

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
16 September 2010 (16.09.2010)

PCT

(10) International Publication Number  
**WO 2010/103294 A1**

(51) International Patent Classification:  
*G01N 33/50* (2006.01)

(21) International Application Number:  
PCT/GB2010/000468

(22) International Filing Date:  
12 March 2010 (12.03.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
0904321.7 12 March 2009 (12.03.2009) GB

(71) Applicant (for all designated States except US): **CAMBRIDGE ENTERPRISE LIMITED** [GB/GB]; The Old Schools, Trinity Lane, Cambridge CB2 1TN (GB).

(72) Inventor; and

(75) Inventor/Applicant (for US only): **RUDD, Christopher** [CA/CA]; Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QP (CA).

(74) Agent: **SUTCLIFFE, Nicholas, R.**; Mewburn Ellis LLP, 33 Gutter Lane, London EC2V 8AS (GB).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO,

DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Declarations under Rule 4.17:**

— of inventorship (Rule 4.17(iv))

**Published:**

— with international search report (Art. 21(3))

— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))

(54) Title: MODULATION OF T-CELL MEDIATED IMMUNE RESPONSES

(57) Abstract: This invention relates to the finding that the binding of SKAP1 to RapL plays a role in the LFA-I mediated adhesion of T cells. Methods are provided for the identification of compounds which alter this binding and which may therefore be useful in the modulation of T-cell immune responses.



WO 2010/103294 A1

Modulation of T-Cell Mediated Immune Responses

This invention relates to T-cell mediated immune responses, and in particular to the identification of compounds which  
5 modulate T-cell adhesion and migration.

Integrins regulate multiple aspects of immunity including migration to sites of inflammation, in lymph nodes and the conjugate formation between T-cells and antigen-presenting  
10 cells (APCs)<sup>1-3</sup>. As a family, they comprise as many as 20  $\alpha/\beta$  hetero-dimers whose binding is affected by localization, conformation and cell surface clustering<sup>2-4</sup>. Immune function is especially regulated by members of the  $\beta 2$  subfamily, in particular lymphocyte function-associated antigen (LFA-1;  
15 CD11 $\alpha$ /CD18 or  $\alpha L\beta 2$ ) as well as certain  $\alpha 4$  integrins such as  $\alpha 4\beta 1$  (VLA-4)<sup>5</sup>. LFA-1 binds to intercellular adhesion molecules (ICAM)-1 and -2 on major histocompatibility complex (MHC) bearing antigen presenting cells (APCs)<sup>2, 6-9</sup>. Initial contact leads to engagement of the antigen-receptor complex (TcR $\zeta$ /CD3)  
20 that induces so-called 'inside-out' signals that fully activate the integrin<sup>2, 3, 10</sup>. LFA-1  $\alpha$  and  $\beta$  subunits create a headpiece formed by a  $\beta$ -propeller domain of the  $\alpha$  subunit that interacts with a dinucleotide fold domain in the  $\beta$  subunit termed the I-like domain<sup>10, 11</sup>. On resting cells, LFA-1 adopts an  
25 inactive bent compact structure with the headpiece being located only 5 nm from the membrane. Chemokine and TcR $\zeta$ /CD3 signalling induces changes in affinity and avidity<sup>3, 10</sup>. Affinity is increased by conformational changes due to the extension and exposure the major I domain ligand binding site<sup>10, 12, 13</sup>. Avidity enhancement is mediated by the clustering of LFA-  
30 1 that increases the valency of binding for ICAM-1. In this manner, LFA-1 has a 10-fold lower dissociation constant ( $k_{off}$ ) for dimeric than monomeric ICAM-1<sup>14</sup>.

Given the central importance of adhesion to immune function, there has been a major interest over the past decade in identifying the 'inside-out' signalling pathway for integrin activation. An array of early TcR $\zeta$ /CD3 induced signalling events influence adhesion including kinases CD4/CD8-p56lck<sup>15</sup>, IL2-inducible T-cell kinase (ITK)<sup>16</sup>, the guanine nucleotide exchange factor Vav-1<sup>17</sup>, phosphatidylinositol 3-kinase (PI 3K)<sup>18, 19</sup>, Rho/Rac GTP binding proteins<sup>20, 21</sup> including Rap1<sup>22-25</sup>, its binding partner RapL (regulator of cell adhesion and polarization enriched in lymphoid tissues)<sup>26, 27</sup> and Riam (Rap1-interacting adaptor molecule)<sup>28</sup> as well as adaptors SLP-76 (76-kD src homology 2 domain-containing leukocyte phosphoprotein)<sup>29</sup>, ADAP (adhesion and degranulation-promoting adaptor protein)<sup>30-32</sup> and SKAP1 (55-kD src kinase-associated phosphoprotein) (also called SKAP-55: src kinase associated phosphoprotein 1 or src family associated phosphoprotein 1)<sup>33-35</sup>. However, it has proved difficult to distinguish specific effectors of LFA-1 adhesion from the generic events needed for cell integrity and function.

20

The present invention relates to the finding that the binding of SKAP1 to RapL plays a key role in the LFA-1 mediated adhesion of T cells. This interaction may therefore be a target for the development of therapeutic molecules which modulate T-cell immune responses.

25

An aspect of the invention provides a method of identifying a compound which modulates LFA-1 mediated T-cell adhesion comprising;

30

determining the effect of a test compound on the binding of an SKAP1 polypeptide to a RapL polypeptide, wherein a compound which affects said binding is a candidate modulator of LFA-1 mediated T-cell adhesion.

The effect of the test compound may be determined by contacting the SKAP1 polypeptide and the RapL polypeptide in the presence and absence of a test compound.

- 5 In some embodiments, the SKAP1 polypeptide and the RapL polypeptide may be contacted under conditions in which they bind together unless a compound which inhibits binding is present. In other embodiments, the SKAP1 polypeptide and the RapL polypeptide may be contacted under conditions in which  
10 they do not bind together unless a compound which promotes binding is present.

- A difference in the binding of the SKAP1 polypeptide and the RapL polypeptide in the presence relative to the absence of a  
15 test compound is indicative that the compound modulates LFA-1 mediated T-cell adhesion.

- For example, an increase in binding in the presence of the compound relative to the absence may be indicative that the  
20 compound increases or enhances LFA-1 mediated T-cell adhesion. A decrease in binding in the presence of said compound relative to the absence may be indicative that the compound inhibits or represses LFA-1 mediated T-cell adhesion.

- 25 A SKAP1 polypeptide may comprise or consist of the amino acid sequence of residues 1 to 104 of the full-length SKAP1 amino acid sequence (SEQ ID NO: 2) or may be a variant thereof. In some embodiments, the SKAP1 polypeptide may comprise or consist of the full-length SKAP1 amino acid sequence or a  
30 fragment, allele, isoform or variant thereof. A suitable SKAP1 polypeptide may include any eukaryotic SKAP1, in particular a mammalian SKAP1 such as human or mouse SKAP1. Preferably, a SKAP1 polypeptide as described herein includes the wild-type residue (i.e. leucine) at a position corresponding to position  
35 25 of the full-length SKAP1 amino acid sequence (SEQ ID NO:2).

The Swiss-Prot database identifiers for the amino acid sequence of human SKAP1 (GeneID 8631; src kinase associated phosphoprotein 1, also known as SKAP55) are Q86WV1.2 GI: 122070147 and (NCBI accession no: NP\_003717.3 GI: 115527074).  
5 Another isoform of human SKAP1 (isoform 2) has the database identifiers NP\_001068567.1 GI: 115527076.

The NCBI database identifiers for the nucleotide coding  
10 sequences of human SKAP1 are NM\_003726.3 GI: 115527073 (isoform 1) and NM\_001075099.1 GI: 115527075 (isoform 2).

The NCBI database identifiers for the amino acid sequence of mouse SKAP1 are NP\_001028358.1 GI: 84794544. The NCBI database  
15 identifiers for the nucleotide sequence of mouse SKAP1 are NM\_001033186.2 GI: 141803165.

An allele or variant of a wild-type SKAP1 sequence may differ from the wild-type sequence by the addition, deletion,  
20 substitution and/or insertion of one or more amino acids, provided the function of binding to RapL is retained.

A RapL polypeptide may comprise or consist of the amino acid sequence of the RapL SARAH domain which is located at residues  
25 223 to 253 of the full-length RapL amino acid sequence (SEQ ID NO: 4); or may be a variant thereof. In some embodiments, the RapL polypeptide may comprise or consist of the full-length RapL amino acid sequence or a fragment, isoform, allele or variant thereof. A suitable RapL may include any eukaryotic  
30 RapL, in particular a mammalian RapL, such as human or mouse RapL.

The Swiss-Prot database identifiers for the amino acid sequence of human RapL (GeneID 83593; also known as RASSF5)  
35 are Q8WWW0.1 GI: 74751587 (NCBI database identifiers

NP\_872604.1 GI: 32996731). Other isoforms of human RapL have the NCBI database identifiers NP\_872605.1 GI: 32996733 (isoform B) and NP\_872606.1 GI: 32996735 (isoform C). The NCBI database identifiers for the nucleotide coding sequences of human RapL are NM\_182663.2 GI: 115430205 (isoform A), NM\_182664.2 GI: 115430204 (isoform B) and NM\_182665.2 GI: 115430207 (isoform C).

The Swiss-Prot database identifiers for amino acid sequence of mouse RapL are Q5EBH1.1 GI: 81882747 (NCBI database identifiers NP\_061220.2 GI: 60097929). The NCBI database identifiers for the nucleotide coding sequence of mouse RapL are NM\_018750.3 GI: 141803301.

An allele or variant of a wild-type RapL sequence as described herein may differ from the wild-type sequence by the addition, deletion, substitution and/or insertion of one or more amino acids, provided the function of binding to SKAP1 is retained.

Preferably, a RapL polypeptide as described herein includes the wild-type residue (i.e. leucine) at a position corresponding to position 224 of the full-length RapL amino acid sequence.

A RapL or SKAP1 polypeptide for use in the methods described herein which comprises an amino acid sequence which is an allele or variant of a wild-type amino acid sequence described herein (e.g. the wild-type RapL SARAH domain; the SKAP1 N terminal domain; or the RapL or SKAP1 full length sequences) may comprise an amino acid sequence which shares greater than 50% sequence identity with the wild-type sequence, greater than 55%, greater than 65%, greater than 70%, greater than 80%, greater than 90%, greater than 95% or greater than 98%.

Sequence identity is commonly defined with reference to the algorithm GAP (Genetics Computer Group, Madison, WI). GAP uses the Needleman and Wunsch algorithm to align two complete sequences that maximizes the number of matches and minimizes the number of gaps. Generally, default parameters are used, with a gap creation penalty = 12 and gap extension penalty = 4. Use of GAP may be preferred but other algorithms may be used, e.g. BLAST (which uses the method of Altschul *et al.* (1990) *J. Mol. Biol.* 215: 405-410), FASTA (which uses the method of Pearson and Lipman (1988) *PNAS USA* 85: 2444-2448), or the Smith-Waterman algorithm (Smith and Waterman (1981) *J. Mol Biol.* 147: 195-197), or the TBLASTN program, of Altschul *et al.* (1990) *supra*, generally employing default parameters. In particular, the psi-Blast algorithm (Nucl. Acids Res. (1997) 25 3389-3402) may be used. Sequence identity similarity may also be determined using Genomequest™ software (Gene-IT, Worcester MA USA).

Sequence comparisons are preferably made over the full-length of the relevant sequence described herein.

As described above, polypeptide fragments which retain all or part of the activity of the full-length protein may be generated and used in the methods described herein, whether *in vitro* or *in vivo*. Suitable ways of generating fragments include, but are not limited to, recombinant expression of a fragment from encoding DNA. For example, fragments may be generated by taking encoding DNA, identifying suitable restriction enzyme recognition sites either side of the portion to be expressed, and cutting out said portion from the DNA. The portion may then be operably linked to a suitable promoter in a standard commercially available expression system. Another recombinant approach is to amplify the relevant portion of the DNA with suitable PCR primers. Small fragments (e.g. up to about 20 or 30 amino acids) may also be

generated using peptide synthesis methods which are well known in the art as further described below.

A fragment of a full-length sequence may consist of fewer amino acids than the full-length sequence. For example a fragment may consist of at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90 or at least 100 amino acids of the full length sequence but 800 or less, 700 or less, 600 or less, 500 or less, 250 or less, 200 or less, 150 or less, or 125 or less amino acids of the full length sequence.

