Subcellular localization of the immunoregulatory protein MACIR by TNPO1 is mediated by a C terminal PY motif

Gavin McGauran[1], Emma Dorris[2], Razvan Borza[3], Niamh Morgan[2], Denis C. Shields[2], David Matallanas[2,3], Anthony G. Wilson[2], David J. O'Connell[1,4]

1. School of Biomolecular & Biomedical Science, University College Dublin, Belfield, Dublin 4, Ireland.
2. School of Medicine & Medical Science, University College Dublin, Belfield, Dublin 4, Ireland.
3. Systems Biology Ireland, University College Dublin, Belfield, Dublin 4, Ireland.
4. BEACON Bioeconomy Research Centre, University College Dublin, Belfield, Dublin 4, Ireland.
Corresponding author e-mail address: david.oconnell@ucd.ie

Abstract

The macrophage immunometabolism regulator gene (MACIR) encodes a 206 amino acid protein lacking homology to any characterized protein sequence. MACIR expression is associated with severity of autoimmune disease pathology and regulates macrophage and fibroblast biology. To identify specific subcellular interactors of this protein with pull down and mass spectrometry we prepared whole cell proteins using a membrane solubilization buffer that enriched MACIR interacting proteins from cell organelles not seen with RIPA cell lysis buffer. A fragment complementation-based pull down approach identified 63 significant interactors including those with the nuclear import protein Transportin-1 (TNPO1) and UNC119 homologs A and B that were then validated by immunoprecipitation. Analysis of mutations in two candidate recognition motifs in the MACIR amino acid sequence confirmed TNPO1 binds via a C terminal PY motif (aa99-119). Understanding the mechanism controlling this interaction is a priority with respect to resolving inflammation in autoimmune disease.

Introduction

Expression of MACIR has been shown to have a significant impact on the immunometabolism of macrophages and fibroblasts in autoimmune conditions. It is highly expressed in the synovium of rheumatoid arthritis patients compared with control synovial tissue where it is predominately expressed by synovial fibroblasts and macrophage and loss of expression contributes to the pathology of inflammatory arthritis in vivo, with inhibition of MACIR in the collagen-induced arthritis model leading to accentuated joint inflammation and tissue damage (1). MACIR expression has been shown to regulate macrophage phenotype. Elevated expression of MACIR occurs in macrophages in response to anti-inflammatory stimulants and is differentially turned over in inflammatory and anti-inflammatory macrophages. Inhibition of expression reduces wound healing/repair–associated functions of macrophages, reduces signaling required for resolution of inflammation, and decreases secretion of anti-inflammatory mediators (2). Comparative database analysis of the primary amino acid sequence of this protein fails to yield useful information aiding prediction of the function lacking significant homology with any related human protein and with secondary structure prediction analysis suggesting the protein is disordered with renderings of the sequence lacking structural domains or significant α-helical or β-sheet content. Identification of function of this protein in the cell then requires the careful identification of the protein:protein interaction networks it participates in and the fine mapping of interactions that regulate the localisation of the protein that may impact on the immunometabolism phenotypes. We have previously developed a calcium-dependent fragment complementation-based affinity pull down system that uses the highly specific recognition between two fragments, EF1 & EF2, of a small calcium binding protein to pull down overexpressed kinase proteins from HEK293T cells and 7 transmembrane proteins from *Escherichia coli* membranes using a membrane solubilizing buffer (MSB) that contains high

concentrations of glycerol and a cocktail of detergents that disrupt cellular and intracellular membranes and stabilises membrane proteins in detergent micelles (3,4). In this study we employ this approach to identify MACIR interacting proteins from all compartments in the cell To further increase the sensitivity of the analysis protein complexes were eluted from the affinity matrix through calcium chelation prior to digestion and MS analysis improving the signal to noise ratio and identification of significant interactions.

Results

Affinity pull down of EF1-MACIR in RIPA & MSB

Western blot analysis of EF2 agarose pull down of EF1-MACIR from normalised total protein lysates shows that substantially more protein is released for pull down in the lysates prepared with MSB buffer than in those prepared with RIPA buffer and the amount of pulled down protein is also significantly enhanced (Figure 1A i & ii). The empty vector control lysate and pull down for MSB is shown for comparison (Figure 1A iii). Mass spectrometry analysis of the pulled down interacting proteins showed that while 9 proteins were identified as significant interactors in RIPA buffer pull downs, these 9 proteins and 54 others were identified using MSB prepared lysates (Figure 1B, Table S1). The top 20 interactors identified by mass spectrometry and quantitative analysis ranked by LFQ ratio over empty vector control signal, showed that in addition to the cytosolic proteins UNC119B and 14-3-3 proteins (YWAH, YWHAE and YWAHG) identified in both buffer prepared lysates, proteins from nuclear and mitochondrial compartments were identified in the MSB samples.

