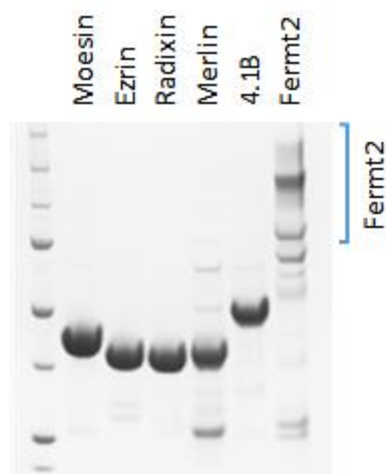

Figure 6. Purification of FERM-domain proteins to serve as a selectivity panel for the development of specific small-molecule ligands.

Future plans

Inhibition of the moesin-CD44 interaction is an ongoing project pursued as part of the TREAT-AD consortium (**https://treatad.org/**), involving scientists from Emory University, University of North Carolina, SAGE Bionetworks, and Washington University. The assay described here has been adapted to high-throughput screens and the development of small molecules is underway.

CONCLUSION

MSN emerged from system analysis as a probably key driver in a protein expression module that is closely correlated with Alzheimer's disease (AD). This module seems to be important in microglia. Complete knockout of moesin may cause pleiotropic effects, which would obscure its utility as a target for treatment of AD. This TEP focusses on a specific interaction of MSN with another AD-linked protein, CD44. The tools presented here provide a foundation for a medicinal chemistry program aiming to develop chemical probes to test the possible roles of MSN in AD.

TEP IMPACT

The assay developed in the TEP has been adapted to high-throughput screens and the development of small molecules is underway.

FUNDING INFORMATION

The work performed at the SGC has been funded by NIH grants 1RF1AG057443-01 and 1U54AG065187-01 and Wellcome grant [106169/ZZ14/Z].

ADDITIONAL INFORMATION

Structure Files

PDB ID	Structure Details
6TXQ	Structure of the FERM domain and helical linker of human moesin at 1.73 Å resolution.
6TXS	Structure of the FERM domain and helical linker of human moesin bound to a CD44 peptide at 2.20 Å resolution.

Materials and Methods

Proteins Expression and Purification

Cloning and expression plasmids

MSNA-c001 (FERM domain, 1-346). Vector: pNIC28-Bsa4 (13) (Genbank EF198106.1, Kanamycin -resistance, IPTG-inducible)

Protein sequence (Tag sequence underlined; * TEV protease cleavage site)

*MHHHHHHSSGVDLGTENLYFQ**SMPKTISVRVTTMDAELEFAIQPNTTGKQLFDQVVKTIGLREVWFFGLQYQDTKGFST
WLKLNKKVTAQDVRKESPLLFKFRAKFYPEDVSEELIQDITQRLFFLQVKEGILNDDIYCPPETAVLLASYAVQSKYGDFNKEVH
KSGYLAGDKLLPQRVLEQHKLNKDQWEERIQVWHEEHRGMLREDAVLEYLKIAQDLEMYGVNYFSIKNKKGSELWLGVDA
LGLNIYEQNDRLTPKIGFPWSEIRNISFNDKKFVIKPIDKKAPDFVFYAPRLRINKRILALCMGNHELYMRRRKPDTIEVQQMK
AQAREEKHQKQMERAMLENEKKKREMAEKEKEKIEREKEE
Predicted mass: 70372.9, after tag removal: 67907.3.

MSNA-c000 (full-length, 1-577)

Vector : pNIC28-Bsa4(13) (Genbank EF198106.1, Kanamycin -resistance, IPTG-inducible)

Protein sequence (Tag sequence underlined; * TEV protease cleavage site)