Methods described herein may be *in vivo* cell-based methods, or *in vitro* non-cell-based methods. The precise format for performing methods of the invention may be varied by those of skill in the art using routine skill and knowledge.

Techniques which are suitable for use in determining the binding between SKAP1 and RapL polypeptides are well-known in the art and include scintillation proximity assay, flow cytometry (e.g. FACS), immunohistochemical staining, immunocytochemical staining, surface plasmon resonance (e.g. BIAcore™), Western Blotting, immunofluorescence, enzyme linked immunosorbent assays (ELISA), radioimmunoassays (RIA), immunoradiometric assays (IRMA) and immunoenzymatic assays (IEMA), including sandwich assays using monoclonal and/or polyclonal antibodies.

For example, binding between SKAP1 and RapL polypeptides may be studied *in vitro* by labelling one with a detectable label and bringing it into contact with the other which has been immobilised on a solid support. This may be performed in the presence of a test compound.



Suitable detectable labels, especially for peptidyl substances include  $^{35}\text{S}$ -methionine, which may be incorporated into recombinantly produced peptides and polypeptides.

Recombinantly produced peptides and polypeptides may also be  
5 expressed as fusion proteins containing a label, for example a fluorescent label, such as GFP or mCherry, or an epitope which can be labelled with an antibody.

In a scintillation proximity assay, a biotinylated protein  
10 fragment may be bound to streptavidin coated scintillant-impregnated beads (for example, produced by Amersham). Binding of radiolabelled peptide is then measured by determination of radioactivity-induced scintillation as the radioactive peptide binds to the immobilized fragment. Agents  
15 that block this binding are inhibitors of the interaction.

A polypeptide may be immobilized using an antibody against that polypeptide which is bound to a solid support or via other technologies which are known *per se*. A preferred *in*  
20 *vitro* interaction may utilise a fusion protein including glutathione-S-transferase (GST). This may be immobilized on glutathione agarose beads. In an *in vitro* format, a test compound can be assayed by determining its ability to diminish the amount of labelled peptide or polypeptide which binds to  
25 the immobilized GST-fusion polypeptide. This may be determined by fractionating the glutathione-agarose beads by SDS-polyacrylamide gel electrophoresis.

Alternatively, the beads may be rinsed to remove unbound  
30 protein and the amount of bound protein determined by counting the amount of label present, for example, using a suitable scintillation counter.

Of course, the person skilled in the art will design any appropriate control experiments with which to compare results obtained in methods of the invention.

5 Methods described herein may also take the form of *in vivo* methods. *In vivo* methods may be performed in a cell line such as a yeast strain, insect or mammalian cell line, for example CHO, HeLa or COS cells, in which the relevant polypeptides or peptides are expressed from one or more vectors introduced  
10 into the cell.

Suitable techniques include the yeast two-hybrid system (e.g. Evan *et al.* *Mol. Cell. Biol.* 5, 3610-3616 (1985); Fields & Song *Nature* 340, 245-246 (1989)). This system may be used to  
15 screen for compounds able to disrupt binding between RapL and SKAP1 polypeptides. For instance, the polypeptides may be expressed in a yeast two-hybrid system (e.g. one as a GAL4 DNA binding domain fusion, the other as a GAL4 activator fusion) which is treated with test substances. The absence of the  
20 end-point which normally indicates interaction between the pathway components (e.g. the absence of a blue colour generated by  $\beta$ -galactosidase), when a test compound is applied, indicates that the compound disrupts interaction between the two components, and may therefore modulate LFA-1  
25 mediated T-cell adhesion as described herein.

Test compounds for use in methods of the invention may be natural or synthetic chemical compounds used in drug screening programmes and may include, for example, small organic  
30 molecules, polypeptides and nucleic acids, such as aptamers. Extracts of plants that contain several characterised or uncharacterised components may also be used. Combinatorial library technology (Schultz, JS (1996) *Biotechnol. Prog.* 12:729-743) provides an efficient way of testing a potentially

vast number of different substances for ability to modulate activity of a polypeptide.

The amount of test substance or compound which may be employed  
5 in a method described herein will normally be determined by trial and error depending upon the type of compound used. Typically, from about 0.1 to 100µM concentrations of putative inhibitor compound may be used, for example from 1 to 10µM. When cell-based methods are employed, the test substance or  
10 compound is desirably membrane permeable in order to access the interacting polypeptides.

One class of putative agents for modulating T-cell mediated immune responses can be derived from the SKAP1 and RapL  
15 polypeptides as described above. In particular, peptide fragments from the N-terminal domain of SKAP1 and the SARAH domain of RapL, in particular peptide fragments comprising L224 of RapL, may be employed. Membrane permeable peptide fragments of from 5 to 40 amino acids, for example, from 6 to  
20 10 amino acids may be tested for their ability to disrupt, for example, the SKAP-55/RapL interaction.

The inhibitory properties of a peptide fragment as described above may be increased by the addition of one of the following  
25 groups to the C terminal: chloromethyl ketone, aldehyde and boronic acid. These groups are transition state analogues for serine, cysteine and threonine proteases. The N terminus of a peptide fragment may be blocked with carbobenzyl to inhibit aminopeptidases and improve stability (Proteolytic Enzymes 2nd  
30 Ed, Edited by R. Beynon and J. Bond, Oxford University Press, 2001).

Antibodies directed to the site of interaction of SKAP1 and RapL form a further class of putative agents for modulating  
35 the LFA-1 mediated T-cell adhesion. For example, a suitable

antibody may bind an epitope within N terminal domain of SKAP1 or the SARA domain of RapL, such as an epitope which comprises residue 224 of RapL. Candidate inhibitor antibodies may be characterised and their binding regions determined to provide single chain antibodies and fragments thereof which are responsible for disrupting the interaction. Suitable antibodies may be obtained using techniques which are standard in the art, including, for example immunising a mammal with a suitable peptide, such as a fragment of RapL or SKAP1, or isolating a specific antibody from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047.

Other candidate compounds for modulating LFA-1 mediated T-cell adhesion may be based on modelling the 3-dimensional structure of RapL and SKAP1, in particular the SARA domain of RapL and the N-terminal domain of SKAP1 either alone or in combination, and using rational drug design to provide candidate compounds with particular molecular shape, size and charge characteristics. For example, a chemical compound may be modelled to resemble the three dimensional structure of the component in an area which contacts another component, and in particular the arrangement of the key amino acid residues as they appear. Techniques for the rational design of compounds that bind to target proteins are well known in the art.

Firstly, the particular parts of a compound that are critical and/or important in modulating the interaction of RapL and SKAP1 are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, e.g. by substituting each residue in turn. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled to according its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray  
5 diffraction data and NMR.

Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in  
10 this modelling process.

In a variant of this approach, the three-dimensional structure of RapL and SKAP1 are modelled. This allows the model to take account of changes conformation on binding in the optimisation  
15 of the lead compound.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can  
20 conveniently be selected so that the modified compound is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. The modified compounds found by this approach can then be screened to see whether they have  
25 the target property, or to what extent they exhibit it. Modified compounds include mimetics of the lead compound.

Further optimisation or modification can then be carried out to arrive at one or more final compounds for further testing,  
30 for example *in vitro*, *in vivo* or clinical testing.

Methods as described herein may comprise the step of identifying a test compound which modulates the binding of the SKAP1 polypeptide to the RapL polypeptide. Compounds which  
35 modulate SKAP1/RapL binding are candidate modulators of LFA-1

mediated T-cell adhesion. For example, a compound which increases SKAP1/RapL binding may be identified as a candidate enhancer or activator of LFA-1 mediated T-cell adhesion and/or a compound which decreases SKAP1/RapL binding may be identified as a candidate inhibitor of LFA-1 mediated T-cell adhesion.

Following identification of a compound which modulates the SKAP1/ RapL binding, a method may further comprise modifying the compound to optimise its pharmaceutical properties. This may be done by modelling techniques as described above.

A test compound identified using one or more initial screens as having ability to modulate e.g. increase or decrease SKAP1/ RapL binding and thereby modulate LFA-1 mediated T-cell adhesion, may be assessed further using one or more secondary screens.

A secondary screen may involve testing for a biological function or activity *in vitro* and/or *in vivo*, e.g. in an animal model. For example, the ability of a test compound to modulate one or more of LFA-1 activation, clustering and/or adhesion; T-cell-APC conjugation; TcR induced RapL-Rap1 complex formation; SKAP1-Rap1-RapL binding to LFA-1; and disruption of T-cell binding to immobilised ICAM1 may be determined.

The ability of a test compound to modulate T-cell mediated immune responses may be determined *in vitro* and/or *in vivo*, e.g. in an animal model. For example, the ability of a test compound to modulate, e.g. enhance or inhibit T-cell adhesion, migration and/or conjugate formation may be determined.

Following identification of a test compound which modulates RapL/SKAP1 binding, the compound may be isolated and/or

purified or alternatively it may be synthesised using conventional techniques of recombinant expression or chemical synthesis. Furthermore, it may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition  
5 such as a medicament, pharmaceutical composition or drug. These may be administered to individuals for the treatment of a disease or medical condition.

Compounds identified as candidate modulators of LFA-1 mediated  
10 T-cell adhesion using any of the methods described herein may be useful in modulating (i.e. increasing or decreasing) T-cell mediated immune responses in a therapeutic context.

A compound which disrupts the SKAP1/RapL interaction and  
15 thereby inhibits LFA-1 mediated T-cell adhesion may be useful in the suppression of T-cell mediated immune responses, for example the treatment or prevention of conditions in which such responses are aberrant or undesirable. For example, a suitable compound may be useful in the treatment or prevention  
20 of graft versus host reaction or rejection after organ or tissue transplantation (e.g. bone marrow transplants); inflammatory diseases and immune diseases.

For example, a compound may be useful in the treatment or  
25 prevention of atopic dermatitis, allergic rhinitis, psoriasis, asthma, allergies, diabetes mellitus or arthritis (including rheumatoid arthritis).

Other inflammatory diseases include ulcerative colitis,  
30 Crohn's disease, chronic obstructive, pulmonary disease, subcutaneous oedema, Behcet's disease, pleuritis, peritonitis, emphysema, pulmonary oedema, cerebral infarction, polyarteritis nodosa, septic shock, acute respiratory distress syndrome, multiple organ failure and systemic inflammatory  
35 reaction syndrome.

- Other immune diseases include spontaneously systemic progressive skin sclerosis, allergic conjunctivitis, autoimmune uveitis, glomerulonephritis, stromal nephritis, acute renal failure, chronic renal failure, systemic lupus erthematosus, allergic encephalomyelitis, multiple sclerosis, myasthenia gravis, Sjogren syndrome and systemic autoimmune disease.
- 10 A compound identified as a candidate inhibitor of LFA-1 mediated T-cell adhesion using any of the methods described herein may also be useful in modulating (i.e. increasing or decreasing) cell adhesion and migration in a therapeutic context. For example, a compound may be useful in the
- 15 treatment of disorders associated with aberrant cell adhesion and migration. For example, a suitable compound may be useful in the treatment or prevention of solid tumours, cancer metastasis and T-cell leukaemias and lymphomas.
- 20 A compound identified as a candidate stimulator of LFA-1 mediated T-cell adhesion, may be useful for example in promoting or enhancing immune responses. A suitable compound may be useful in the treatment of clinical disorders characterised by general or specific immunodeficiency,
- 25 including HIV infection and congenital immunodeficiency diseases, infectious diseases, including bacterial viral, fungal and parasitic infections and cancers, e.g. to promote a immunotherapeutic T-cell responses against malignant cells. A suitable compound may be also useful in the development of
- 30 vaccines, for example by increasing or enhancing immune responses to vaccine antigens.

Methods of the invention may comprise formulating said test compound in a pharmaceutical composition with a



pharmaceutically acceptable excipient, vehicle or carrier for therapeutic applications, as discussed above.

A pharmaceutical composition may include, in addition to said  
5 compound, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or one or more other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other  
10 material may depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, topical or intraperitoneal routes.

Pharmaceutical compositions for oral administration may be in  
15 tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose  
20 or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or  
25 injection at a particular site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for  
30 example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The  
5 actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes  
10 account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition,  
15 Osol, A. (ed), 1980.

SKAP1 and RapL polypeptides as described above may also be useful in methods for the diagnosis and detection of immunodeficiency in individuals.

