Human Kalirin/RAC1 GEF/GTPase complex

A Target Enabling Package (TEP)

Gene ID / UniProt ID / EC	KALRN: 8997/ P97924 RAC1: 5879 / P63000
Target Nominator	SGC Internal Nomination
SGC Authors	Janine L. Gray, Carmen Jimenez Antunez, Tobias Krojer, Michael Fairhead, Nicola Burgess-Brown, Frank von Delft and Paul E. Brennan
Collaborating Authors	Roger Goody[1], Romain Talon[2]
Target PI	Paul Brennan (SGC Oxford)
Therapeutic Area(s)	Neurological Disorders
Disease Relevance	KALRN has been linked to neurological disorders
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USEFUL LINKS

GeneCards	Open Targets	OMIM	IMPC	ChEMBL	Genomics england
KALRN / RAC1	KALRN / RAC1	KALRN / RAC1	KALRN / RAC1	KALRN / RAC1	RAC1

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SUMMARY OF PROJECT

We have cloned, expressed, purified and crystallised the first DH GEF domain of Kalirin (gene: KALRN) in complex with RAC1 (gene: RAC1) as part of a programme to explore a new way of targeting GTPases. Fragment screening and X-ray crystallography has identified binders within the orthosteric binding site. A nucleotide exchange assay has been developed and GEF activity established.

SCIENTIFIC BACKGROUND

RAC1 is a member of the RHO subfamily of the RAS small GTPases. These are guanine nucleotide dependent molecular switches fundamental to numerous cell signalling pathways and cellular processes. KALRN is a guanine nucleotide exchange factor (GEF), a type of regulatory protein which activates RAC1 by catalysing the exchange of GDP for GTP (1). Within full length Kalirin(2), there are two GEF domains, DH1 and DH2, which target two individual members of the RHO subfamily of small GTPases, proteins RAC1 and RHOA (2). Kalirin is found predominantly in the brain. The most abundant isoform is Kalirin-7, a truncation of full length Kalirin which contains a single RAC1 DH GEF domain. It was found that through activation of RAC1, Kalirin mediates dendritic spine formation and actin remodelling. Changes in KALRN expression have therefore been linked to a number of psychiatric and neurodegenerative disorders associated with dendritic spine pathology (2-4).

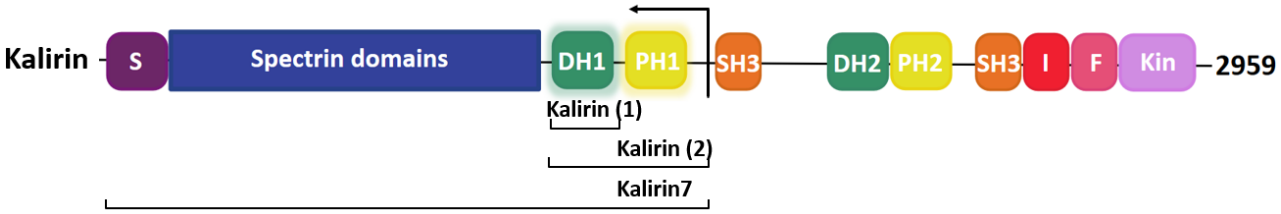


Fig. 1. Domain sequence of full length Kalirin.

Disease	Genetic association	Postmortem evidence	Molecular association	Animal models
Schizophrenia	GWAS (5,6) Rare missense mutations (7)	↓ mRNA (8) ↓ kalirin-7 protein (9) ↑ kalirin-9 (10)	DISC1 (11,12) NRG1 /ErbB4 (13,14) 5-HT_{2A} (15) NMDAR (12) PSD-95 (12) PAK	KALRN KO (16) Kal7 KO (17)
Alzheimer's disease	--	↓ mRNA (18) ↓ protein (18,19)	iNOS (20) PAK (21) EphB2 (22)	--
ADHD	GWAS (23)	--	Cadherins (24)	--
Addiction	--	--	--	Kal7 KO, addiction in mouse model (25,26)
Huntington's disease	--	--	HAP1 (27)	--
Parkinson's disease			Synphillin-1 (28)	
Stress	--	--	--	Chronic restraint stress in mice (29)
Ischemic stroke	Case-control (30)	--	--	Mouse model of ischemia (31)

Table 1 Reproduced from Remmers et al., 2014 (2). The literature mentioned within can be found in the references.

Objectives of this TEP:

- Demonstrate that Kalirin/RAC1 can be expressed, purified and crystallised as recombinant proteins
- Identify nucleotide phosphate competitive chemical starting points for a Kalirin/RAC1 inhibitor
- Develop an assay to assess Kalirin/RAC1 activity

RESULTS – THE TEP

Purified proteins

Using *E. coli* as the expression host, we expressed and purified samples of the 1st DH GEF domain of KALRN (Kalirin (1)), the 1st DH and PH domains of KALRN (Kalirin (2)) (**Fig. 1**) and truncated (by 15 residues) full length RAC1 (RAC1).

Structures

We have obtained 12 high resolution diffraction crystal structures of Kalirin(1)/RAC1 either as a holo-GDP bound structure (1.64 Å resolution, PDB ID 5O33, **Fig. 2**) or with fragments bound (1.9-2.3 Å resolution, PDB IDs 5QQD, 5QQE, 5QQF, 5QQG, 5QQH, 5QQI, 5QQJ, 5QQK, 5QQL, 5QQM, 5QQN).