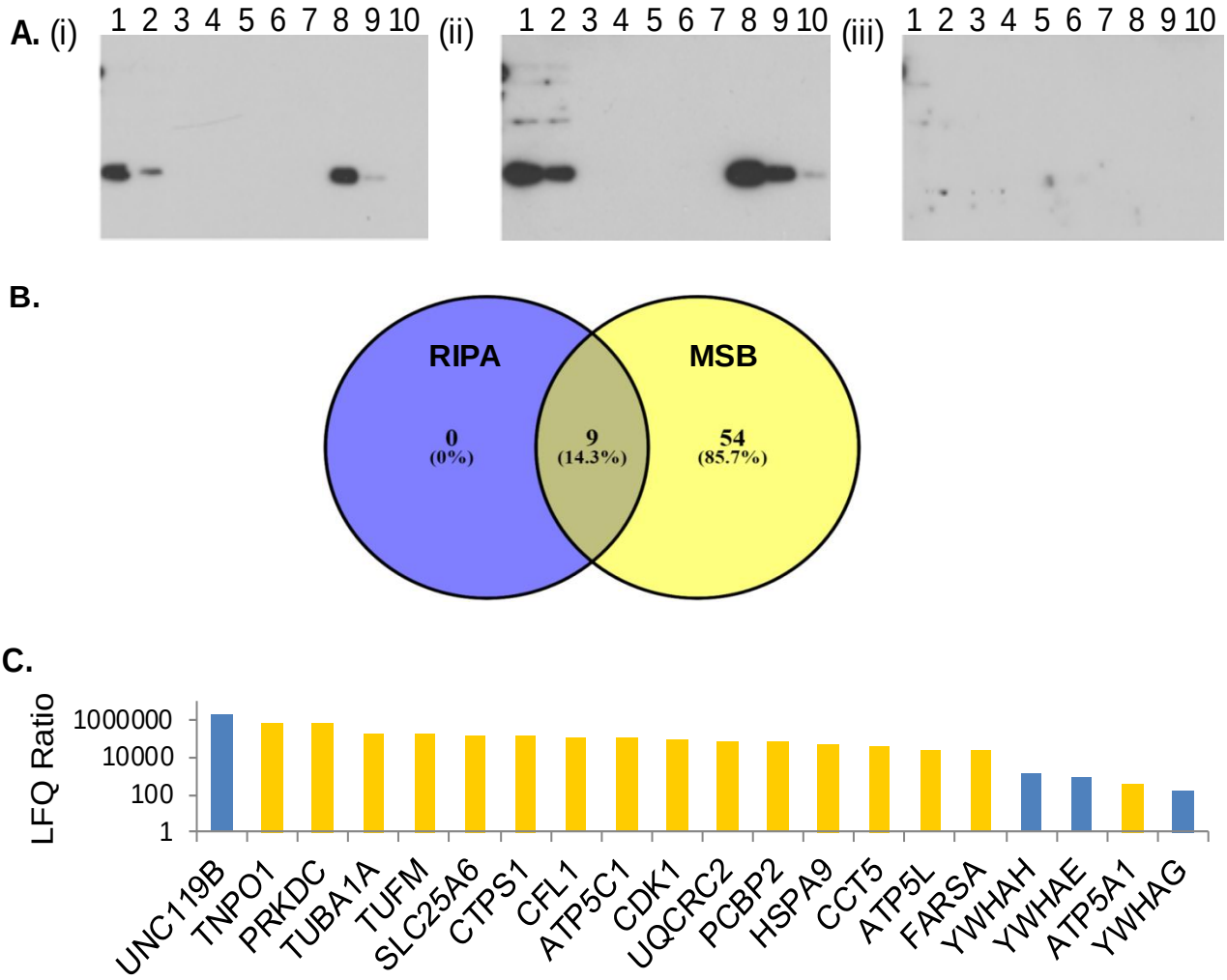


Figure 1. Proteomic analysis of C5orf30 binding interactions. (A) EF2 agarose pull down and elution of (i) EF1-tagged MACIR with RIPA buffer (ii) EF1-tagged MACIR with MSB (iii) EF1-tagged empty vector control with MSB (1:Input, 2:Unbound, 3-7:washes, 8-10:Eluates), probed with anti-EF1 polyclonal antibody. (B) Venn diagram of significant interactors identified with mass spectrometry in RIPA and MSB samples. (C) Top 20 C5orf30 interactors ranked by LFQ intensity ratio with proteins identified in MSB alone identified in yellow and in both buffers in blue.