20

A method of detecting immunodeficiency in an individual may comprise

contacting one or more T cells obtained from an individual with a first member of a binding pair consisting of  
25 a SKAP1 polypeptide and RapL polypeptide,

and determining binding of the first member to the other member of the binding pair in the one or more T cells.

The absence of binding of the first member in the one or more  
30 T cells or a reduction or impairment in binding of the members of the binding pair relative to controls (e.g. T cells from a healthy individual) may be indicative of the presence of immunodeficiency in the individual.

Immunodeficiency may include a primary immune deficiency, such as leukocyte adhesion deficiency (LADS), or a reduced or weakened response to immunotherapies, such as vaccination.

- 5     Suitable SKAP1 polypeptide and RapL polypeptides for use as the first member of the binding pair are described above.

Preferably, the first member of the binding pair is labelled with a detectable label. Suitable labels are well known in the  
10     art and include fluorescent labels such as fluorescein, rhodamine and derivatives thereof.

One or more T cells may be obtained from the individual by taking a blood sample then isolated the T cells using standard  
15     techniques. The T cells may then be permeabilised to allow the labelled SKAP1 polypeptide or RapL polypeptide to enter the cell. Suitable permeabilisation techniques are well known in the art.

- 20     The T cells may be incubated with the labelled SKAP1 polypeptide or RapL polypeptide and then treated to remove unbound labelled polypeptide. For example, permeabilised T cells may be incubated with the labelled SKAP1 polypeptide or RapL polypeptide for 0.5 hours or more, 1 hour or more, or 2  
25     hours or more, then washed to remove unbound labelled polypeptide.

The presence of labelled polypeptide bound to SKAP1 or RapL within the T cells after washing may then be determined using  
30     standard techniques.

The invention also provides labelled SKAP1 polypeptide and RapL polypeptides for use in methods of detecting immunodeficiency and kits containing labelled SKAP1  
35     polypeptide and RapL polypeptides.

Various further aspects and embodiments of the present invention will be apparent to those skilled in the art in view of the present disclosure. All documents mentioned in this specification are incorporated herein by reference in their entirety.

"SKAP1" and SKAP-55" are synonyms for the same protein and both names are used interchangeably in this specification. However, the name "SKAP1" has been officially approved by the HUGO gene nomenclature committee.

"and/or" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. For example "A and/or B" is to be taken as specific disclosure of each of (i) A, (ii) B and (iii) A and B, just as if each is set out individually herein.

Unless context dictates otherwise, the descriptions and definitions of the features set out above are not limited to any particular aspect or embodiment of the invention and apply equally to all aspects and embodiments which are described.

Certain aspects and embodiments of the invention will now be illustrated by way of example and with reference to the figures described below.

Figure 1 shows that the RapL-Rap1 complex fails to form in SKAP-55<sup>-/-</sup> primary T cells. T-cells were purified from splenocytes from wild-type SKAP-55<sup>+/+</sup> (WT) and SKAP-55<sup>-/-</sup> (KO) cells were lysed, subjected to precipitation with anti-Rap1 followed by blotting with anti-RapL (upper panel), anti-SKAP-55 (middle panel) and anti-Rap1 (lower panel). SKAP-55<sup>+/+</sup> wild-type (lanes 1,2,5,6,9). SKAP-55<sup>-/-</sup> T-cells (lanes 3,4,7,8). Resting: lanes 1,3,5,7. Anti-CD3 ligated:

lanes 2,4,6,8,9.

Figures 2 to 5 show that SKAP-55 (SKAP1) coordinates RapL-RapV12 complex formation.

5

Figure 2 shows that anti-Rap1V12 co-precipitates RapL and SKAP-55. RapLV5, Rap1-myc and SKAP-55-GFP were co-expressed in Jurkat T-cells by transfection followed by cell lysis and precipitation using anti-V5 (V5-Rap1V12) (lanes 3-10) or rabbit anti-mouse (lane 11). Blotting was carried out using either a combination of anti-V5 (RapL), anti-SKAP-55 and anti-myc (Rap1V12). Separated fractions: cytosol: lanes 3-6; membranes: lanes 7-11. Resting control: lanes 3,5,7,9,14,16; anti-CD3 ligated: lanes 4, 6, 8, 10, 11.

15

Figure 3 shows that anti-RapL co-precipitates Rap1V12 and SKAP-55. SKAP-55-GFP, RapL-V5 and/or Rap1V12-myc were co-expressed in Jurkat cells followed by precipitation with anti-V5 (RapL) and blotting with anti-myc (Rap1V12) (lanes 1-6) or anti-V5 and anti-GFP (lanes 8-17). Cell lysates: lanes 1,2 and 8, 9. Anti-V5 (RapL) precipitations: lanes 3-6 and 10-16. Rabbit anti-mouse control (lanes 7 and 17). Resting cells: lanes 3, 5, 8,10, 12, 14, 16; anti-CD3 ligation (lanes 1, 2, 4, 6, 8, 9, 11, 13, 15, 17, 18).

25

Figure 4 shows that anti-SKAP-55 co-precipitates RapL. SKAP-55-GFP, RapL-V5 and/or Rap1V12-myc were co-expressed in Jurkat cells followed by precipitation with anti-SKAP-55 and blotting with anti-V5 (RapL). Resting cells: lanes 6-8; anti-CD3 ligated cells (lanes 1-5, 9). Cell lysates: lanes 1,2. Precipitations: anti-SKAP-55: lanes 3-8, rabbit anti-mouse control (lane 9).

30

Figure 5 shows that SKAP-55 induced RapL-Rap1 binding requires active Rap1V12 and not Rap1V17 and cannot be substituted by

35

SLP-76 or ADAP. HASKAP-55, HA-SLP-76 or HA-ADAP were co-expressed with V5-RapL and/or Myc-Rap1V12 in Jurkat cells followed by precipitation with anti-SKAP-55, anti-SLP-76 or anti-ADAP followed by blotting with anti-HA (upper panel);  
5 anti-V5 (middle panel) or anti-myc (lower panel). Cell lysates: lane 1-6; anti-SKAP-55 precipitation: lanes 7-9; anti-SLP-76 precipitation: lane 10; anti-ADAP precipitation: lane 11.

10 Figures 6 to 9 show that the SKAP-55 N-terminus (N-SKAP-55) binds to the RapL coiled-coil domain.

Figure 6 shows that SKAP-55 binds to RapL. SKAP-55-GFP, RapL-V5 and/or Rap1V12-myc were expressed in Jurkat cells followed  
15 by precipitation with anti-SKAP-55 and blotting with anti-V5 (RapL). Anti-SKAP-55 precipitations: lanes 1-4; mouse Ig control: lane 5. SKAP-55 transfectant: lane 1; SKAP-55 and RapL transfectant: lanes 2; SKAP-55 and Rap1V12: lanes 3; SKAP-55 and RapL and Rap1V12: lanes 4,5.

20 Figure 7 shows that N-SKAP-55 binds RapL. Bacterially expressed, purified GST fused full-length SKAP-55 and fragments were incubated with lysates of 293T cells that had been transfected with cDNA of V5-RapL full-length or V5-RapL deletion mutant for pull-down assays. The V5-RapL protein that  
25 associated with GST-SKAP-55, or its sub-domains were detected by immunoblotting with anti-V5 antibodies. Upper panel: diagrammatic depiction of GST-SKAP-55 and the GSTSKAP-55 fragments. Lower panels: Upper lower panel: anti-V5-RapL blot; middle lower panel: anti-V5-RapLΔ2 blot; lower panel: Lane 1:  
30 cell lysate; lane 2: GST; lane 3: GST-SKAP-55 WT; lane 4: N-terminal GST-SKAP-55; lane 5: GST-SK RapL; lane 6: GST-N-PH-SK SKAP-55.

Figure 8 shows that N-SKAP-55 binds RapL coiled-coil (SARAH) domain (C-RapL). Purified GST-SKAP-55 and fragments were incubated with RapL-SARAH from 293T transfected cells followed by precipitation with Glutathione beads and blotting with anti-V5 (RapL). Upper panel: diagrammatic depiction of V5-RapL and V5-RapL-SARAH. Lower panels: Upper lower panel: anti-V5-RapL blot; lower panel: anti-GST blot. Lane 1: cell lysate; lane 2: GST; lane 3: GSTSKAP- 55 WT; lane 4: GST-N-PH-SK SKAP-55; lane 5: N-terminal GST-SKAP-55; lane 6: GST-SK RapL; lane 7: GST-SH3 SKAP-55.

Figure 9 shows Isothermal titration calorimetry (ITC) analysis of the binding between N-SKAP-55 and GST-CRapL. The status of the purification of proteins is detected by silver staining.

Figures 10 to 12 show that the RapL-L224A mutant selectively ablates SKAP-55 binding without affecting MST1 binding.

Figure 10 shows that RapL-L224A mutant ablates binding to full length SKAP-55. V5 tagged RapL, RapLΔC, RapLΔC2, RapLΔC3, RapL-L224A or RapL-L253A were co-expressed with GFP-SKAP-55 in 293T cells followed by precipitation with anti-SKAP-55 and blotting with anti-V5 (upper panel) and anti-GFP (lower panel). Upper panel: diagrammatic depiction of V5-RapL full-length and V5-RapL regions. Lower panel: Lysates (upper and lower panels, lanes 1-6) and anti-SKAP-55 (upper and lower panels, lanes 7-13). Lanes 1,7: WT RapL; lanes 2,8: RapLΔC; lanes 3, 9: RapLΔC2; lanes 4, 10: RapLΔC3; lanes 5, 11: RapLL224A; lanes 6, 12: RapL-L253A; lane 13: rabbit anti-mouse control.

Figure 11 (top panel) shows GST pull downs which confirm that RapL-L224A mutant ablates binding to N-SKAP-55. GST-SKAP-55 and fragments were used to pull-down RapL (upper top panel), RapL-L224A (middle top panel) or RapL-L253A (lower top panel) from 293T transfected cells followed by precipitation with

Glutathion beads and blotting with anti-V5 (RapL). Lane 1: cell lysate; lane 2: GST; lane 3: GST-SKAP-55 WT; lane 4: GST-SK; lane 6: GST-N-PH-SK.

5 Figure 11 (bottom panel) shows RapL-L224A mutant binds to MST-1. V5 tagged RapL, RapLAC, RapLAC2, RapLAC3, RapL-L224A or RapL-L253A were co-expressed with Myc tagged-MST-1 in 293T cells followed by precipitation with anti-Myc and blotting with anti-V5 (upper panel) or anti-Myc (lower panel). Anti-Myc  
10 precipitations: lanes 1-6; rabbit anti-mouse precipitation: lane 7. WT (lanes 1,7); RapLAC (lanes 2); RapLAC2 (lanes 3); RapLAC3 (lanes 4), RapL-L224A (lanes 5) or RapLL253A (lanes 6).

15 Figure 12 shows that RapL-L224A mutant fails to support Rap1V12-RapL complex formation. RapL-V5, RapL-L224A-V5, Rap1-myc and SKAP-55-GFP were coexpressed in Jurkat T-cells by transfection followed by stimulation for 10min with anti-CD3, cell lysis and precipitation using anti-myc  
20 (Rap1V12) (lanes 1-5), anti-SKAP-55 (lanes 8,9) or anti-V5 (lanes 10,11). Samples were blotted with a combination of anti-V5/Myc (lanes 1-5) or anti-V5 (lanes 6-12). Cell lysates (lanes 1,2), anti-Myc precipitations (lanes 3,4), anti-SKAP-55 precipitations (lanes 8,9) and anti-V5 precipitations (lanes  
25 10, 11). Rabbit anti-mouse (lanes 5 and 12).

Figure 13 to 15 show that loss of SKAP-55-RapL binding disrupts Rap-1/RapL colocalization.

30 Figure 13 shows that anti-CD3 induced SKAP-55, RapL-L224A and Rap1 colocalization, an event disrupted by the L224A mutation. RapL-CFP or RapLL224A plus SKAP-55-GFP and Rap1V12-mcherry were expressed in Jurkat cells followed by incubation on anti-CD3 coated cover slips and imaging using a Weiss confocal  
35 microscope over 0-240 seconds. Upper panels: SKAP-55, RapL and



Rap1V12; Lower panels: SKAP-55, RapL-L224A and Rap1V12. Left lower inserts represent magnification (100X) of localized box.