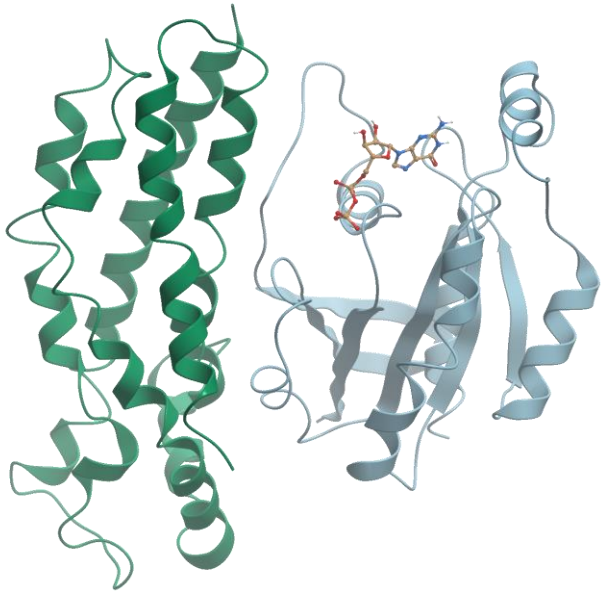


Fig. 2 Structure of GDP-bound Kalirin(1)/RAC1 (Kalirin(1) green; RAC1 light blue; GDP, brown), PDB ID: 5O33

Chemical Matter

A fragment soaking campaign using the XChem fragment library on GDP-bound crystals delivered 1 hit which displaced GDP. Docking and subsequent soaking of related compounds found a further 10 analogues.