*MHHHHHHSSGVDLGTENLYFQ**SMPKTISVRVTTMDAELEFAIQPNTTGKQLFDQVVKTIGLREVWFFGLQYQDTKGFST
WLKLNKKVTAQDVRKESPLLFKFRAKFYPEDVSEELIQDITQRLFFLQVKEGILNDDIYCPPETAVLLASYAVQSKYGDFNKEVH
KSGYLAGDKLLPQRVLEQHKLNKDQWEERIQVWHEEHRGMLREDAVLEYLKIAQDLEMYGVNYFSIKNKKGSELWLGVDA
LGLNIYEQNDRLTPKIGFPWSEIRNISFNDKKFVIKPIDKKAPDFVFYAPRLRINKRILALCMGNHELYMRRRKPDTIEVQQMK
AQAREEKHQKQMERAMLENEKKKREMAEKEKEKIEREKEELMERLKQIEEQTKKAQQELEEQTRRALELEQERKRAQSEAE
KLAKERQEAEEAKEALLQASRDQKKTQEQLALEMAELTARISQLEMARQKKESEAVEWQQKAQMVQEDLEKTRAELKTAM
STPHVAEPAENEQDEQDENGAEASADLRADAMAKDRSEEERTTEAEKNERVQKHLKALTSELANARDESKKTANDMIHAE
NMRLGRDKYKTLRQIRQGNTKQRIDEFESM
Predicted mass: 43541.1, after tag removal: 41075.4.

Protein expression (Both constructs)

1. Transform the plasmids in the E. coli strain BL21(DE3)-R3-pRARE, a phage-resistant variant of Rosetta 2 (MSD). Plate on LB-agar plates containing kanamycin (50 µg/ml) and chloramphenicol, (34 µg/ml). Pick several colonies together and use to inoculate liquid cultures in the same medium; after overnight incubation, Store at -80°C after addition of 15% (v/v) glycerol.
2. For expression, inoculate an overnight culture of LB+kan +chlp at 37°C. Use 10 ml of the overnight culture to inoculate a 1L culture containing Terrific Broth (TB) with kanamycin only. The cultures were grown at 37°C with vigorous aeration in 2.5L Tunair flasks until reaching OD600 of between 1.5-3. Shift the cultures to 18°C; after 30 minutes, add 0.3 mM IPTG (from a 1.0M stock) and continue incubation for 16 hours at 18°C.

3. Harvest the cells by centrifugation (JLA8.1000 rotor, 4000 RPM, 25 min), Safely discard the medium and scrape the cell pellets with a rubber spatula into 50-ml tubes, which are frozen and kept at -80°C.

Purification

Buffers

- Lysis buffer: 50 mM HEPES (pH 7.5), 500 mM NaCl, 10 mM imidazole, 5% glycerol, 1 mM TCEP
- W30 Buffer: 50 mM HEPES (pH 7.5), 500 mM NaCl, 30 mM imidazole, 5% glycerol, 1 mM TCEP
- Elution Buffer (EB): 50 mM HEPES (pH 7.5), 500 mM NaCl, 300 mM imidazole, 5% glycerol, 1 mM TCEP.
- SEC buffer: 50 mM HEPES (pH 7.5), 500 mM NaCl, 10% glycerol, 1 mM TCEP
- Ni-sepharose beads, equilibrated in Lysis buffer.

Procedure

1. Thaw the cell pellet and suspend in 40 ml/Litre of culture of Lysis buffer. Lyse the cells by sonication on ice (20 min, 5s on, 10s off, 35% amplitude) with occasional stirring.
2. Centrifuge the lysate (25 min, 67000 g). Decant the supernatant carefully. Save 0.1 ml for analysis,
3. Add 0.6 ml of Ni-sepharose beads in 50-ml falcon tubes. Mix by rotation for 1 hr at <7°C.
4. Spin 700g/5 min/4°C. Decant lysate (FT) and wash beads with 100 ml LB. Spin, decant (W1), and wash pellets with 50 ml lysis buffer. Spin, decant (W2), and add 1 ml LB. Transfer beads to gravity column in cold room.
5. Wash column with 20 ml W30 (keep W30 eluate).
6. Elute protein with 3x 10 ml EB (E1, E2, E3).
7. If the protein is to be used for crystallization, the N-terminal tag is cleaved using TEV protease. Skip this step if the tag is to be retained. The protein is combined in a dialysis tube with His-tagged TEV protease at a 1:20 mass ration (TEV : MSN) and placed in 1-2 litres of SEC buffer, at 4°C overnight.