Figure 14 (Left panels) show PPC analysis of the co-localization of RapL/RapL-L224A with Rap1V12 and SKAP-55. Pearson's correlation coefficients (PCCs) were determined using Volocity software (Improvision) of cells in panel a. Upper left panel: histogram showing average PPC values for RapL-Rap1V12 co-localization in cells expressing RapL/Rap1V12, SKAP-55/RapL/Rap1V12 or SKAP-55/RapLL224A/Rap1V12 under resting conditions or in response to plate-bound anti-CD3. Lower left panel: as above, but showing average PPC values for SKAP-55/RapL co-localization.

Figure 14 (right panels) show that L224A mutation disrupts RapL, SKAP-55 and Rap1 colocalization in Jurkat cells on ICAM1 coated plates stimulated with soluble anti-CD3. RapL/RapL-L224A-CFP, SKAP-55-GFP and Rap1V12-mcherry were co-expressed in Jurkat cells responding to plate-bound anti-CD3. Instead of an image at the interface on plates (panel b), images were taken from above the cells. RapL-CFP, SKAP-55-GFP and Rap1V12-mcherry formed clusters in vesicular-like structures in a region proximal to lamellapodia-like extensions. Upper right panels: images of polarized T-cells over 0-240 seconds expressing RapLCFP, SKAP-55-GFP and Rap1V12-mcherry (upper panels) or RapL-L224A-CFP, SKAP-55-GFP and Rap1V12-mcherry. Lower right panels: histograms of PPC values over a time-course for RapL-Rap1V12 (upper panel); SKAP55-RapL (middle panel) and SKAP-55-Rap1V12 (lower panel).

30

Figure 15 shows L224A mutation prevents SKAP-55, RapL and Rap1 co-localization in pSMAC-like structure. RapL/RapL-L224A-CFP, SKAP-55-GFP and Rap1V12-mcherry were co-expressed in Jurkat cells responding to platebound anti-CD3 (as in figure 13).

Upper panel: panels showing pSMAC-like circular structure for

35

RapL-CFP (panel a); SKAP-55-GFP (panel b); Rap1V12mcherry (panel c) and overlay (panel d). Lower panel: Panels showing pSMAC like structure for RapL-L224A-CFP (panel a); SKAP-55-GFP (panel b); Rap1V12mcherry (panel c) and overlay (panel d).

5

Figure 16 to 18 show that SKAP-55-Rap1-RapL is required for binding to LFA-1.

Figure 16 shows anti-RapL co-precipitation of LFA-1 in T-cells and its disruption with the L224A mutation of RapL. Left panel: Co-localization of RapL but RapL-L224A with CD18. Upper panel: images of cells at 240sec: lower panel: histogram of average PCC values. Right panels: Upper panel: SKAP-55, RapL/Rap1V12 (lanes 1-4,9) or RapL/Rap1V12 (lanes 5-8) were expressed in Jurkat cells followed by precipitation with anti-V5 (RapL) and blotting with anti-CD11a. Cytosol: lanes 1,2,5,6; membrane fraction: lanes 3,4,7,8,9. Resting: lanes 1,3,5,7. Ant-CD3 ligated: 2,4,6,8,9. Lane 9: rabbit anti-mouse. Lower panel: SKAP-55, RapL WT/Rap1V12 (lanes 1,3,5) or SKAP-55, RapLL224A/Rap1V12 (lanes 2,4) were expressed in Jurkat cells followed by precipitation with anti-V5 (RapL) and blotting with anti-CD11a. Cytosol: lanes 1,2 ; membrane fraction: lanes 3,4,5. Ant-CD3 ligated: 1-5. Lane 5: rabbit antimouse control precipitation.

25

Figure 17 shows anti-SKAP-55 co-precipitation of LFA-1 in T-cells as detected by ant-CD18 blotting. SKAP-55 and RapL (lanes 1,3,5,7) or SKAP-55, RapL and Rap1V12 (lanes 2,4,6,8,9) were expressed in Jurkat cells followed by precipitation with anti-SKAP-55 and blotting with anti-CD11a. Lysates: lanes 1-4; anti-SKAP-55 precipitations: lanes 5-8 ; anti-mouse control: lane 9. Cytosolic fraction: lanes 3,4; 7,8; membrane fraction: lanes 1,2,5,6,9.

30

Figure 18 shows loss of SKAP-55 in *SKAP-55*<sup>-/-</sup> primary T-cells disrupts the association of endogenous Rap1 and RapL with LFA-1. Left panels: Jurkat cells either unstimulated or anti-CD3 stimulated for 10min were precipitated with anti-SKAP-55 or anti-CD18 followed by blotting with anti-CD18 (upper panel), anti-SKAP-55 (middle panel) and anti-RapL (lower panel). Resting: lanes 1,3,5. Anti-CD3 ligated: lanes 2,4,6. Lysates: lanes 1,2. Anti-SKAP precipitations: lanes 3,4; anti-CD18 precipitations: lanes 5,6. Right panel: T35 cells were purified from splenocytes from wild-type *SKAP-55*<sup>+/+</sup> (WT) and *SKAP-55*<sup>-/-</sup> cells, were lysed and precipitated with anti-CD18 followed by anti-SKAP-55 blotting (upper panel), anti-RapL blotting (middle panel) and anti-Rap1 blotting (lower panel). Left panel: *SKAP-55*<sup>+/+</sup> or *SKAP-55*<sup>-/-</sup> T-cells from splenocytes were left unstimulated or stimulated with anti-CD3 for 10min. Cells were then lysed and subjected to precipitated with anti-CD18 followed by blotting with anti-SKAP-55 (upper panel), anti-RapL (middle panel) or anti-Rap1 (lower panel). *SKAP-55*<sup>+/+</sup> (lanes 9,10) and *SKAP-55*<sup>-/-</sup> cells (lanes 11, 12). Resting: lanes 9, 11. Anti-CD3 ligated: lanes 8,10,12. Rabbit anti-mouse control: lane 8.

Figures 19 and 20 show L224A disrupts anti-CD3 induced ICAM-1 binding and T cell/APC conjugate formation.

25

Figure 19 upper panel shows that L224A disrupts anti-CD3 induced ICAM-1 binding. Jurkat cells were transfected with vector, *SKAP-55*, RapL, RapLAC2 or RapL-L224A and assessed for binding to ICAM-1 immobilized on micro-titer plates in response to anti-CD3 ligation. Values expressed as a percentage of the total number of overlaid cells.

30

Figure 19 lower panel shows that L224A disrupts T-cell-APC conjugation. T8.1 cells transfected with RapL-WT or RapL-L224A were cultured with L625 antigen presenting cells in the

35

presence or absence of tetanus toxoid (TTox) peptide and assessed for conjugate formation as previously described<sup>34, 70, 71</sup>. Values expressed as a function of the T-cell/APC ratio.

5

Figure 20 shows a model of SKAP-55 (SKAP1) regulation of the Rap1-RapL complex and binding to LFA-1. SKAP-55 (SKAP1) is needed for the binding of Rap1 to RapL. This involves the binding of N-SKAP-55 to the coiled coil SARAH domain of RapL leading to the formation of a complex comprised of at least SKAP-55-RapL and Rap1. Rap1 must be in a GTP-bound, while an additional signal is needed from the TcR. Formation of the SKAP-55-RapL-Rap1 complex then binds via RapL to the cytoplasmic tail of LFA-1 that induces clustering and increased adhesion.

10  
15

Figure 21 shows Western blot data which demonstrate that the PH domain of SKAP1 is needed for RapL translocation to the membrane. Cytosolic and membrane fractions of SKAP1<sup>+/+</sup> and SKAP1<sup>-/-</sup> T-cells were purified and subjected to blotting with anti-SKAP1 (upper panel); anti-Rap1 (upper middle panel); anti-RapL (lower middle panel) and anti-actin (lower panel). SKAP1<sup>+/+</sup> wild-type (lanes 1-5). SKAP1<sup>-/-</sup> T-cells (lanes 6-10). Resting: lanes 2,4,7,9. Anti-CD3 ligated: lanes 3,5,8,10.

20  
25

Figure 22 shows Western blot data which demonstrate that the inhibition of PI3K blocks RapL translocation to the membrane.

Figure 23 shows Western blot data which demonstrate that mutation of the L25 residue in N-SKAP1 prevents binding to RapL. GST-WT SKAP1 or GST-L25A and GST-L30A mutants were co-transfected with V5-RapL in Jurkat cells followed by anti-CD3 ligation (1 $\mu$ g/ml) for 5 min and precipitation with anti-GST followed by blotting with anti-V5 (upper panel) and anti-GST

30

(lower panel). Lane 1: lysate; lane 2: GST control; lane 3: GST WT SKAP1; lane 4: GST-L24A and lane 5: GST-L30A.

Figure 24 shows T-cell motility in the presence and absence of SKAP1 in murine lymph node slices.

## Experiments

### 1. Materials and Methods

#### 1.1 Cells and antibodies

10 T cells were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum and 1% (wt/vol) penicillin streptomycin, as previously reported<sup>50</sup>. The murine hybridoma T8.1-expressing human CD4 and a chimeric human mouse TCR specific for Ttox (830-843), as well as L625.7 cells, were  
15 gifts of O. Acuto (Institute Pasteur, Paris, France)<sup>34, 51, 70</sup>. Anti-SKAP-55 were purchased from Transduction Laboratories; anti-V5 was purchased from Invitrogen; anti-myc antibody from Upstate Biotechnology; anti-GFP from Santa Cruz Biotechnology; anti-human CD3 from American Type Culture Collection (ATCC),  
20 anti-mouse CD3 (2C11; hamster anti-mouse CD3), and anti-CD11a (anti-LFA-1) were purchased from BD Biosciences; and Alexa-488-conjugated secondary Abs were purchased from Molecular Probes. Ttox (residues 830-843) was obtained from Research Genetics.

25

#### 1.2 Generation of plasmids and mutagenesis.

Full-length human SKAP-55 (SKAP1) cDNA were cloned into the pSRa expression vector and cloned in-frame with the NH2 terminus of the GFP gene in the pSRa-GFP vector (Promega).  
30 DNA-Sequences encoding N (residues 1 to 104), SK (residues 209 to 285), SH3 (residues 285 to 359) and N-PH-SK (residues 1 to 359) domains of SKAP-55 were amplified by PCR from plasmid containing full-length human SKAP-55 cDNA and subcloned into the pGEX-2T vector (GE Healthcare). Full-length human RapL  
35 cDNA was cloned into the pcDNA3.1-V5 expression vector

(Invitrogen). Deletion mutants were generated by standard PCR-based cloning techniques. RapL L224A and L253A mutants were generated by site-direct mutagenesis using the Quick Change protocol and Pfu Ultra® II Fusion HS DNA Polymerase (Stratagene). Full-length human Rap1V12 and Rap1N17 were cloned into myc-expression vector. SKAP-55 and RapL were also cloned into pSR alpha HAGFP and CFP for C-terminal tagging (Statagene). Rap1 was tagged with mCherry and also cloned into pCDNA3 for expression studies.

10

T-cells were purified from SKAP-55+/+ and SKAP-55-/- spleens as previously described. Jurkat T cells were co-transfected expression plasmids by microporation (Digital Bio Technology) followed by subsequently culture in RPMI with 5 % FCS medium lacking penicillin and streptomycin. For live cell imaging, poly-L-lysine (Sigma) treated chambered cover-slides (LabTek) were coated with 10 µg/ml mAb OKT3. Images of the contact area between cells and mock treated and OKT3-coated glass surface were acquired by resonance scanning confocal microscopy (TCS SP2 RS, Leica, Heidelberg, Germany) using excitation wavelengths of 514 nm for EYFP and 594 nm for mRFP and a 63x water immersion objective (NA=1.2). Images were processed with Leica confocal software (LCS; Leica Microsystems), Volocity (Improvision), and ImageJ (National Institutes of Health) software). Live-cell images were acquired at 37°C, 5% CO<sub>2</sub>. Pearson's correlation coefficients (PCCs) for co-localization was determined using Volocity software (Improvision).