Immunoprecipitation validation of TNPO1 & UNC119 interactions with FLAG-MACIR

Immunoprecipitation of overexpressed FLAG-tagged MACIR with anti-FLAG M2 sepharose beads confirmed the specific co-precipitation of TNPO1 (Figure 2A i), UNC119A and B (Fig 2A ii) from HEK293T cell lysates. Anti-C5orf30 and anti-FLAG antibody western blots confirmed specificity (Fig 2A ii). Given the

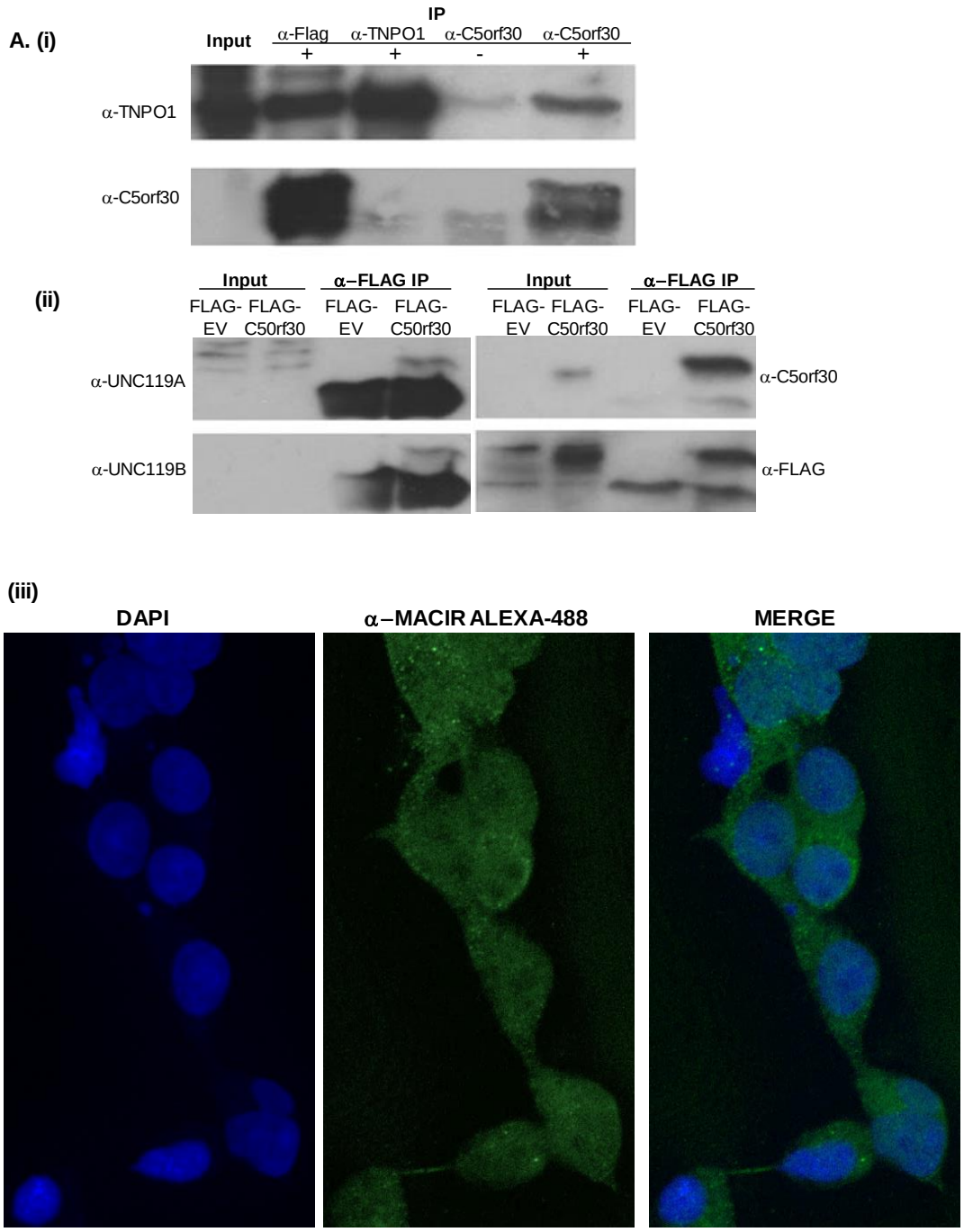


Figure 2. Validation of MACIR binding interactions and identification of nuclear localization of endogenous MACIR in HEK293T cells. (A) (i) Immunoprecipitation (IP) of FLAG-C5orf30, TNPO1 and C5orf30 and immunodetection with anti-TNPO1 and anti-C5orf30 antibodies (ii) anti-FLAG IP of FLAG-C5orf30 and probing with anti-UNC119A and UNC119B antibodies (iii) anti-FLAG IP and probing with anti-C5orf30 and anti-FLAG antibody controls.