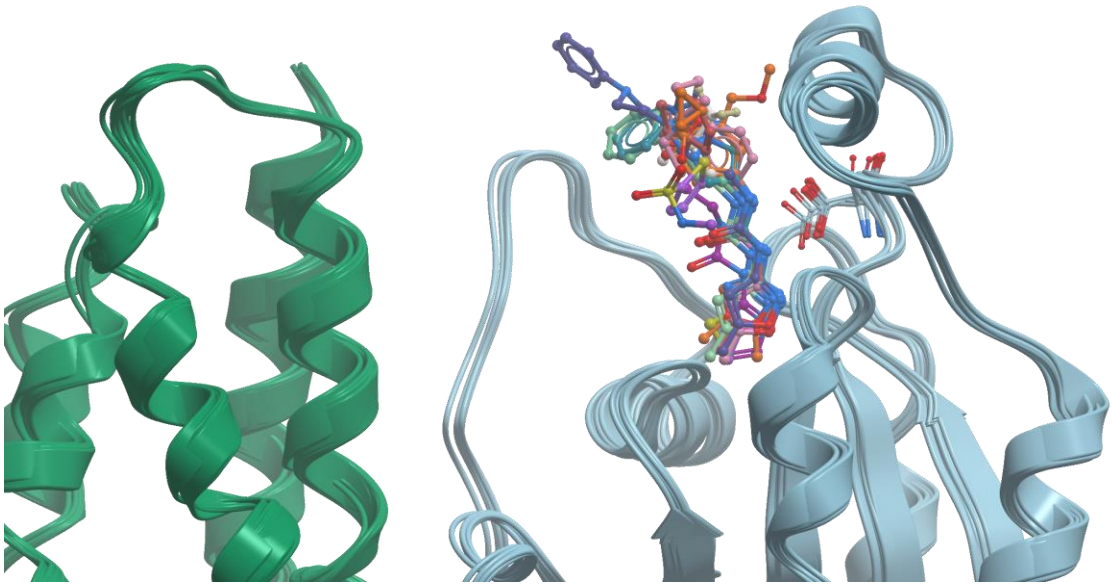
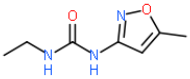
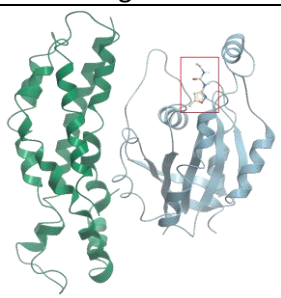
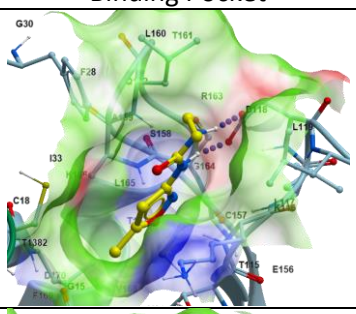
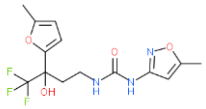
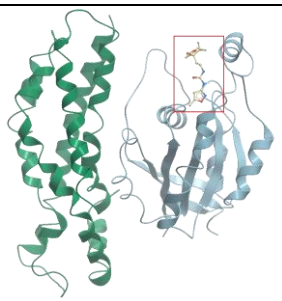
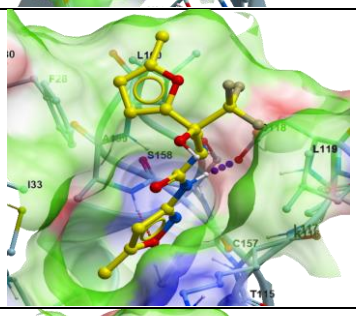
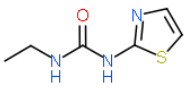
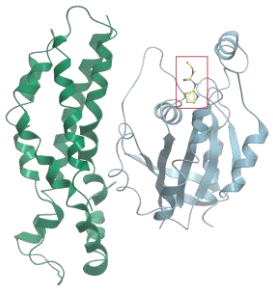
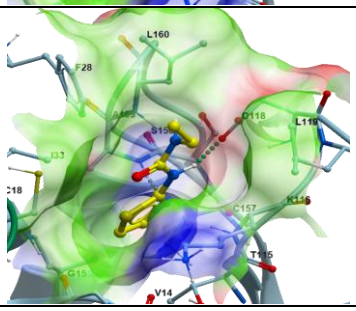
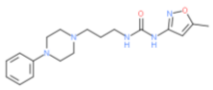
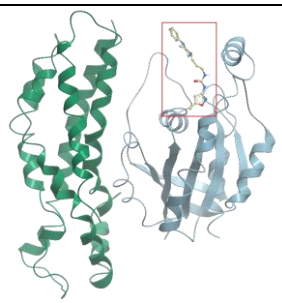
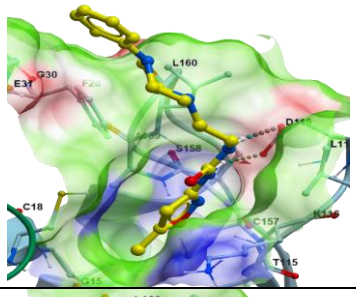
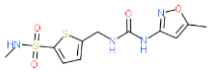
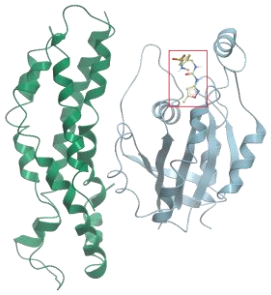
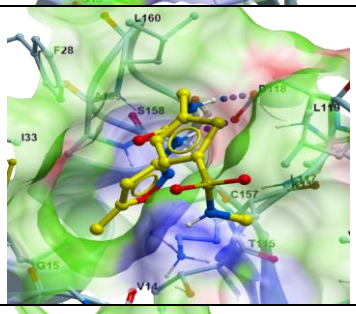
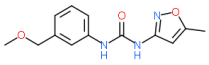
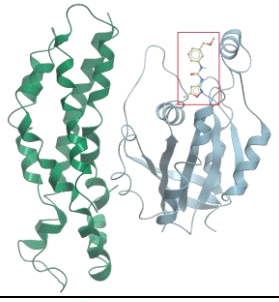
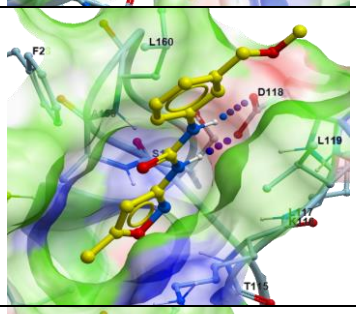
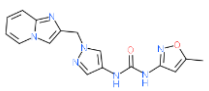
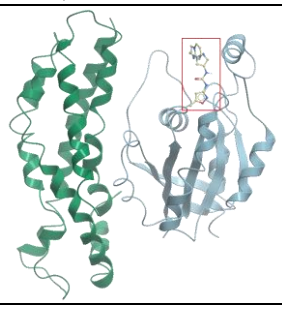
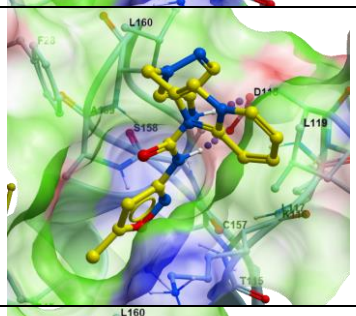
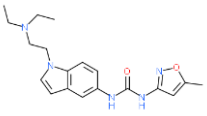
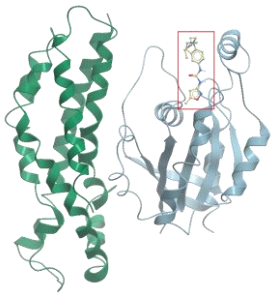
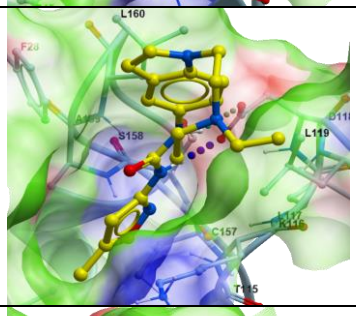
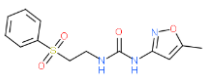
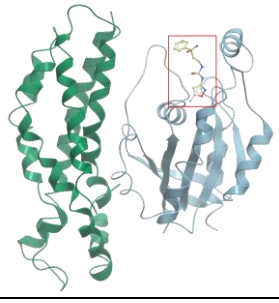
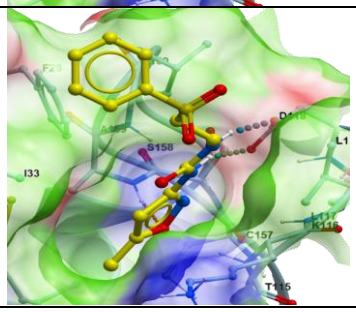


Fig. 3 Overlaid structures of fragment hits in the GDP binding site of Kalirin/RAC1 (Kalirin(1) green, RAC1 light blue, PDB IDs 5QQD, 5QQE, 5QQF, 5QQG, 5QQH, 5QQI, 5QQJ, 5QQK, 5QQL, 5QQM, 5QQN)