15

20

25

30

### 1.3 Immunoprecipitation and Immunoblotting.

Cell lysis, immunoprecipitation, and detection were performed as described previously<sup>50, 84</sup>. Cells were harvested and lysed with 200 µl of lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, 1mM leupeptin, 1 mM pepstatin,

and 1% aprotinin). Immunoprecipitation was carried out by incubation of the lysate with the antibody for 1 h at 4 °C, followed by incubation with 50 µl of glutathione-Sepharose beads (50% w/v) for 1 h at 4 °C. For immunoblotting, the immunoprecipitates were separated by SDS-PAGE and transferred onto nitrocellulose filters (Schleicher and Schuell). Filters were blocked with 5% (w/v) skimmed milk for 1 h in Tris-buffered saline (Tris buffered saline) pH 8.0 and then probed with the indicated antibody or GST fusion proteins followed by binding with an anti-GST monoclonal antibody. Bound antibody was revealed with horseradish peroxidase-conjugated rabbit anti-mouse antibody using enhanced chemiluminescence (ECL, Amersham Biosciences). For the Far Western technique, ADAP-SH3c was expressed with a T7 tag, and binding to SDS-PAGE-separated and blotted GST fusion proteins was detected with anti-T7 antibody followed by chemiluminescence development.<sup>50</sup>

#### 1.4 Expression and Purification of GST Fusion Proteins

Plasmids were transformed into the DH5 strain of *Escherichia coli* and induced with isopropyl-D thiogalactopyranoside to produce GST fusion proteins as described<sup>57</sup> (provided by Immune Ventures Inc., Cambridge). Expression of recombinant GST-proteins was induced in *Escherichia coli* BL21 cells at 37 °C for 2 h by addition of 1 mM IPTG<sup>29</sup>. GST-fused proteins were incubated with lysates of RAPL-transfected cells in TBSN buffer (20 mM Tris pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EGTA, 0.5 mM Na<sub>3</sub>VO<sub>4</sub>, 20 mM p-nitrophenyl phosphate) supplemented with protease inhibitors (Complete Mini EDTA-free Protease Inhibitor Cocktail, Roche Diagnostics) at 4 °C for 2 h. GST, GST-full-length SKAP-55 (SKAP1) and GST-fused subdomains of SKAP-55 were adsorbed to glutathione-Sepharose 4B (GE Healthcare) for an additional 1 h. The bound proteins were washed three times, boiled for 5 min in SDS sample buffer, resolved by SDS-PAGE and transferred to Immobilon-P

membrane (Millipore). Western blotting was performed as previously described.

#### 1.5 Isothermal Titration Calorimetry.

5 For ITC analysis, recombinant GST-NSKAP-55 and GST-C-RapL was bacterially expressed as described<sup>57, 85, 86</sup>. ITC analysis was performed using the VP ITC (Microcal, Northampton, MA, U.S.A.) as described<sup>51, 87, 88</sup>. Protein was diluted from stock solution to the concentration required for the ITC experiment (4-10  $\mu$ M)  
10 and dialyzed against the ITC buffer 7.5 (10 mM Na,K phosphate buffer, pH 7.5, 150 mM NaCl). The 'heat of dilution' control experiments were performed and the residual heats determined were subtracted from the heats of binding prior to data analysis (as shown in Fig 3 right hand panel). All binding  
15 data were analysed by fitting the binding isotherm to a simple independent binding-site model using Origin software provided with the ITC (MicroCal Inc.). GST was cleaved with Factor X according to the manufacturer's instruction.

#### 20 1.6 T Cell Motility Assays

Inguinal mouse LNs were embedded in 4 percent low-gelling-temperature agarose (type VII-A; Sigma-Aldrich) prepared in PBS. 320- $\mu$ m slices were then sliced with a vibratome (VT 1000S; Leica) in a bath of ice-cold PBS. Slices were then submerged  
25 in RPMI 1640 plus 10% FCS at 4°C and transferred to 0.4- $\mu$ m organotypic culture inserts (Millicell; Millipore) in 35-mm Petri dishes containing 1 ml RPMI 1640 plus 10% FCS in an incubator at 37°C/5% CO<sub>2</sub>. After washing, cells were labelled with secondary antibody anti-rat (IgH-L) Alexa633 for 2h at  
30 37°C. CD4+ T cells were purified by positive selection (Dynabeads mouse CD4 (L3T4), Detachabead mouse CD4). Lymphocytes were incubated with CFSE (Sigma, Poole, UK) to label cells in suspension (at 20 x 10<sup>6</sup> cells per ml in 0.1% FBS/PBS) at a final concentration of 1 $\mu$ M for 8 min at room  
35 temperature. Between 10<sup>5</sup> and 5 x 10<sup>5</sup> lymphocytes in 10-20  $\mu$ l of



RPMI 1640 plus 10% FCS were plated onto the cut surface of each slice. Slices were incubated for 1 h at 37°C/5% CO<sub>2</sub>, gently washed to remove the residual cells that had not penetrated the tissue, and kept in the incubator before the imaging experiment. Imaging experiments were performed with an inverted Zeiss LSM510 confocal microscope equipped with a chamber thermostated at 37°C. Images were acquired every 10 sec during 20 min using a 20X phase objective on a Zeiss LSM510 microscope. Cell motility was analyzed with Zeiss LSM confocal software and Volocity software (Improvision) and documented in terms of mean average speed (uM/min).

## 2. Results

### 2.1 Rap-1-RapL complex does not form in SKAP-55<sup>-/-</sup> primary T-cells

TcR-driven Rap1-RapL complex formation was initially assessed in freshly isolated primary T-cells from spleens of SKAP-55<sup>+/+</sup> and SKAP-55<sup>-/-</sup> mice<sup>33</sup>. T-cells were stimulated with anti-CD3, were lysed and subjected to precipitation with anti-Rap1, followed by blotting with anti-RapL or SKAP-55 (Fig. 1). Anti-Rap1 co-precipitated RapL from SKAP-55<sup>+/+</sup> T-cells (upper panel, lanes 5, 6), and this increased with anti-CD3 ligation at 5 minutes (lane 6 vs. 5) and 15 minutes post-activation (data not shown). This confirmed that anti-CD3 ligation induces binding of Rap1 to RapL in wild-type T cells<sup>22, 26, 27</sup>. By contrast, anti-Rap1 failed to co-precipitate RapL from resting or activated SKAP-55<sup>-/-</sup> T-cells (lanes 7, 8). Immunoblotting of lysates confirmed the presence of equal levels of RapL and Rap1 in SKAP-55<sup>+/+</sup> and SKAP-55<sup>-/-</sup> T-cells (upper and lower panels, lanes 1-4, respectively). These data showed that SKAP-55 (SKAP1) expression is needed in coupling the TCR/CD3 complex to the induction of Rap1-RapL complex formation in primary T-cells.

Given this role, it was also possible that SKAP-55 binds to the Rap1-RapL complex. Indeed, anti-Rap1 co-precipitated SKAP-55 from wild-type cells (middle panel, lanes 5,6) and this increased with anti-CD3 ligation (lane 6 vs. 5). Anti-SKAP-55 blotting confirmed the presence of SKAP-55 in lysates from SKAP-55++, but not SKAP-55-/- T-cells (middle panel, lanes 1,2 and 3,4, respectively). These findings show SKAP-55 can be co-precipitated with Rap1 and RapL in response to anti-CD3 ligation.

10

## 2.2 SKAP-55 (SKAP1) coordinates RapL-Rap1 binding

To extend this observation to co-expression studies, V5-tagged RapL, myc-tagged Rap1V12 and GFP-tagged SKAP-55 were expressed in Jurkat T-cells, lysed, subjected to anti-myc (Rap1V12) precipitation and blotted with anti-SKAP-55, anti-V5 (Rap1) and anti-myc (Rap1V12) (Fig. 2). Rap1V12 is the constitutively active form of Rap1. Contrary to a previous report<sup>26, 37</sup>, co-expression of RapL and Rap1 alone failed to allow for co-precipitation of RapL (V5) with anti-myc (Rap1). Specifically, RapL was absent from cytosolic (lanes 3,4) and membrane fractions (lanes 7,8) as well as from resting (lanes 3, 7), or anti-CD3 ligated cells (lanes 4, 8). It was also not observed with different concentrations of anti-CD3 antibody (1-10µg/ml; data not shown). In marked contrast, the mere expression of SKAP-55 with RapL and Rap1-V12 resulted in anti-myc (Rap1V12) coprecipitation of RapL as well as SKAP-55 (lane 10). This occurred in the membrane (lane 10), but not the cytosolic fraction (lane 6) and required anti-CD3 ligation (lane 10 vs. 9). Rabbit anti-mouse served as a negative control (lane 11). The identity of each band was confirmed by blotting with individual antibodies and the expression of endogenous SKAP-55 is low in these cells. These data confirmed that TCR/CD3 induced Rap1-RapL complex formation required SKAP-55.

The same result was obtained when anti-V5 (RapL) was used to precipitate protein (Fig. 3). While co-expression of Rap1 and RapL failed to allow for anti-V5 (RapL) co-precipitation of Rap1 (lanes 3,4), the co-expression of SKAP-55 allowed for co-precipitation of Rap1V12 (lane 6) (lowest band under the light chain of IgG). Anti-CD3 ligation was needed (lane 6 vs. 5). Rabbit anti-mouse served as a negative control (lane 7). Anti-myc (Rap1V12) and anti-V5 (RapL) also co-precipitated SKAP-55 under conditions of SKAP-55 co-expression as detected by anti-GFP or anti-SKAP-55 (Fig. 2, lane 10 and Fig. 3, lane 7, respectively). This also required anti-CD3 ligation (Rap1V12 and GFP-tagged SKAP-55 were expressed in Jurkat T-cells, lysed, subjected to anti-myc (Rap1V12) precipitation and blotted with anti-SKAP-55, anti-V5 (Rap1) and anti-myc (Rap1V12) (Fig. 2). Rap1V12 is the constitutively active form of Rap1. Contrary to a previous report<sup>26, 37</sup>, co-expression of RapL and Rap1 alone failed to allow for co-precipitation of RapL (V5) with anti-myc (Rap1). Specifically, RapL was absent from cytosolic (lanes 3,4) and membrane fractions (lanes 7,8) as well as from resting (lanes 3, 7), or anti-CD3 ligated cells (lanes 4, 8). It was also not observed with different concentrations of anti-CD3 antibody (1-10µg/ml). In marked contrast, the mere expression of SKAP-55 with RapL and Rap1-V12 resulted in anti-myc (Rap1V12) coprecipitation of RapL as well as SKAP-55 (lane 10). This occurred in the membrane (lane 10), but not the cytosolic fraction (lane 6) and required anti-CD3 ligation (lane 10 vs. 9). Rabbit anti-mouse served as a negative control (lane 11).

The identity of each band was confirmed by blotting with individual antibodies and the expression of endogenous SKAP-55 (SKAP1) is low in these cells. These data confirmed that TCR/CD3 induced Rap1-RapL complex formation required SKAP-55 (SKAP1).

The same result was obtained when anti-V5 (RapL) was used to precipitate protein (Fig. 3). While co-expression of Rap1 and RapL failed to allow for anti-V5 (RapL) co-precipitation of Rap1 (lanes 3,4), the co-expression of SKAP-55 allowed for co-precipitation of Rap1V12 (lane 6) (lowest band under the light chain of IgG). Anti-CD3 ligation was needed (lane 6 vs. 5). Rabbit anti-mouse served as a negative control (lane 7). Anti-myc (Rap1V12) and anti-V5 (RapL) also co-precipitated SKAP-55 under conditions of SKAP-55 co-expression as detected by anti-GFP or anti-SKAP-55 (Fig. 2, lane 10 and Fig. 3, lane 7, respectively). This also required anti-CD3 ligation (Fig. 2, lane 10 vs. 9 and Fig. 3, lane 7 vs. 6, respectively). Conversely, anti-SKAP-55 was also able to co-precipitate RapL (Fig. 4, lane 5) under conditions of V5-RapL, myc-Rap1V12 and GFP-SKAP-55 co-expression with anti-CD3 ligation (lane 5 vs. 8).

Importantly, active Rap1V12 but not inactive Rap1N17 supported anti-SKAP-55 co-precipitation of RapL (Fig. 5, middle panel, lane 8 vs. 9), and neither SLP-76 nor ADAP (the binding partner of SKAP-55) could substitute for SKAP-55 (lanes 10, 11 vs. 8). In this instance, SKAP-55, ADAP and SLP-76 were tagged with HA. This observation indicated that SKAP-55 plays a specific role in promoting Rap1-RapL complex formation that cannot be replaced by SLP-76 or ADAP. Moreover, this data indicates that complex formation depends on active Rap1.

Expression of the various proteins was confirmed by blotting of cell lysates (panels, lanes 1-6), or in anti-SKAP-55 (upper panel, lanes 7-9), anti-SLP-76 (lane 10) or anti-ADAP precipitations (lane 11). Taken together, these observations confirmed using a different approach that SKAP-55 was needed for Rap1-RapL complex formation in T-cells, and that SKAP-55 becomes part of the complex in response to anti-CD3 ligation.