TNPO1 has two candidate consensus binding sites in the primary sequence of C5orf30 (Fig 2A (iii)), previously described in other substrate proteins (12,13). We aimed to identify the specific site in C5orf30 through mutation of proline/tyrosine (PY) residues to alanine/glutamine with analysis of TNPO1 co-immunoprecipitation. FLAG-tagged C5orf30 P116A/Y117Q and C5orf30 P142A/Y143Q mutants showed that binding to the C5orf30 P116/Y117Q mutant is lost while binding to P142A/Y143Q mutant is maintained, confirming that TNPO1 binds to C5orf30 via the recognition motif [R/K/H-X(2-5)-P-Y], following a hydrophobic motif (f-G/A/S-f-f, where f is a hydrophobic residue) (aa98-117).

A.

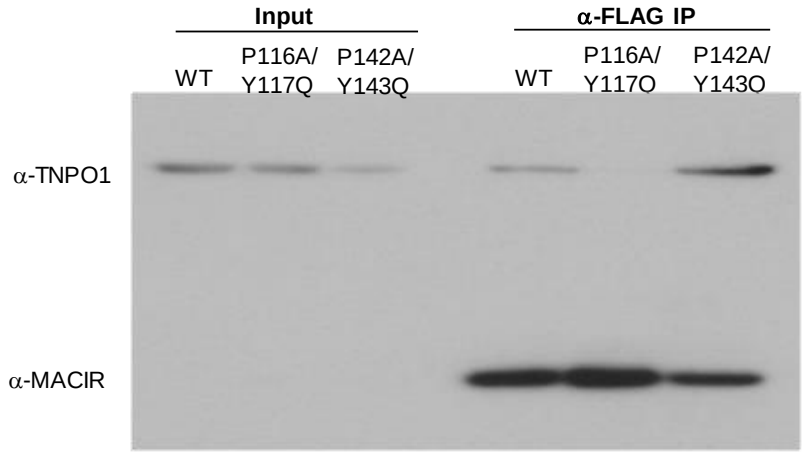
```
        10         20         30         40         50         60
MEVDINGESR STLTTLPFPG AEANSPGKAE AEKPRCSSTP CSPMRRTVSG YQILHMDSNY

        70         80         90        100        110        120
LVGFTTGEEL LKLAQKCTGG EESKAEAMPS LRSKQLDAGL ARSSRLYKTR SRYYQPYEIP

       130        140        150        160        170        180
AVNGRRRRRM PSSGDKCTKS LPYEPYKALH GPLPLCLLKG KRAHSKSLDY LNLDKMIKEP

       190        200    206
ADTEVLQYQL QHLTLRGDRV FARNNT
```

B. (i)



(ii)

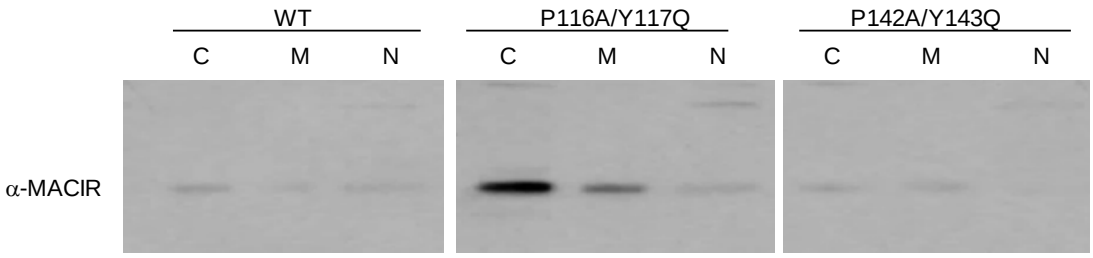


Figure 3. Mutational analysis of candidate TNPO1 recognition sites in MACIR. (A) Candidate transportin recognition sites studied with specific residues identified in the consensus sequence motif in bold and mutated PY residues shown in red. (C) Analysis of C5orf30 P116A/Y117Q and P142A/Y143Q mutant protein binding to Transportin (i) Anti-

FLAG IP of FLAG-C5orf30 and probing with anti-TNPO1 and anti-C5orf30 (ii) Probing of cell fractions C (cytosol), M (membrane) and N (nuclear) with anti-C5orf30.

We also identified an increase in cytosolic levels of the P116A/Y117Q mutant protein appearing to indicate a specific dysregulation of the subcellular localization of the P116A/Y117Q mutant (Fig 2B (ii)).