PDBID	Ligand	Binding Location	Binding Pocket	Resolution (Å)
5QQD	 Z56880342 (Original hit)			1.91
5QQE	 SG000012 (follow-up)			1.95
5QQF	 SG000013 (follow-up)			2.26

5QQG	 SG000055 (follow-up)			2.23
5QQH	 SG000059 (follow-up)			2.09
5QQI	 SG000070 (follow-up)			2.08
5QQJ	 SG000086 (follow-up)			1.9
5QQK	 SG000089 (follow-up)			2.24
5QQL	 SG000096 (follow-up)			2.25

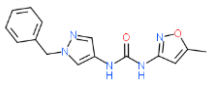
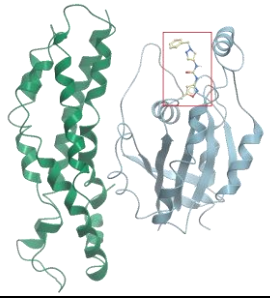
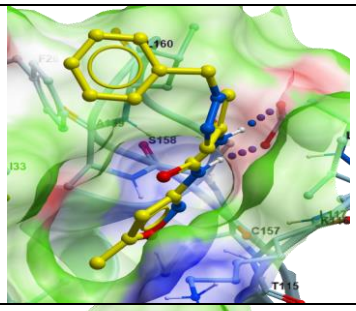
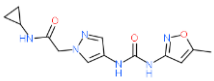
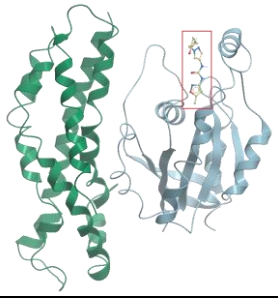
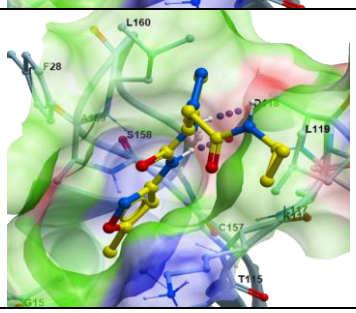
5QQM	 SG000098 (follow-up)			2.02
5QQN	 SG000112 (follow-up)			2.26

Table 2 Chemical structures of the fragment hit and follow-up compounds

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.

Nucleotide exchange activity assay

A nucleotide exchange assay was developed for Kalirin/RAC1 to measure the exchange rate of the GDP inside the pocket with GTP in solution (**Fig. 4**).

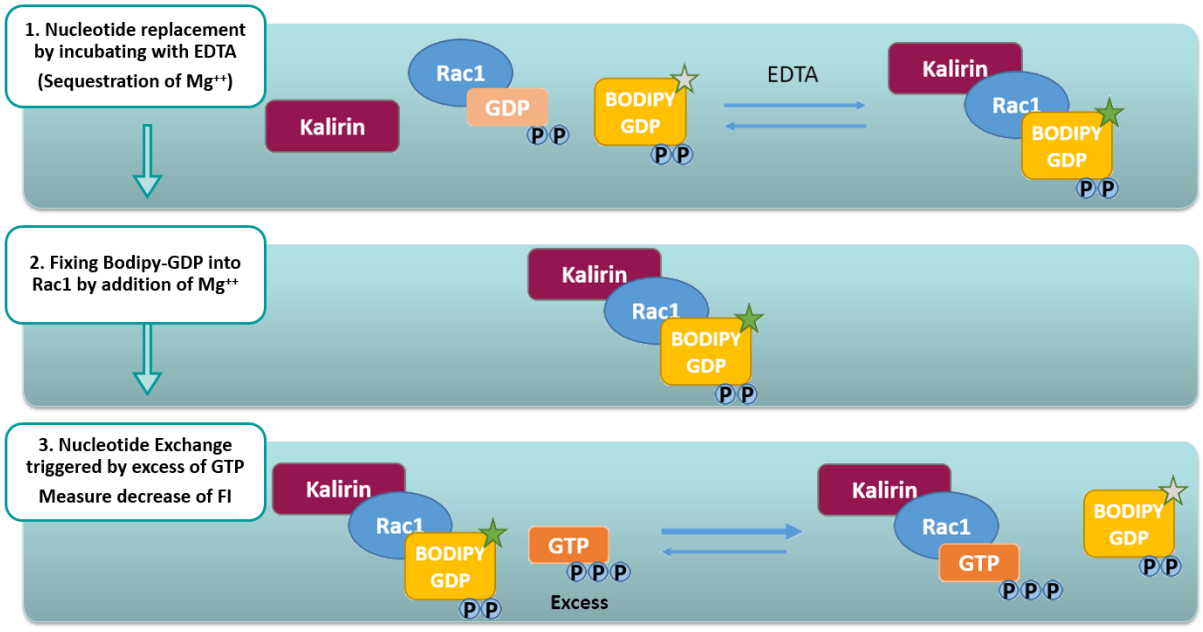


Fig. 4 The nucleotide exchange assay. **(1)** Loading BODIPY-GDP into RAC1. **(2)** Fixing BODIPY-GDP into the pocket. **(3)** Nucleotide exchange reaction of bound BODIPY-GDP with GTP in solution.