### 2.3 SKAP-55 (SKAP1) N-terminal domain binds RapL C-terminal coiled-coil (SARAH) domain

Unlike with Rap1V12-RapL co-expression, SKAP-55 and RapL co-expression often resulted in a low level of co-precipitation (an example in Fig. 6, lane 2). This binding increased with Rap1V12 co-expression (lane 4), but provided indication that SKAP-55 might bind directly to RapL. To confirm and define the sites of binding, GST fusion proteins encoding different regions of SKAP-55 were used in a pull-down assay from lysates of cells transfected with wild-type V5-tagged RapL, or a truncated version of RapL lacking the C-terminal coiled coiled SARAH domain (Fig. 7). The pull-down assay using glutathione sepharose beads was followed by blotting with anti-V5 as previously described<sup>50, 57</sup>. The GST fusion proteins included full-length SKAP-55, N-terminal (N-SKAP-55; residues 1-104), SK region (SK; residues 209-285), or N plus PH and SK regions (N-PH-SK; residues 1-285) (see upper diagram). While WT, N and N-PH-SK readily precipitated V5 tagged RapL (upper panel, lanes 3,4,6), GST alone and GST-SK failed to precipitate the protein (lanes 2 and 5, respectively). Anti-GST blotting confirmed expression of the constructs (lower panel). These data indicated that the N-terminus of SKAP-55 (N-SKAP-55) interacts with RapL. In this assay, deletion of the C-terminal coiled-coiled (SARAH) domain of RapL (i.e. RapL-ΔC2) completely abrogated binding to WT, N and N-PH-SK RapL (middle panel, lanes 3-6), suggesting that N-SKAP-55 binds to the RapL SARAH domain (i.e. C-RapL).

To test this directly, GST-SKAP-55 domains were incubated with lysates of cells expressing V5-C-RapL and assessed for binding (Fig. 8). Full length SKAP-55 WT, N and N-PH-SK precipitated C-RapL (lanes 3,4,5), while GST alone, GST-SK or GST-SH3 failed to precipitate the protein (lanes 2,6 and 7, respectively). Anti-GST blotting confirmed expression of the

constructs (lower panel). These data indicated that N-SKAP-55 interacts with coiled-coil C-RapL SARAH domain. Unlike the C-terminal SARAH domain of RapL, the N-terminus of SKAP-55 does not form a coiled-coil domain using protein sequence-structure programs (i.e. 3D-ALI, BIOSCAN etc.)<sup>54, 55, 63, 64</sup>. By contrast, residues 12-64 of the SKAP-55 homologue SKAP-55R/Hom (SKAP2) does form a coiled-coil domain<sup>54, 55</sup>, that under certain conditions can dimerize<sup>63</sup>. However, molecular modelling of the SKAP-55 regions based on the structure of the N/PH domains of SKAP-55R/Hom<sup>63</sup> predicts that N-SKAP-55 would form two similar alpha helical structures. One or both alpha helices with alternating leucines within N-SKAP-55 could possibly form the basis of a binding region for the RapL coiled-coil alpha helix. To assess directly the interaction between N-SKAP-55 and C-RapL, individual N-SKAP-55 and C-RapL domains were purified as GST-fusion proteins followed by Factor X cleavage and purification of individual domains (see *Material and Methods*). Silver staining of material showed the presence of individual subunits without contaminating material.

An interaction between these proteins was then examined using isothermal titration calorimetry (ITC), a method that measures stoichiometry, binding constants and thermodynamic parameters in a single interaction (Fig. 9). The ITC data determined at 25°C clearly demonstrated that the SARAH RapL Cterminal coiled-coil domain interacts directly with N-SKAP-55 with a stoichiometry which approximates to unity ( $n = 0.85$ ). The equilibrium dissociation constant,  $K_d$ , of 0.6  $\mu$ M corresponds to a change in free energy of binding ( $\Delta G$ ) of -8.5 kcal/mol. The change in enthalpy of binding is relatively small ( $\Delta H = -2.3$  kcal/mol) and hence, at the experimental temperature, the interaction is entropy driven ( $T \Delta S = 6.2$  kcal/mol). This is likely to indicate that the interaction is accompanied by a significant contribution from the release of solvent molecules

from the surface area that is buried on forming the complex. These observations clearly indicated that the N-SKAP-55 binds directly and on a one-to one basis to the C-RapL SARAH domain.

5    2.4 RapL-L224A mutation that selectively disrupts SKAP-55 binding without affecting MST-1 binding

N-SKAP-55 binding to C-RapL was next confirmed by deletion and mutation analysis (Fig. 10). RapL mutants tested included RapLAC (residues 1-222; lacking the entire SARAH-coiled-coil domain), RapLAC2 (residues 1-243; lacking part of the SARAH domain), RapLAC3 (residues 1-254; C-terminal and outside the SARAH domain) or, alternatively, full length RapL with point mutations at residues L224A (RapL L224A) and L253A (RapL L253A) within the coiled-coil domain (see upper diagram). While anti-SKAP-55 precipitated wild-type and the RapLAC3 mutant (lanes 7 and 10, respectively), it failed to precipitate the RapLAC2 and RapLAC mutants (lanes 8 and 9, respectively). Each of the V5-tagged constructs and GFP-SKAP-55 was expressed as seen in cell lysates (upper and lower panels, lanes 1-6).

The same result was obtained in GST pull-down assays (Fig. 11). WT V5-RapL, V5-RapL-L224A and V5-RapL-L253A were expressed and assessed for binding to the different regions of SKAP-55. While the N-SKAP-55 precipitated WT RapL and the L253A mutant (upper and lower panels, lane 4), it failed to precipitate the L224A single site mutant (middle panel, lane 4). These data showed by two independent approaches that the RapL-L224A mutation disrupts SKAP-55 N-terminal binding. While N-SKAP-55 bound to coiled-coil domain of RapL, the serine-threonine kinase MST-1 has also been reported to bind to the same region in RapL27. MST-1 binds via its coiled-coiled domain, and until now, had been the only protein reported to bind the RapL SARAH domain<sup>27</sup>. To define the function of the SKAP-55-RapL interaction, and to distinguish

it from the MST-1-RapL interaction, it was therefore necessary to identify a RapL mutant that would abrogate SKAP-55 binding without affecting the binding of MST1. To assess MST-1 binding to RapL, myc tagged MST-1 and V5-tagged RapL were co-expressed followed by an antimyc (MST-1) precipitation and anti-V5 blotting for RapL (Fig. 11). Co-expression of MST-1 and RapL showed clear binding (lane 1), consistent with previous reports<sup>27</sup>. However, unlike SKAP-55, MST-1 also bound to the L224A mutant (lane 5 vs. 1, 4, 6). By contrast, as a control, deletion of the coiled-coil domain in RapL $\Delta$ C and RapL $\Delta$ C2 eliminated MST-1 binding confirming binding to the coiled-coil domain (lanes 2,3 vs. 1). Rabbit anti-mouse served as a negative control (lane 7). This observation made the important finding that MST1 binds to the RapL-L224A mutant that is defective in binding to SKAP-55.

Given this, it was next important to assess whether the L224A mutation could disrupt Rap1-RapL complex formation. RapL or RapL-L224A was coexpressed with Rap1V12 in the presence or absence of SKAP-55 and examined for Rap1 precipitation of RapL (Fig. 12). While co-expression of SKAP-55, RapL and Rap1V12 allowed anti-myc (Rap1v12) precipitation of V5-RapL (lane 3), RapL-L224A failed to support this (lane 4). Blotting against cell lysates confirmed the presence of Rap1V12 and RapL in cell lysates (lanes 1,2). Anti-SKAP-55 also co-precipitated RapL from SKAP-55, RapL, Rap1 (upper panel, lane 8), but not SKAP-55, RapL-L224A, Rap1 transfected cells (lane 9). Anti-RapL blotting confirmed the presence of RapL expression in both sets of cell lysates (upper panel, lanes 6,7). Conversely, anti-V5 (RapL) precipitated SKAP-55 from SKAP-55, RapL and Rap1 expressing cells (lower panel, lane 10), but not from cells expressing SKAP-55, RapL-L224A and Rap1 (lane 11). These data indicated that N-SKAP-55 binding to the RapL-L224 site is the key event needed to regulate Rap1- RapL complex formation in T-cells.



### 2.5 SKAP-55-RapL regulates RapL-Rap co-localization

Identification of the RapL-L224 mutant then allowed an examination of the role of N-SKAP-55-C-RapL binding in intracellular localization and immune function (Figs 13 to 5 15). Cells transfected with RapL-CFP, SKAP-55-GFP and Rap1V12-mcherry were seeded on cover-slips with immobilized anti-CD3 antibody and ICAM-1 and imaged at the contact area by confocal immunofluorescence microscopy, as previously described<sup>66,67</sup>. Cells with only low-moderate intensities of expression were 10 imaged to avoid problems with protein over-expression. Using three-color immunofluorescence, RapL-CFP, SKAP-55-GFP and Rap1V12-mcherry were seen localized in clusters in resting and anti-CD3 ligated T-cells over 0-240 seconds (Fig. 13, upper panels). Anti-CD3 did not appear to alter the number or size 15 of the clusters. Further, RapL-L224A also localized in clusters (lower panel). In addition, SKAP-55-GFP and Rap1V12-mcherry were also found in clusters in cells co-transfected with the RapL-L224A mutant. No obvious difference was noted in the average size or number of clusters in RapL or RapL-L224A 20 transfected cells. Further, although difficult to visualize by eye, all clusters migrated with similar speeds along the peripheral contact region in RapL and RapL-L224A of anti-CD3 ligated transfectants as assessed by Volocity software analysis (Improvision).

25 The single consistent difference between RapL and RapL-L224A was in colocalization with RapL and SKAP-55 (Fig. 14). This was again determined by the derivation of Pearson's correlation coefficients (PCCs) using Volocity software. 30 RapL and Rap1V12 showed low PCC values in resting and anti-CD3 ligated cells (i.e. average PCC=0.09 and 0.12, respectively) in the absence SKAP-55 (upper panel). By contrast, while SKAP-55 co-expression had little effect on Rap1-RapL co-localization in resting cells (i.e. PCC=0.14), it 35 significantly increased RapLRap1V12 co-localization in anti-

CD3 activated cells (i.e. average PCC value=0.34). Similar values have been reported for other co-localized proteins such as cytokines and Rab3d68. This indicated that SKAP-55 is needed for RapLRap1V12 co-localization, an observation

5 consistent with the co-precipitation results (Figs. 1, 2). In striking contrast, RapL-L224A failed to support RapLRap1V12 co-localization in resting, or anti-CD3 ligated cells (i.e. average PCC=0.1 and 0.13, respectively). This was observed over the entire time-course of imaging. Further, similar

10 differences were noted in the SKAP-55-RapL co-localization (lower panel). The limited colocalization in resting cells increased with anti-CD3 ligation (i.e. average PCC=0.23 and 0.55, respectively). However, little SKAP-55 co-localization with RapL-L224A was observed in response to anti-CD3 (i.e.

15 average PCC=0.2), or over a time-course. Similar observations were made when soluble anti-CD3 was used to activate T-cells on plates coated with ICAM-1 (Fig. 14). In this instance, cells imaged from above showed RapL-CFP, SKAP-55-GFP and Rap1V12-mcherry clusters in vesicular-like structures at the

20 polarized edge of cells next to membrane protrusions (upper panels). RapL and Rap1V12 co-localized in response to anti-CD3 as seen over the time course (PPC values from 0.14 to 0.6) (lower histogram). Similarly, RapL and Rap1V12 co-localized with SKAP-55 (i.e. PPC values of 0.6), while RapL-L224A failed

25 to support co-co-localization of any of the proteins (i.e. PPC values of 0.1-0.18). Similarly, SKAP-55 failed to co-localize with RapL (0.6 vs. 0.15) or RapV12 (.06 vs. 0.15). These data showed that although WT and L224A RapL can both be found in vesicular structures, N-SKAP-55 binding to C-RapL still

30 determined co-localization in anti-CD3 activated T-cells. Incubation of cells on cover-slips coated with immobilized anti-CD3 eventually (i.e. 5-15min) led to the formation of a peri-cellular region in many cells that resembled the peripheral supra-molecular complex (pSMAC) as described by

35 others<sup>6, 69</sup>. The pSMAC is enriched with LFA-1 molecules, while

the cSMAC is comprised of the TcR, CD28 and other signaling proteins<sup>2, 6</sup>. In this case, imaging was conducted at the contact area between the T-cell and anti-CD3 covered cover-slip surface (as in panel a) (Fig. 15). RapL and SKAP-55 showed close co-localization in this region (panels a, b). In the case of Rap1V12, the pattern showed two areas, one enriched in the ring together with lesser amounts in the inner region (panel c). By contrast, co-expression of RapL-L224A with SKAP-55 and Rap1V12 showed a loss of the SMAC-like region, instead showing a diffuse pattern for RapL-L224, SKAP-55 and Rap1V12 at the interface (lower panels a, b, c). N-SKAP-55 binding to C-RapL therefore regulates the ability of the three components to co-localize in clusters and in the pSMAC-like formation.