Discussion

C5orf30 was expressed with a 43 amino acid EF1 tag in HEK293T cells and a 37 amino acid EF2 affinity ligand coupled to agarose was used in calcium-dependent fragment complementation-based pull down, using either RIPA buffer with 150 mM NaCl and detergents Deoxycholic acid (1%) and NP-40 (1%) or membrane solubilisation buffer (MSB) we previously employed in the pull down of EF1-tagged cannabinoid receptor CB_2 from *Escherichia coli* membranes (3). MSB Proteins were eluted from the agarose using calcium chelation to dissociate the EF1-EF2 complex and prepared for mass spectrometry with a Nanoflow Ultimate 3000 LC and Q-Exactive instrument (Thermo Fisher Scientific). MS data was analyzed with MaxQuant software (version 1.5.3.30) dividing the average LFQ intensities found in the EF1-tagged C5orf30 samples by those identified in the EF1-tagged empty vector control. An LFQ intensity ratio greater than 2 with a p-value of less than 0.05 following t-test analysis was considered significant (4).

Figure 1A shows affinity pull down of EF1-C5orf30 with EF2-agarose in both buffer conditions with anti-EF1 western blotting of PAGE separated protein complexes. The empty vector control confirmed the specificity of the pull down approach (Fig 1A (i)), while total signal for EF1-tagged C5orf30 pulled down from RIPA buffer was lower than that from the MSB sample (Fig 1A (ii) & (iii)). Specific interactors of C5orf30 were significantly enriched in the membrane solubilised preparation with 63/255 proteins scoring above the threshold compared with 9 significant proteins in the RIPA buffer preparation (Table S1). The highest ranked interactions in this dataset were identified in MSB samples where nuclear and mitochondrial proteins dominated (Fig 1B) with UNC119B identified in both buffers. 14-3-3 protein isoforms were also identified in both buffers and these may interact with C5orf30 via predicted binding motifs at known phosphorylation sites (S38 & S49) in the C5orf30 protein (5,6). C5orf30 interactor CDK1 phosphorylates the transcription factor FOXO1 disrupting binding to 14-3-3 proteins promoting nuclear accumulation in neuronal cells (7) and 14-3-3 proteins regulate TNPO1 subcellular localization in a phospho-specific manner (8). While the interaction with TNPO1 has not been reported previously, the interaction with UNC119 proteins confirms a reported interaction in HEK293T cells (9). UNC119B is required for localization of certain myristoylated proteins (10), while UNC119A cargo localization regulates T-cell and macrophage activities (11).

Thus, C5orf30 interacts specifically with regulators of subcellular localization and nuclear and mitochondrial proteins. Characterization of these interactions will determine what function this protein may have in subcellular compartments. Therapeutic agents that help to block localization to particular sub-compartments could hold promise in the treatment of inflammatory diseases such as rheumatoid arthritis.

Materials and Methods

Chemicals and reagents.

Primers were purchased from MWG Eurofins Genomics. Restriction enzymes were obtained from New England Biolabs. SulfoLink coupling resin was from Thermo Scientific and EF2 peptide conjugation was performed in-house. Monoclonal antibody C5orf30 was from Sigma and polyclonal Calbindin D9k (H-60) against EF1 peptide was from Santa Cruz. Cholesteryl hemisuccinate Tris salt (CHS) and detergents 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and n-dodecyl-β-D-maltoside (DDM) were bought from Anatrace. In all cases, sequencing was done by MWG Eurofins Genomics.

Cloning.

The C5orf30 gene for the constructs EF1-N-C5orf30 and EF1-C-C5orf30 were prepared by PCR using a pcDNA3.1(+) with the gene for C5orf30 as the template. The forward primer was designed as follows: 5'-GGATCCGAAGTCGATATTAATG-3' with a BamHI restriction site included to allow for cloning into the expression vector. The reverse primer was designed as follows: 5'-AAGCTTTGTATTATTCCTAGC-3' with a HindIII restriction site included for cloning into the expression vector.

Cell culture and DNA transfection.

HEK293T cells were seeded at 800,000 cells per 10 cm3 dish with Dulbecco's Modified Eagle Media containing 10% foetal bovine serum (FBS) supplemented with penicillin and streptomycin. Cells were grown to 60-80% confluency prior to transfection. Prior to transfection, media was removed, and cells were washed with Phosphate Buffered Saline (PBS). 10% FBS containing media supplemented without penicillin and streptomycin was added and cells were incubated at 37°C. Transfection was performed as per manufacturer's protocol using 5 µg DNA and 2.4µl TransIT-2020 per µg of DNA made up in 1ml serum-free media. The TransIT-2020 reagent:DNA complex mix was incubated for 20 minutes at room temperature. Samples were then added drop-wise to pre-warmed media containing 10% FBS without penicillin and streptomycin and agitated. Cells were incubated at 37°C for 24 hours.