This procedure measures the exchange reaction rate of the guanine nucleotide bound to the GTPase RAC1 with GTP in solution. It can be used to compare the exchange activity of RAC1 when it is either in complex with its GEF interacting partner Kalirin or alone. The assay makes use of a fluorescent labelled GDP analogue containing a boron-dipyrromethene moiety attached to position 2' or 3' (BODIPY-GDP, ex/em: 485/510 nm).

The fluorescence yield from BODIPY-GDP is enhanced when it is bound to RAC1 and decreases upon release. This can be used to measure the nucleotide exchange rate through changes in the fluorescence intensity.

The assay has three phases: **(1)** nucleotide replacement, **(2)** BODIPY-GDP fixing, and **(3)** nucleotide exchange (**Fig. 4**). During the nucleotide replacement, EDTA sequesters the Mg^{++} ion, essential for GDP binding, and GDP is displaced by labelled BODIPY-GDP. When equilibrium is reached, an excess of Mg^{++} is added to complex the remaining EDTA and fix the fluorescent nucleotide inside the pocket. Lastly, GTP is added to trigger the dissociation of BODIPY-GDP and RAC1, decreasing the fluorescence intensity signal (**Fig. 5**)

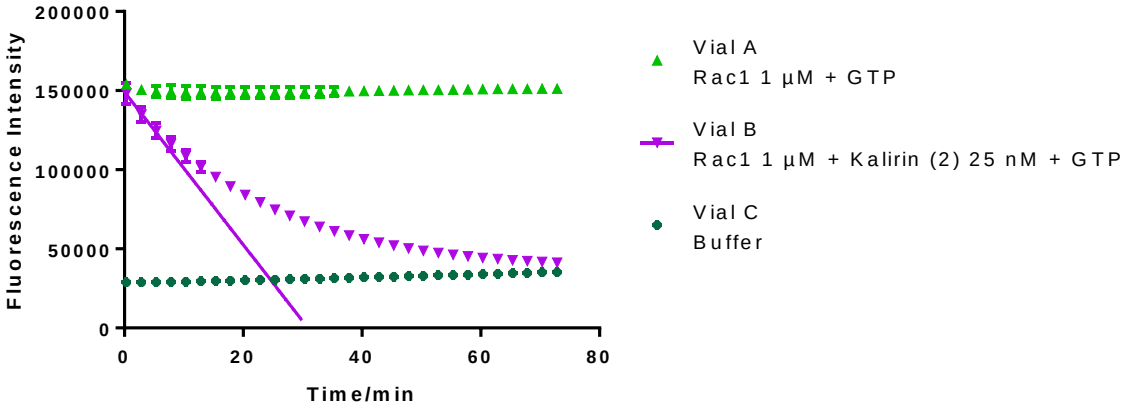


Fig. 5. The nucleotide exchange assay shows that SGC-expressed Kalirin is active as a GEF. Solid line represents initial rate of Kalirin (2)/RAC1.

Future work

- Develop chemical probes for Kalirin/RAC1 based on TEP fragment hits
- Express and screen other GEF/GTPase targets

Collaborations

- A collaboration with Paul Harrison (University of Oxford) is planned to test compounds binding to Kalirin/Rac1 in cellular models of schizophrenia.
- Nir London, received a grant from the Israeli Cancer Research Fund to develop RAC1 inhibitors in collaboration with the Oxford team (https://www.icrfonline.org/scientists/nir-london-phd/).

CONCLUSION

We have generated protein, an assay, crystal structures and chemical matter that has been shown to bind to the guanine nucleotide binding pocket of the Kalirin/RAC1 complex.

TEP IMPACT

Publications arising from this work:

- Gray, J.L., von Delft, F. and Brennan, P.E. Targeting the Small GTPase Superfamily through their Regulatory Proteins. (2019) Angew. Chem. Int. Ed.

FUNDING INFORMATION

The work at the SGC has been supported by Wellcome (grant 106169/ZZ14/Z), the Innovative Medicines Initiative Joint Undertaking (IMI JU) (grant 115766), Alzheimer's Research UK (grant ARUK-2018DDI-OX), National Institutes of Health (NIH) (grant 1RF1AG057443-01) and NDM Prize Graduate Studentship (Nuffield Department of Medicine, University of Oxford and Diamond Light Source Ltd, UK)

ADDITIONAL INFORMATION

Structure Files

PDB ID	Structure Details	Compound ID
5O33	Structure of GDP-bound Kalirin/RAC1	GT000001f
5QQD	Kalirin/RAC1 in complex with fragment	Z56880342
5QQE	Kalirin/RAC1 in complex with fragment	SG000012
5QQF	Kalirin/RAC1 in complex with fragment	SG000013
5QQG	Kalirin/RAC1 in complex with fragment	SG000055
5QQH	Kalirin/RAC1 in complex with fragment	SG000059
5QQI	Kalirin/RAC1 in complex with fragment	SG000070
5QQJ	Kalirin/RAC1 in complex with fragment	SG000086
5QQK	Kalirin/RAC1 in complex with fragment	SG000089
5QQL	Kalirin/RAC1 in complex with fragment	SG000096
5QQM	Kalirin/RAC1 in complex with fragment	SG000098
5QQN	Kalirin/RAC1 in complex with fragment	SG000112

Materials and Methods

Protein expression and purification

Kalirin (1)

Vector: pNIC28-10His
Cell line: BL21(DE3)-R3-pRARE2
Tags and additions: N-terminal, TEV protease cleavable decahistidine tag