15

#### 2.6 N-SKAP-55-C-RapL binding regulates RapL binding to LFA-1

Initially, co-localization studies were conducted with Jurkat cells co-transfected with RapL/RapL-L224A-CFP, SKAP-55-GFP and LFA-1 (CD11a)-mcherry and examined by immunofluorescent microscopy<sup>66</sup>. The transfected CD11a-mcherry was confirmed to be active in transfection-ICAM-1 binding assays (data not shown). RapL co-localized with CD11a in response to anti-CD3 ligation as judged by PCCs (i.e. average PCC from 0.14 to 0.45) (lower left histogram). By contrast, RapL-L224A failed to colocalize (i.e. approx. 0.18).

25

Binding between RapL and LFA-1 was assessed biochemically by anti-V5- RapL precipitation from cells expressing SKAP-55, RapL or RapL-L224A and Rap1V12 followed by blotting with anti-CD18 (Fig. 16). CD18 (or ITGB2) is the beta 2 subunit that pairs with CD11a of LFA-1. Significantly, anti-V5-RapL failed to precipitate CD18 from cells transfected with RapL and Rap1V12 (upper right panel, lanes 5-8), neither from the cytosolic (lanes 5,6) nor membrane fractions (lanes 7,8). By contrast, SKAP-55 co-expression with RapL and Rap1V12 allowed

35

anti-RapL co-precipitation of CD18 from the membranes of cells in response to anti-CD3 (lane 4). Rabbit anti-mouse served as a negative control (lane 9).

5 Unlike with wild-type RapL (lower panel, lane 3), co-expression of RapL-L224A with SKAP-55 and Rap1V12 failed to co-precipitate LFA-1 from the membrane fraction (lane 4). Rabbit anti-mouse served as a negative control (lane 5). Similarly, anti-SKAP-55 co-precipitated CD18 from membranes of  
10 anti-CD3 activated cells expressing RapL, Rap1 and SKAP-55 (Fig. 17, lane 6). Lastly, the complex could also be precipitated from primary SKAP-55+/+, but not from SKAP-55-/- T-cells (Fig. 18). Anti-SKAP-55 co-precipitated endogenous CD18 in a manner dependent on anti-CD3 ligation in SKAP-55+/+  
15 T cells (upper panel, lane 3 vs. 4). Conversely, anti-CD18 co-precipitated SKAP-55 (middle panel, lane 6 vs. 5). Further, both anti-SKAP-55 and anti-CD18 co precipitated RapL and this was increased with anti-CD3 ligation (lower panel, lanes 4 vs. 3 and 6 vs. 5, respectively). Rabbit anti-mouse served as a  
20 positive control (lane 7).

In a comparison of primary SKAP-55+/+ vs. SKAP-55-/- primary T-cells, anti-CD18 co-precipitated SKAP-55 (upper panel, lanes 10 vs. 9), RapL (middle panel, lanes 10 vs. 9) and Rap1 (lower  
25 panel, lanes 10 vs. 9). By contrast, anti-CD18 co-precipitated little if any of these proteins from SKAP-55-/- cells (all panels, lanes 11, 12). Collectively, these observations clearly indicated that SKAP-55 expression is needed for the binding of the SKAP-55-RapL-Rap1 complex to LFA-1.

30

#### 2.7 RapL224A disrupts LFA-1-ICAM1 binding and T-cell/conjugate formation

Lastly, to test whether SKAP-55-RapL binding was needed for LFA-1 mediated adhesion, T-cells were cultured on tissue

culture plates coated with ICAM-1 in the presence or absence of soluble anti-CD3 and assessed for binding, as previously described<sup>34, 35, 51</sup> (Fig. 19). In these experiments, RapL and SKAP-55 was used to transfect cells, although the additional co-expression of Rap1V12 gave similar results. Anti-CD3 increased binding of vector-transfected cells to ICAM-1 on plates by 3-4 fold, while co-expression of SKAP-55 and RapL increased adhesion further. However, neither RapLΔC2 lacking RapL coiled-coil (SARAH) domain, nor the RapL-L224A mutant supported an increase in binding. In most experiments, L224A acted as a dominant negative in inhibiting anti-CD3 induced adhesion. In keeping with this, RapL-L224A also failed to support conjugate formation between T-cells and APCs (Fig. 19). T8.1 cells used for this analysis express a TcR that reacts with Ttox peptide as presented by I-Ak on L625 cells<sup>70, 71</sup>. The T/APC ratio corresponds to the number of adherent T cells in a given field (i.e. at least three fields with greater than 60APCs) divided by the number of APCs in the same field. While the addition of Ttox peptide increased conjugate formation by 2-fold (i.e. from 1.8 to 4.0 T/APC ratio) in the GFP transfected control, SKAP-55/RapL co-expression increased conjugate formation by 10 fold (i.e. from 1.8 to 12.0 T/APC ratio). By contrast, the RapLL224A mutant failed to support an increase in conjugation. These data showed that SKAP-55 binding to RapL is needed for T-cell-APC conjugation.

### 2.8 PH domain of SKAP1 is required for translocation of RapL

The effect of the PH domain of SKAP1 (SKAP55) on RapL translocation to the membrane was investigated. While anti-CD3 induced WT Flag-tagged SKAP1 translocation to the membrane fraction (Fig 21 upper panel, lanes 5 vs. 4), V5-tagged RapL failed to translocate in the presence of SKAP1-R131M (Fig 21 middle upper panel, lanes 9, 10). The PH domain of SKAP1 is therefore needed to shuttle RapL to the membrane where it can interact with Rap1.

### 2.9 RapL translocation requires phosphoinositide 3-kinase

T-cells were ligated with anti-CD3 for 10min in the absence or presence of phosphoinositide 3-kinase inhibitors LY294002 and wortmannin. Cytosolic and membrane fractions of T-cells were then purified and subjected to blotting with anti-SKAP1, RapL, Rap1 and actin (Fig 22 upper panels). While anti-CD3 induced the appearance of SKAP1 in the membrane fraction of untreated cells, incubation with LY294002 or wortmannin was found to inhibit the translocation from the cytosol (Fig 22 lanes 11, 12). Lower panel is a positive control showing that PI 3K inhibitors inhibit ATK (PKB) as shown by the reduced phosphorylation of the AKT target GSK3 (Fig 22; lanes 3,4 vs. 2). This data shows that RapL translocation to the membrane requires phosphoinositide 3-kinase.

### 2.10 L25 of N-SKAP1 interacts with RapL

The effect of mutation of the L25A and L30A residues on N-SKAP1 binding to RAPL was investigated. While anti-GST was found to co-precipitate V5-RapL in GST-WT and GST-L30A transfected cells, anti-GST failed to precipitate V5-RapL in GST-L25A transfected cells (Fig 23). Anti-GST blotting confirmed the expression of GST-SKAP1, SKAP1-L25A and SKAP1-L30A. Mutation of L25 therefore disrupts the GST precipitation of V5 RapL (Fig 23; lane 4 vs. 3 and 5). These data confirm that the N-terminus of SKAP1 interacts with RapL and residue L25 of N-SKAP1 is needed for the interaction.

### 2.11 SKAP1 required for T cell slowing in response to SEA

The effect of RAPL on the TCR induced 'slowing' of T-cell motility in the presence and absence of SKAP1 was investigated in lymph node slices (Fig 24). T-cells from *Skap1*<sup>+/+</sup> vs. <sup>-/-</sup> mice were layered on nodes and followed over time in the presence of SEA super-antigen. In the absence of SKAP1, RAPL did not cause 'slowing' of T-cell motility. The poor ability

of *Skap1*<sup>-/-</sup> cells to 'slow' in response to SEA relative to *Skap1*<sup>+/+</sup> T-cells is consistent with the notion that SKAP1 and SKAP1 binding to RapL regulates the slowing of T-cells and the ability of T-cells to interact with dendritic cells for a response to antigen.

Ligation of the antigen-receptor generates key 'inside-out' signals that upregulate LFA-1 adhesion on T-cells<sup>1-5</sup>. This pathway is of central importance to multiple immune functions including antigen-presentation, migration in lymph nodes and to sites of inflammation<sup>48</sup>. Despite this, the 'inside-out' pathway has yet to be clearly defined. To date, two modules have been defined that include Rap1 and binding partners RapL and Riam<sup>26-28, 37</sup>, and another involving immune cell adaptors SLP-76, ADAP and SKAP1 (SKAP-55)<sup>30, 31, 34, 45, 48, 61, 74</sup>. In this study, we have defined a novel pathway that integrates the two modules by showing that SKAP1 is an obligatory upstream regulator of TCR induction of the Rap1-RapL pathway, the formation of a SKAP1-RapL-Rap1 complex and the binding of the complex to LFA-1 in T-cells. The Rap1-RapL complex and binding to LFA-1 failed to occur in *SKAP1*<sup>-/-</sup> T-cells, and in co-expression studies involving Rap1-RapL expression without SKAP1. N-SKAP1 clearly bound to the C-RapL SARAH domain (i.e. K<sub>d</sub>= 0.6uM), and a RapL mutant (L224A) that selectively disrupted SKAP1 (without affecting MST-1 binding) abrogated RapL-Rap1 complex formation, binding to LFA-1, co-localization, as well as anti-CD3 induced LFA-1 adhesion and T-cell-APC conjugate formation. Overall, our identification of a novel pathway that accounts for 'inside-out' signalling will have important implications for multiple aspects of T-cell immunity.

In this study, the requirement for SKAP1 in anti-CD3 driven Rap1-RapL binding was clear as demonstrated by the absence of complex formation in *SKAP1*<sup>-/-</sup> T-cells, and in co-expression

studies with Rap1 and RapL lacking the adaptor. In all cases, complex formation was restored with the simple expression of SKAP1, while specificity was shown by the fact that neither ADAP nor SLP-76 could substitute for SKAP1 in promoting Rap1-RapL binding. Further, the mutation of single amino acid in the RapL SARAH domain (i.e. L224A) was sufficient to interfere with SKAP1 binding, and potentially blocked Rap1-RapL co-localization, complex formation, LFA-1 binding and the induction of LFA-1 clustering and adhesion.

10

This SKAP1-RapL-Rap1 pathway can now account for SKAP1 effector function in adhesion where  $\beta$ -2 adhesion is defective in *SKAP1*<sup>-/-</sup> T-cells<sup>33, 48</sup>. In this case, two immune cell specific molecules and domains appear to have evolved to couple the TCR/CD3 complex in an 'inside-out' signalling pathway to the Rap1 and RapL pathway for adhesion. One prediction is that the SKAP1 homologue SKAP-55R/Hom (SKAP2) will play an analogous role in B cells.

15

SKAP1 regulation of the RapL-Rap1 module and LFA-1 adhesion in T-cells may control multiple aspects of T-cell immunity where TCR ligation is needed to up-regulate adhesion. Expression of RapL-L224A mutant disrupted ICAM1 binding and conjugate formation between T-cells and antigen presenting cells (APCs) (Fig. 20). Migration in sites of inflammation and peripheral tissues lymph nodes is also likely to be influenced by SKAP1-RapL complex formation. Importantly, we also showed that SKAP1 N-terminal region binds directly to the coiled-coil SARAH domain of RapL. Binding was established using a variety of techniques including co-precipitation, GST-pull down assays, site-directed mutagenesis and isothermal titration calorimetry of isolated protein domains. N-SKAP1 binding to the C-RapL SARAH domain of RapL was found to have an equilibrium dissociation constant of 0.6 $\mu$ M and a stoichiometry close to 1 (0.85). This slight discrepancy may be related to water or

30

35



other contaminants. N-SKAP1 therefore binds on a one-to-one basis to CRapL, while Rap1-GTP also binds to the RBD domain of RapL with a one-to-one stoichiometry. Different regions in RapL therefore bind to SKAP1 and Rap1 leading to the formation of a trimeric complex. Consistent with this, antibodies to SKAP1, RapL and Rap1 precipitated each other in reciprocal manner.