Harvesting and lysis of cells:.

Media was discarded and cells were washed twice with PBS. Cells were gently harvested in 750 µl of RIPA (20 mM Tris-HCl, 150 mM NaCl, 1% Deoxycholic acid, 1% NP-40, 2 mM CaCl2, 2.5 mM Sodium Pyrophosphate, 1 mM β-Glycerophosphate, 2 mM Sodium Fluoride, 1 mM Sodium Orthovanadate and Protease Inhibitors (Roche)) or high detergent buffer (50 mM Tris-HCl, 200 mM NaCl, 0.5% CHAPS, 0.1% CHS, 1% DDM, 30% Glycerol, 2 mM

CaCl2, pH 7.5 and Protease Inhibitors (Roche)) per 10 cm^3 dish using a cell scraper. Cell pellets were mechanically disrupted via aspiration through a 21-gauge needle in the lysis buffer. Cells were centrifuged at 10,000 x g for 10 minutes at 4°C, retaining the supernatant. The total protein concentration was determined using bicinchoninic acid (BCA) assay (Pierce) according to the manufacturer's protocol.

Pull-down of EF1-C5orf30 using EF2 agarose beads

EF2 agarose and control beads were collected by centrifugation at 1,000 x g for 1 minute at 4°C. Beads were washed five times in 1 ml lysis buffer depending on the protein being purified. 1.5 mg of lysate samples were precleared by incubation at 4°C for 1 hour using non-conjugated beads. The agarose beads were collected by centrifugation at 5,000 x g at 4°C for 1 minute and the supernatant was retained. The precleared lysate was incubated overnight at 4°C with EF2 agarose beads or non-conjugated beads as a negative control. Following incubation, samples were collected by centrifugation at 5,000 x g for 1 minute at 4°C. Samples were washed five times in lysis buffer. Following wash procedure, EF1-C5orf30 was eluted from the EF2-agarose beads using elution buffer by resuspension and allowing samples to incubate for 5 minutes at room temperature. Beads were collected by centrifugation at 5,000 x g for 1 minute and the supernatant was collected. Elution was repeated 5 times. Samples were stored at -80°C prior to further analysis.

Immunoprecipitation validation:

0.5-1.5 mg cell lysates were used for the immunoprecipitation procedure. Protein G agarose beads (Santa Cruz Biotechnology) or FLAG beads (Sigma) were collected by centrifugation at 10,000 x g for 30 seconds and washed three times in lysis buffer. 30 µl of FLAG beads or 30 µl protein G agarose beads were used per 0.5-1.5 mg of sample. The appropriate amount of antibody and equivalent amount of rabbit or mouse IgG control antibody was used per sample as per instructed by the manufactures data sheet. Samples were incubated on rotation for 1-4 hours at 4˚C. After incubation samples were washed with lysis buffer three

times. The beads containing the protein of interest were resuspended in 2x laemmli loading buffer and boiled for 5 minutes. Protein samples were analysed by Western blotting probing with the appropriate antibody.

Mass Spectrometry Sample preparation:

Samples were mixed 400 µl of 8 M urea in 0.1 M Tris-HCl, pH 8.9 using a clean eppendorf. Samples are then added Vivacon spin ultracentrifugation units and centrifugated at 14,00 x g for 15 minutes at 20°C for 15 minutes. 200 µl of 8 M urea in 0.1 M Tris-HCl, pH 8.9 is added to the filter unit and it is centrifugated again at 14,000 x g for 15 minutes at 20°C. The flow through waste is discarded from the collection tube. 100 µl of 0.05 M iodoacetamide prepared of 8 M urea in 0.1 M Tris-HCl, pH 8.9 is added to the filter units. The samples are mixed for 1 minute at 600 rpm in a thermo-mixer and incubated in darkness for 20 minutes. Following the 20 minutes, the filter units are centrifuged at 14,000 x g for 10 minutes at 20°C. The filter units are washed twice with 100 µl of 8 M urea in 0.1 M Tris-HCl, pH 8.9 and the flow-through is discarded from the collection tube. The filter units are then washed twice with 100 µl of 0.05 M NH4HCO3. The filter units are transferred to new collection tubes. 40 µl of 0.05 M NH4HCO3 with trypsin (enzyme to protein ratio 1:50) was added to the filter units mixed at 600 rpm in a thermo-mixer for 1 minute. Then these were incubated on a wet chamber overnight at 37°C. The next day filter units are centrifugated at 14,000 x g for 15 minutes at 20°C to collect digest peptides. The filter units are washed once with 40 µl of 0.05 M NH4HCO3 to make a total volume of 80 µl per sample. The samples are measured by NanoDrop™2000 to determine a concentration.