Final protein sequence:
MHHHHHHHHHHSSGVDLGTENLYFQ*SMRKKEFIMAELLQTEKAYVRDLHECLETYLWEMTSGVEEIPPGILNKEHIIFGNI
QEIYDFHNNIFLKELEKYEQLPEDVGHCFVTWADKFQMYVTYCKNKPDSNQLILEHAGTFFDEIQQRHGLANSISSYLIKPVQ
RVTKYQLLLKELLTCCEEGKGELKDGLEVMLSVPKKANDAMHV
(underlined sequence contains vector encoded His-tag and TEV protease cleavage site*)

Protein Expression

A 10 mL overnight culture grown in LB containing kanamycin (final concentration 50 µg/mL) and chloramphenicol (34 µg/mL) at 37 °C was used to inoculate 1 L of AIM-TB (ForMedium) with 0.01% Antifoam 204 and antibiotics in a 2.5 L baffled flask (Ultrayield, Thomson). Cells were grown for 4 hours at 37 °C 250 rpm before the temperature reduced to 25 °C 250 rpm and shaken overnight. Cells were harvested by centrifugation at 4,000 x g for 20 minutes at 4 °C and the pellet stored at -20 °C.

Cell Lysis

Lysis Buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 0.5 mM TCEP, 0.5 mg/mL Lysozyme, 0.1 µg/mL Benzonase and protease inhibitors (Calbiochem EDTA-free Protease Inhibitor Cocktail Set III)

The cell pellet was resuspended in lysis buffer (20 mL per 5g of pellet), Triton X-100 to a final concentration of 2% added and the cells frozen overnight at -80 °C. The cells were thawed in a room temperature water bath, imidazole added to a final concentration of 20 mM and the cell debris was removed by centrifugation for 1 hour at 4000 x g.

Column 1: His GraviTrap columns (3 x 1 mL volume of Ni-Sepharose 6 FF in a gravity-flow column)

Wash Buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 20 mM Imidazole, 0.5 mM TCEP
Elution Buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 500 mM Imidazole, 0.5 mM TCEP

The clarified cell extract was added to 3 x 1 mL of His GraviTrap columns pre-equilibrated with Wash buffer. The columns were then washed with 2 x 10 mL Wash Buffer. The protein was eluted with 2.5 mL Elution Buffer after applying column 1 directly onto column 2.

Column 2: PD-10 desalting columns (3 x 8.3 mL volume of Sephadex G-25 resin in a gravity-flow column)
Wash Buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 20 mM Imidazole, 0.5 mM TCEP

Following elution of the protein from the His GraviTrap column directly onto the PD-10 column, the HisGraviTrap column was removed and the protein eluted from the PD-10 column using 3.5 mL of Wash Buffer.

Tag cleavage
1 mg of TEV protease was added to every 10 mg of the eluted protein and the digestion was performed overnight at 4 °C.

Column 3: His GraviTrap columns (3 x 1 mL volume of Ni-Sepharose 6 FF in a gravity-flow column)
Wash Buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 20 mM Imidazole, 0.5 mM TCEP

The TEV cleaved protein was applied to a HisGraviTrap column pre-equilibrated in Wash Buffer to remove TEV protease, His-tag and uncleaved protein. The columns were then washed with 2.5 mL of Wash Buffer for a final pool of 6 mL. Protein fractions were combined and concentrated to ~ 30 mg/mL using a 10 kDa MWCO concentrator.

Column 4: Yarra SEC 2000 300x21.2 mm column (300x21.2 mm column, 104 mL volume) (Gel filtration)
GF buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP

The protein was loaded onto the column pre-equilibrated in GF buffer, the peak corresponding to the target protein was taken, concentrated to 12 mg/ml and stored at -80 °C.

Kalirin (2)

Vector: pNIC28-Bsa4
Cell line: BL21(DE3)-R3-pRARE2
Tags and additions: N-terminal, TEV protease cleavable hexahistidine tag

Final protein sequence:
MHHHHHHSSGVDLGTENLYFQ*SMDREVKLRDANHEVNEEKRKSARKKEFIMAELLQTEKAYVRDLHECLETYLWEMTSG
VEEIPPGILNKEHIIFGNIQEIYDFHNNIFLKELEKYEQLPEDVGHCFVTWADKFQMYVTYCKNKPDSNQLILEHAGTFFDEIQQ
RHGLANSISSYLIKPVQRVTKYQLLLKELLTCCEEGKGELKDGLEVMLSVPKKANDAMHVSMLEGFDENLDVQGELILQDAFQ
VWDPKSLIRKGRERHLFLFEISLVFSKEIKDSSGHTKYVYKNKLLTSELGVTEHVEGDPCKFALWSGRTPSSDNKTVLKASNIETK
QEWIKNIREVIQERIIHLKGAL
(underlined sequence contains vector encoded His-tag and TEV protease cleavage site*)

Protein Expression
A 10 mL overnight culture grown in LB containing kanamycin (final concentration 50 µg/mL) and chloramphenicol (34 µg/mL) at 37 °C was used to inoculate 1 L of AIM-TB (ForMedium) with 0.01% Antifoam 204 and antibiotics in a 2.5 L baffled flask (Ultrayield, Thomson). Cells were grown for 4 hours at 37 °C 250 rpm before the temperature reduced to 25 °C 250 rpm and shaken overnight. Cells were harvested by centrifugation at 4,000 x g for 20 minutes at 4 °C and the pellet stored at -20 °C.