   Then, pass the protein solution through a Ni-Sepharose column equilibrated with SEC buffer using gravity flow. Wash the beads successively with 10 ml each of Lysis buffer, W30 buffer, and Elution buffer and collect each effluent. Analyze by gel electrophoresis and/or intact MS to locate the cleaved protein.
8. Concentrate the protein (cleaved or tagged) to <1 ml using a centrifugal concentrator with MWCO of 30 kDa.
9. Purify the protein further by Size-exclusion chromatography (SEC) on a HiLoad Superdex S200 HR 16/60 column in SEC buffer at 1 ml/min. Identify the fractions containing pure MSN protein by SDS-PAGE, pool and concentrates as required. Snap-freeze in thin-walled PCR tubes in liquid N2, and store at -80°C.

Crystallisation and structure determination

6TXQ

The protein was mixed 1:1.1 with CD44 peptide (SRRRCGQKKKLVINSGNGAVEDY). The protein (13.6 mg/ml) was then crystallized in a sitting drop by mixing with 0.1-0.2M ammonium acetate, 0.1M tris pH 8.5, 32-34% propan-2-ol and incubating at 20°C. One or more rounds of seeding were required to obtain well-diffracting crystals; the peptide could not be seen in the refined structure.

Data was collected on beamline I03 at Diamond Light Source to a resolution of 1.73 Å. The data was processed using Dials, scaled with Aimless and the structure determined by molecular replacement with Phaser using the earlier moesin structure **1E5W** as a model. The structure was refined with Refmac to final R / R_{free} of 20.0%/23.3%.

6TXS

Moesin was mixed with a shorter CD44 peptide (QKKKLVIN) and crystallized in
0.2M potassium thiocyanate, 0.1M bis-tris pH 7.0, 10% ethylene glycol, 20% PEG3350 at 20°C.

Data was collected on beamline I04 at Diamond Light Source to a resolution of 2.20 Å. The data was processed using Dials, scaled with Aimless and the structure determined by molecular replacement with Phaser using the earlier moesin structure **1E5W** as a model. The structure was refined with Refmac to final R / R_{free} of 23.3%/27.3%.

Assays

Reagents

Protein: MSNA-c001 (20 mg/ml; 487 µM)
Peptide b-CD44(672-691): biotin-SRRRCGQKKKLVINSGNGAVEDY (10 mM)
Competitor peptide CD44(672-691): SRRRCGQKKKLVINSGNGAVEDY (10 mM)
Assay Buffer (AB): 25 mM HEPES (pH 7.5), 200 mM NaCl, 0.1% BSA, 0.05% Tween-20. Filter.
Donor Reagent: LANCE Eu-W1024 Anti-6xHis (Perkin Elmer AD0205; 10ug) at 0.625 µM.
Acceptor Reagent: Streptavidin-XL665 (Cisbio 610SAXLF; 1000 tests) at 200 ug/ml or 3.3 µM.

Procedure for titration of competitor, unlabelled peptide

Perform assay in triplicates in black 384-well plates:

1. Dilute protein to 4x concentration in AB (20 nM; 1/25000).
2. For competition experiments: Dilute CD44(672-691) to 4x concentration in AB. Do a 15-point dilution series from 8 µM to 0.5 nM final, and a no-peptide control.
3. Dilute b-CD44(672-691) to 4x concentration in AB (240 nM; 1/40000).
4. Prepare a mix of Donor and Acceptor to 4x concentration in AB: Eu-anti-6His to 1 nM final, SA-XL665 to 10 nM final.
   Note: Do not refreeze SA-XL665. Discard leftovers.
5. Add 5 ul of MSN-c001 to wells.
6. Add 5 ul of CD44(672-691) to wells. Incubate 30 min at RT.
7. Add 5 ul of b-CD44(672-691) to wells. Incubate 30 min at RT.
5. Add 5 ul of diluted donor and acceptor (4x). Spin plate. Incubate for 1 h.
7. Read on PheraStar FSX at 620 and 660 nm.

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