ZipTip activation:
c18 ZipTips were first equilibrated by aspirating 10 µL wetting solution (50% acetonitrile in 0.1% trifluoroacetic acid), the waste was discarded. This step was repeated once more before centrifugation of the samples by 10,000 rpm for 5 min. Protein from the tryptic digest supernatants was aspirated and dispensed a total of 3-7 times for simple mixtures, or up to 10 times for more complex samples. Washing solution (0.1% trifluoroacetic acid in ddH2O)

was aspirated and dispensed twice through the ZipTip. 10 µL of elution solution (50% acetonitrile in 0.1% trifluoroacetic acid) was dispensed into a clean eppendorf followed by aspiration and dispensing of the sample through the ZipTip at least 3 times without the introduction of air. Samples were evaporated on a CentriVap Concentrator for 10-15 min, allowing for a small volume (6 µL) to remain. The sample was finally resuspended in 20 µL of 0.1% formic acid and centrifuged for 5 min at 15,000 rpm. 16 µL of this sample was transferred to a clean mass spectrometry vial for analysis.

Mass spectrometry:

Mass spectrometry was performed using a Ultimate3000 RSLC system that was coupled to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific). Following tryptic digest, the peptides were loaded onto a nano-trap column (300 µm i.d x 5mm precolumn that was packed with Acclaim PepMap100 C18, 5 µm, 100 Å; Thermo Scientific) running at a flow rate of 30 µl/min in 0.1% trifluoroacetic acid made up in HPLC water. The peptides were eluted and separated on the analytical column (75 µm i.d. × 25 cm, Acclaim PepMap RSLC C18, 2µm, 100 Å; Thermo Scientific) after 3 minutes by a linear gradient from 2% to 30% of buffer B (80% acetonitrile and 0.08% formic acid in HPLC water) in buffer A (2% acetonitrile and 0.1% formic acid in HPLC water) using a flow rate if 300 nl/min over 150 minutes. The remaining peptides were eluted using a short gradient from 30% to 95% in buffer B for 10 minutes. The mass spectrometry parameters were as follows: for full mass spectrometry spectra, the scan range was 335-1500 with a resolution of 120,000 at m/z=200. MS/MS acquisition was performed using top speed mode with 3 seconds cycle time. Maximum injection time was 50 ms. The AGC target was set to 400,000, and the isolation window was 1 m/z. Positive Ions with charge states 2-7 were sequentially fragmented by higher energy collisional dissociation. The dynamic exclusion duration was set at 60 seconds and the lock mass option was activated and set to a background signal with a mass of 445.12002.

Analysis of mass spectrometry data:

The analysis was performed using MaxQuant (version 1.5.3.30). Trypsin was set to be the digesting enzyme with maximal 2 missed cleavages. Cysteine carbamidomethylation was set for fixed modifications, and oxidation of methionine and N-thermal acetylation were specified as variable modifications. The data was then analysed with the minimum ratio count of 2. The first search peptide was set to 20, the main search peptide tolerance to 5 ppm and the 2re-quantify2 option was selected. For protein and peptide identification the Human subset of the SwissProt database (Release 2015_12) was used and the contaminants were detected using the MaxQuant contaminant search. A minimum peptide number of 1 and a minimum of 6 amino acids was tolerated. Unique and razor peptides were used for quantification. The match between run option was enabled with a match time window of 0.7 minutes and an alignment window of 20 minutes. A statistical analysis of the cut off ratio and two-sample t-test was done to determine interactors.

Acknowledgements

We thank the Health Research Board & Arthritis Ireland and Science Foundation Ireland award 16/RC/3998 (BEACON).