Cell Lysis
Lysis Buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 0.5 mM TCEP, 0.5 mg/ml Lysozyme, 0.1 µg/ml Benzonase and protease inhibitors (Calbiochem EDTA-free Protease Inhibitor Cocktail Set III)

Cell pellet was resuspended in lysis buffer (20 mL per 5g of pellet), Triton X-100 to a final concentration of 2% added and the cells frozen overnight at -80 °C. The cells were thawed in a room-temperature water bath, imidazole added to a final concentration of 20 mM and the cell debris was removed by centrifugation for 1 hour at 4000 x g.

Column 1: His GraviTrap columns (3 x 1 mL volume of Ni-Sepharose 6 FF in a gravity-flow column)
Wash Buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 20 mM Imidazole, 0.5 mM TCEP
Elution Buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 500 mM Imidazole, 0.5 mM TCEP

The clarified cell extract was added to 3 x 1 mL of His GraviTrap columns pre-equilibrated with Wash buffer. The columns were then washed with 2 x 10 mL Wash Buffer. The protein was eluted with 2.5 mL Elution Buffer after applying column 1 directly onto column 2.

Column 2: PD-10 desalting columns (3 x 8.3 mL volume of Sephadex G-25 resin in a gravity-flow column)
Wash Buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 20 mM Imidazole, 0.5 mM TCEP

Following elution of the protein from the His GraviTrap column directly onto the PD-10 column, the HisGraviTrap column was removed and the protein eluted from the PD-10 column using 3.5 mL of Wash Buffer.

Tag cleavage
1 mg of TEV protease was added to every 10 mg of the eluted protein and the digestion was performed overnight at 4 °C.

Column 3: His GraviTrap columns (3 x 1 mL volume of Ni-Sepharose 6 FF in a gravity-flow column)
Wash Buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 20 mM Imidazole, 0.5 mM TCEP

The TEV cleaved protein was applied to a HisGraviTrap column pre-equilibrated in Wash Buffer to remove TEV protease, His-tag and uncleaved protein. The columns were then washed with 2.5 mL of Wash Buffer for a final pool of 6 mL. Protein fractions were combined and concentrated to ~ 30 mg/mL using a 30 kDa MWCO concentrator.

Column 4: Yarra SEC 2000 300x21.2 mm column (300x21.2 mm column, 104 mL volume) (Gel filtration)
GF buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP

The protein was loaded onto the column pre-equilibrated in GF buffer, the peak corresponding to the target protein was taken, concentrated to 20 mg/ml and stored at -80 °C.

RAC1

Vector: pNIC-NStIIT
Cell line: BL21(DE3)-R3-pRARE2
Tags and additions: N-terminal, TEV protease cleavable Strep-tag II

Final protein sequence
HMSSGASWSHPQFEKGGGSGGGSGGAAWSHPQFEKGSGVDLGTENLYFQ*SMQAIKCVVVGDGAVGKTCLLISYTTNAF
PGEYIPTVFDNYSANVMVDGKPVNLGLWDTAGQEDYDRLRPLSYPQTDVFLICFSLVSPASFENVRAKWYPEVRHHCPNTPI
ILVGTKLDLRDDKDTIEKLKEKKLTPITYPQGLAMAKEIGAVKYLECSALTQRGLKTVFDEAIRAVL
(underlined sequence contains vector encoded Strep-tag and TEV protease cleavage site*)

Protein Expression
A 10 mL overnight culture grown in LB containing kanamycin (final concentration 50 µg/mL) and chloramphenicol (34 µg/mL) at 37 °C was used to inoculate 1 L of AIM-TB (ForMedium) with 0.01% Antifoam 204 and antibiotics in a 2.5 L baffled flask (Ultrayield, Thomson). Cells were grown for 4 hours at 37 °C 250 rpm

before the temperature reduced to 25 °C 250 rpm and shaken overnight. Cells were harvested by centrifugation at 4,000 x g for 20 minutes at 4 °C and the pellet stored at -20 °C.

Cell Lysis
Lysis Buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 0.5 mM TCEP, 0.5 mg/ml Lysozyme, 0.1 µg/ml Benzonase and protease inhibitors (Calbiochem EDTA-free Protease Inhibitor Cocktail Set III)

Cell pellet was resuspended in lysis buffer (20 mL per 5g of pellet), Triton X-100 to a final concentration of 2% added and the cells frozen overnight at -80 °C. The cells were thawed in a room temperature water bath and the cell debris was removed by centrifugation for 1 hour at 4000 x g.

Column 1: Strep-Tactin columns (3 x 1 mL volume of Strep-Tactin XT resin in a gravity-flow column)
Wash Buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 0.5 mM TCEP
Elution Buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 50 mM D-Biotin, 0.5 mM TCEP

The clarified cell extract was added to 3 x 1 mL of His GraviTrap columns pre-equilibrated with Wash buffer. The columns were then washed with 2 x 10 mL Wash Buffer. 2 mL of Elution Buffer was applied to each column. After 10 minutes and 20 minutes, this was repeated to result in a 6 mL pool of eluted protein.

Tag cleavage
1 mg of TEV protease was added to every 10 mg of the eluted protein and the digestion was performed overnight at 4 °C.