1. Muthana M, Hawtree S, Wilshaw A, Linehan E, Roberts H, Khetan S, Adeleke G, Wright F, Akil M, Fearon U, Veale D, Ciani B, Wilson AG. C5orf30 is a negative regulator of tissue damage in rheumatoid arthritis. Proc Natl Acad Sci U S A. 2015 Sep 15;112(37):11618-23.
2. Dorris ER, Tazzyman SJ, Moylett J, Ramamoorthi N, Hackney J, Townsend M, Muthana M, Lewis MJ, Pitzalis C, Wilson AG. The Autoimmune Susceptibility Gene C5orf30 Regulates Macrophage-Mediated Resolution of Inflammation. J Immunol. 2019 Feb 15;202(4):1069-1078.
3. Mhurchú NN, Zoubak L, McGauran G, Linse S, Yeliseev A, O'Connell DJ. Simplifying G Protein-Coupled Receptor Isolation with a Calcium-Dependent Fragment Complementation Affinity System. Biochemistry. 2018 Jul 31;57(30):4383-4390.
4. Turriziani B, Garcia-Munoz A, Pilkington R, Raso C, Kolch W, von Kriegsheim A. On-beads digestion in conjunction with data-dependent mass spectrometry: a shortcut to quantitative and dynamic interaction proteomics. Biology (Basel). 2014 Apr 16;3(2):320-32.
5. Madeira F, Tinti M, Murugesan G, Berrett E, Stafford M, Toth R, Cole C, MacKintosh C, Barton GJ. 14-3-3-Pred: improved methods to predict 14-3-3-binding phosphopeptides. Bioinformatics. 2015 15;31(14):2276-83.
6. Hornbeck PV, Kornhauser JM, Tkachev S, Zhang B, Skrzypek E, Murray B, Latham V, Sullivan M. PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse. Nucleic Acids Res. 2012;40(Database issue):D261-70.
7. Yuan Z, Becker EB, Merlo P, Yamada T, DiBacco S, Konishi Y, Schaefer EM, Bonni A. Activation of FOXO1 by Cdk1 in cycling cells and postmitotic neurons. Science. 2008 Mar 21;319(5870):1665-8.
81. Faul C, Hüttelmaier S, Oh J, Hachet V, Singer RH, Mundel P. Promotion of importin alpha-mediated nuclear import by the phosphorylation-dependent binding of cargo protein to 14-3-3. J Cell Biol. 2005 May 9;169(3):415-24.

9. Huttlin EL, Bruckner RJ, Paulo JA, Cannon JR, Ting L, Baltier K, Colby G, Gebreab F, Gygi MP, Parzen H, Szpyt J, Tam S, Zarraga G, Pontano-Vaites L, Swarup S, White AE, Schweppe DK, Rad R, Erickson BK, Obar RA, Guruharsha KG, Li K, Artavanis-Tsakonas S, Gygi SP, Harper JW. Architecture of the human interactome defines protein communities and disease networks. Nature 2017 May 25;545(7655):505-509.
10. Wright KJ, Baye LM, Olivier-Mason A, Mukhopadhyay S, Sang L, Kwong M, Wang W, Pretorius PR, Sheffield VC, Sengupta P, Slusarski DC, Jackson PK. An ARL3-UNC119-RP2 GTPase cycle targets myristoylated NPHP3 to the primary cilium. Genes Dev. 2011 25:2347-2360.
11. Gorska MM1, Goplen N, Liang Q, Alam R. Uncoordinated 119 preferentially induces Th2 differentiation and promotes the development of asthma. J Immunol. 2010 15;184(8):4488-96.
12. Lee BJ, Cansizoglu AE, Süel KE, Louis TH, Zhang Z, Chook YM. Rules for nuclear localization sequence recognition by karyopherin beta 2. Cell. 2006 Aug 11;126(3):543-58.
13. Imasaki T, Shimizu T, Hashimoto H, Hidaka Y, Kose S, Imamoto N, Yamada M, Sato M. Structural basis for substrate recognition and dissociation by human transportin 1. Mol Cell. 2007 Oct 12;28(1):57-67.

Figure 1. Proteomic analysis of C5orf30 binding interactions. (A) EF2 agarose pull down and elution of (i) EF1-tagged empty vector control (1:Input, 2:Unbound, 3-7:washes, 8-10:Eluates) (ii) EF1-tagged C5orf30 with RIPA buffer and (iii) EF1-tagged C5orf30 with MSB, probed with anti-EF1 polyclonal antibody. (B) Top 20 C5orf30 interactors ranked by LFQ intensity ratio with proteins identified in MSB alone identified in red and in both buffers in blue.

Figure 2. Validation of human C5orf30 binding interactions. (A) (i) Immunoprecipitation (IP) of FLAG-C5orf30, TNPO1 and C5orf30 and immunodetection with anti-TNPO1 and anti-C5orf30 antibodies (ii) anti-FLAG IP of FLAG-C5orf30 and probing with anti-UNC119A and UNC119B antibodies (iii) anti-FLAG IP and probing with anti-C5orf30 and anti-FLAG antibody controls.

Figure 3. Mutational analysis of candidate TNPO1 recognition sites in MACIR. (A) Candidate transportin recognition sites studied with specific residues identified in the consensus sequence motif in bold and mutated PY residues shown in red. (C) Analysis of C5orf30 P116A/Y117Q and P142A/Y143Q mutant protein binding to Transportin (i) Anti-FLAG IP of FLAG-C5orf30 and probing with anti-TNPO1 and anti-C5orf30 (ii) Probing of cell fractions C (cytosol), M (membrane) and N (nuclear) with anti-C5orf30.