Column 2: His GraviTrap columns (3 x 1 mL volume of Ni-Sepharose 6 FF in a gravity-flow column)
Wash Buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 20 mM Imidazole, 0.5 mM TCEP

The TEV cleaved protein was applied to a HisGraviTrap column pre-equilibrated in Wash Buffer to remove TEV protease. The columns were then washed with 2.5 mL of Wash Buffer. Protein fractions were combined and concentrated to ~ 30 mg/mL using a 10 kDa MWCO concentrator.

Column 3: Yarra SEC 2000 300x21.2 mm column (300x21.2 mm column, 104 mL volume) (Gel filtration)
GF buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP

The protein was loaded onto the column pre-equilibrated in GF buffer, the peak corresponding to the target protein was taken and concentrated to 15 mg/ml and stored at -80 °C.

Kalirin(1)/RAC1 GDP-free complex formation

GDP removal
HPLC buffer: 100 mM Potassium phosphate pH 6.5, 10 mM Tetrabutyl phosphonium bromide, 5% methanol
Equipment: Agilent 1200 series HPLC. Reverse-phase C-18 5 µM (250 x 4.6 mm)
10 x Exchange Buffer: 2M ammonium phosphate, 10 µM zinc chloride

Kalirin (1) and RAC1 were mixed in a 1:1 molar ratio for an hour and concentrated to 60 mg/mL (138 µL) using a 30 kDa MWCO concentrator. 40 µL of 10 mM β,γ-Methyleneguanosine 5'-triphosphate sodium salt (GppCp, Sigma) and 4 Units of Alkaline phosphatase (Roche) were added. 20 µL of the 10x exchange buffer was added (final concentration 1 x) and the mixture incubated at 4 °C for 3 hours (Final concentration of Kalirin(1)/RAC1 is 1 mM). Degradation of GDP to GMP and guanosine was monitored by HPLC. Following complete degradation of GDP, 0.008 Units of snake venom phosphodiesterase I (Sigma) was added. The reaction was left overnight at 4 °C and the degradation of GppCp to GMP and guanosine monitored by HPLC. The protein was diluted to 30 mg/mL, flash frozen in liquid nitrogen and stored at -80 °C.

Column 1: Yarra SEC 2000 300x21.2 mm column (300x21.2 mm column, 104 mL volume)

GF buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP

The protein was thawed and loaded onto the column pre-equilibrated in GF buffer, the peak corresponding to the complex taken, concentrated to 10.4 mg/mL and stored at -80 °C.

Protein crystallisation

Kalirin(1)/RAC1 was crystallised by mixing 75nL of 10.4 mg/mL protein in 10mM HEPES pH 7.5, 500mM NaCl, 5% Glycerol 0.5 mM TCEP with 75nL of reservoir solution containing 0.1M bis-tris pH 5.5, 24% PEG3350. Crystals appeared overnight from sitting drop plates at 20 °C. Kalirin(1)/RAC1 crystallised in space group P65 2 2 with unit cell dimensions of a=63 Å, b=63 Å, c=346 Å, corresponding to one Kalirin(1)/RAC1 molecule in the asymmetric unit. The crystals typically diffract between 1.7 and 2.3 Å.

Nucleotide exchange assay

Equipment

PHERAstar FSX Plate reader (BMG Labtech, Germany)

Consumables

Microplate, 384 well, PS, flat bottom, small volume, non-binding, black, 784900. (Grenier Bio-One, UK)

Chemicals and reagents

$MgCl_2$ (magnesium chloride) M8266. (Sigma-Aldrich, UK)
GTP (guanosine 5'-triphosphate disodium salt trihydrate) JBS-NU-1012-100. (Enzo Life Sciences, UK)

Buffers

Dilution buffer (DB): 20 mM TRIS HCl pH7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.1% BSA, 1 mM DTT
Exchange buffer (EB): 20 mM TRIS HCl pH7.5, 50 mM NaCl, 2 mM EDTA, 0.1% BSA, 1 mM DTT, 0.75 µM BODIPY™ FL GDP

Protein and peptide

RAC1 c001 (RAC1), SGC: stored in aliquots at -80°C.
Kalirin c002 (Kalirin (2)), SGC: stored in aliquots at -80°C.

RAC1 was prepared in a stock solution at 50 µM in DB, Kalirin (2) was prepared in stock solution at 1.25 µM in DB. Three vials were prepared: 2 µL of RAC1, 2 µL of Kalirin (2) and 63.5 µL of EB were mixed into Vial A; 2 µL of RAC1, 2 µL of Kalirin (2) and 63.5 µL of EB were mixed into Vial B; 4 µL of DB and 63.5 µL of EB were mixed into Vial C. The vials were incubated at room temperature for 20 minutes, then 7.5 µL of 50 mM $MgCl_2$ in DB was added to each vial and mixed. To prepare the assay plate, 15 µL of Vial A was dispensed into row A columns 1-4, 15 µL of Vial B was dispensed into row B columns 1-4 and 15 µL of Vial C was dispensed into row C columns 1-4. The Pherastar FSX was used to dispense 5 µL of 1.8 mM GTP in DB into each well. The plate was then shaken for 10 seconds and FI signal was measured every 30 seconds for 75 minutes. FI 485/520, Gain 300. FI data was analysed in Prism 7 (Version 7.04). The enzyme rate was calculated as the slope obtained by fitting a straight line in a range from 0-5 minutes, units are ΔRFU/time.

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