Further, anti-CD11a could also precipitate SKAP1, RapL and Rap1 from cells. The dissociation constant of 0.6 $\mu$ M for N-SKAP1-C-RapL binding is in the range of other interactions involved signal transduction such as in the case of SH3 domain binding to proline-based ligands<sup>75</sup>.

The unique nature of SKAP1-RapL binding allowed the identification of a single site mutation in RapL (i.e. L224A) that disrupted SKAP1 binding without affecting the binding of Mst-1. Loss of SKAP1 binding in turn had a major effect on multiple aspects of 'inside-out' signalling. These included a marked loss of Rap1-RapL co-localization and binding, LFA-1-ICAM-1 adhesion and T-cell-APC conjugate formation as well as Rap1-RapL-SKAP1 complex binding to LFA-1. The potency of the effect of a single mutation within a single molecule on a variety of events underscores the importance of the SKAP1-RapL interaction to T-cell biology. The mutant also distinguishes the role of C-RapL binding to SKAP1 from its binding to Mst-1, a kinase that also co-localizes with RapL in vesicular compartments at the leading edge of moving cells and that is required for cell polarization and integrin LFA-1 clustering adhesion<sup>2,26,27</sup>. Our imaging studies also showed that SKAP1, RapL and Rap1 clusters, often localized in vesicular structures in T-cells. RapL localization in vesicles as been observed by others<sup>26,27</sup>. SKAP1, RapL and Rap1 co-clusters in vesicles were generally found at the frontal concentric area of the cell next to extending membrane extensions, but not in the

extensions themselves. Interestingly, mutation of the SKAP1 binding site on RapL did not affect the presence of SKAP1, RapL and Rap1 in these clusters, but rather markedly reduced their more intimate co-localization as determined by Pearson's correlation coefficients following TCR ligation. Rap1-RapL, SKAP1-RapL and SKAP1-Rap1 were affected such that the PCC values were similar to those observed in resting cells. This is consistent with anti-CD3 induction of binding between these proteins as detected in biochemical assays (Figs. 1-15). Co-localization in pSMAC-like structures was also disrupted. In our model, N-SKAP1 binding to the coiled-coil domain of RapL is the crucial step in the regulation of the TCR mediated 'inside-out' signalling for LFA-1 adhesion (see model Fig. 20). The most straightforward model involves TCR induced ADAP phosphorylation at YDDV sites<sup>49-50, 59</sup> that allows SLP-76 binding, together with ADAP binding to the SH3 domain of SKAP1<sup>45-48, 61</sup>. Concurrent with this, TCR ligation would convert Rap1 to a GTP-bound form that binds to RapL under the obligatory regulation by SKAP1. Rap1 binding to GTP is itself has not been found altered by reduced SKAP1 expression<sup>58</sup>. Optimal SKAP1-RapL binding also required active Rap1.

Rap1-GTP binding to the RBD domain of RapL may work by potentially opening and stabilizing access to the C-terminus of RapL. Conversely, N-SKAP1 binding to C-RapL may help stabilise access of Rap1-GTP to the RBD domain of RapL. Intramolecular binding within NorelA has been reported with the interaction of Ras/Rap1-GTP with the RA domain of mNorel that is weakened significantly by the binding of the C1 domain to the RA domain<sup>79</sup>. Taken together, this leads to the formation of a minimal trimeric complex that binds to the  $\alpha$ L chain of LFA-1. The complex must be quite stable since it could withstand co-precipitation in detergent. RapL has been reported to bind to K1097/K1099 residues in  $\alpha$ L tail<sup>76 26</sup>. While previous reports indicated that this might occur in a manner involving only

Rap1 and RapL tail<sup>76 26</sup>, our findings now demonstrate that this is orchestrated upstream by SKAP1.

Anti-CD3 ligation was needed for SKAP1 regulation of 'inside-out' pathway. In no experiment was SKAP1 able to induce Rap1V12-RapL complex formation or it's binding to LFA-1 without anti-CD3 engagement. This is consistent with specificity in the role for SKAP1 in linking the TCR complex with LFA-1 adhesion.

The SKAP1 PH domain should bind phosphatidylinositol (3,4,5)-trisphosphate (PI-3,4,5-P3) for membrane localisation and does not have myristylation sites etc. The PH domain may also account for the need of an additional TCR signal for SKAP1-RapL function where complex formation in the presence of constitutively active Rap1 (i.e. Rap1V12) still required anti-CD3 ligation.

We inactivated the PH domain (i.e. SKAP1-R131M) and showed that RapL was unable to translocate in the presence of SKAP1-R131M (Fig. 21, lanes 9, 10). Rap1 was constitutively associated with membranes in both WT and SKAP1-R131M transfected cells (lower middle panel, lanes 4,5,9,10).

Inhibitors of PI 3K, wortmanin and LY294002 also blocked SKAP1 and RapL translocation (Fig. 22; upper and upper middle panels). We had also identified a specific SKAP1 site that binds RapL. GST tagged SKAP1 and mutants L25A and L30A were co-expressed with V5 RapL and assessed for co-precipitation of RapL (Fig. 23). This clearly showed that the mutation of leucine 25 to alanine (SKAP1-L25A) disrupts RapL binding. We have therefore identified key residues in SKAP1 and RapL that are responsible for the interaction.

Lastly, we have examined the T-cell motility of *SKAP1*<sup>+/+</sup> and -/- T-cells in lymph node slices (Fig. 24). In wild-type cells, the addition of SEA reduced the motility in the nodes, while *SKAP1* knock-out cells did not slow in response to SEA.

5

This is an important result, showing that *SKAP1* is needed for the slowing of T-cells in response to TCR ligation. *SKAP1* (upstream of RapL) is therefore needed for slowing of T-cell motility which in turn is needed for interactions with

10 antigen-presenting cells and an optimal T-cell response.

References

1. Butcher, E.C. *Cell* 67, 1033-1036 (1991).
2. Dustin, M.L. et al *Nat Immunol* 5, 363-372 (2004).
3. Hogg, N et al *J Cell Sci* 116, 4695-4705 (2003).
- 5 4. Carman, C.V. et al *Curr Opin Cell Biol* 15, 547-556 (2003).
5. Hynes, R.O. *Cell* 110, 673-687 (2002).
6. Kupfer, A. *Immunity* 25, 11-13 (2006).
7. Kupfer, A. & Kupfer, H. *Semin Immunol* 15, 295-300 (2003).
8. Dustin, M.L. *Semin Immunol* 17, 400-410 (2005).
- 10 9. Davis, D.M. et al *Trends Immunol* 25, 323-327 (2004).
10. Takagi, J. et al *Immunol. Rev* 186, 141-163 (2002).
11. Takagi, J. et al *Cell* 110, 599-511 (2002).
12. Lupher, M.L., Jr. et al. *J Immunol* 167, 1431-1439 (2001).
13. Xiong, J.P. et al. *Science* 294, 339-345 (2001).
- 15 14. Jun, C.D. et al. *J Biol Chem* 276, 29019-29027 (2001).
15. Rudd, C.E. et al. *Immunol Today* 15, 225-234 (1994).
16. Labno, C.M. et al. *Curr Biol* 13, 1619-1624 (2003).
17. Krawczyk, C., A. et al. *Immunity* 16, 331-374 (2002).
18. Shimizu, Y. et al *J Cell Biol* 131, 1867-1880 (1995).
- 20 19. Zell, T. et al *J Immunol* 156, 883-886 (1996).
20. Collins, T.L. et al *Immunol Today* 18, 221-225 (1997).
21. Mor, A. et al *Immunol Rev* 218, 114-125 (2007).
22. Katagiri, K. et al *J Biol Chem* 279, 11875-11881 (2004).
23. Katagiri, K. et al. *Mol Cell Biol* 20, 1956-1969 (2000).
- 25 24. Shimonaka, M. et al. *J Cell Biol* 161, 417-427 (2003).
25. Bivona, T.G. et al. *J Cell Biol* 164, 461-470 (2004).
26. Katagiri, K. et al *Nat Immunol* 4, 741-748 (2003).
27. Katagiri, K. et al *Nat Immunol* 7, 919-928 (2006).
28. Lafuente, E.M. et al. *Dev Cell* 7, 585-595 (2004).
- 30 29. Kuhne, M.R. et al. *J Immunol* 171, 860-866 (2003).
30. Griffiths, E.K. et al *Science* 293, 2260-2263 (2001).
31. Peterson, E.J. et al. *Science* 293, 2263-2265 (2001).
32. Geng, L. et al *PNAS U S A* 98, 11527-11532 (2001).
33. Wang, H. et al. *Mol Cell Biol* 27, 6863- 6875 (2007).
- 35 34. Wang, H. et al. *Nat Immunol* 4, 366-374 (2003).

35. Jo, E.K. et al J Exp Med 201, 1733-1739 (2005).
36. Schneider, H. et al Adv Exp Med Biol 584, 115-126 (2006).
37. Katagiri, K. et al. Nat Immunol 5, 1045-1051 (2004).
38. Reedquist, K.A. et al. J Cell Biol 148, 1151-1158 (2000).
- 5 39. Caron, E. et al Curr Biol 10, 974-978 (2000).
40. Sebzda, E. et al Nat Immunol 3, 251-258 (2002).
41. Menasche, G. et al. Mol Cell Biol 27, 4070-4081 (2007).
42. Tommasi, S. et al. Oncogene 21, 2713-2720 (2002).
43. Ura, S. et al PNAS U S A 98, 10148-10153 (2001).
- 10 44. Ura, S. et al Genes Cells 6, 519-530 (2001).
45. Rudd, C.E. Cell 96, 5-8 (1999).
46. Jordan, M.S. Nat Immunol 4, 110-116 (2003).
47. Samelson, L.E. Annu Rev Immunol 20, 371- 394 (2002).
48. Wang, H. et al Trends Cell Biol. 18 :486-93 (2008).
- 15 49. Veale, M. et al. J Biol Chem 274, 28427-28435 (1999).
50. Raab, M. et al J Biol Chem 274, 21170-21179 (1999).
51. Wang, H. et al. J Exp Med 200, 1063-1074 (2004).
52. da Silva, A.J. et al. PNAS U S A 94,7493-7498 (1997).
53. Musci, M.A. et al. J Biol Chem 272, 11674-11677 (1997).
- 20 54. Marie-Cardine, A. et al. JBC 272, 16077-16080 (1997).
55. Liu, J. et al. PNAS U S A 95, 8779-8784 (1998).
56. Marie-Cardine, A. et al. JBC 273, 25789-25795 (1998).
57. Kang, H. et al. EMBO J 19, 2889-2899 (2000).
58. Kliche, S. et al. Mol Cell Biol 26, 7130-7144 (2006).
- 25 59. Geng, L. et al J Immunol 163, 5753-5757 (1999).
60. Duke-Cohan, J.S. et al J Biol Chem 281 13743-13750 (2006).
61. Peterson, E.J. et al. Science 293 (2001).
62. Mueller, K.L. et al J Immunol 179, 3559-3569 (2007).
63. Swanson, K.D et al Mol Cell 32, 564-575.
- 30 64. Marie-Cardine, A. et al FEBS Lett 435, 55-60 (1998).
65. Alva, V. et al Protein Pept Lett 15, 33-38 (2008).
66. Schneider, H. et al Eur J Immunol 31, 2042-2050 (2001).
67. Bunnell, S.C. et al. J Cell Biol 158, 1263-1275 (2002).
68. Huse, M. et al Nat Immunol 7, 247-255 (2006).
- 35 69. Bromley, S.K. et al. Annu Rev Immunol 19, 375-396 (2001).

70. Blank, U. et al Eur J Immunol 23, 3057-3065 (1993).  
71. Michel, F. et al Cell Immunol 173, 165-175 (1996).  
72. Schneider, H. et al PNAS U S A 102, 12861-12866 (2005).  
73. Schneider, H. et al Eur J Immunol. 38, 40-47.  
5 (2007).  
74. Singer, A.L. et al Science 296, 1639-1640 (2002).  
75. Pawson, T. & Nash, P Genes Dev 14, 1027-1047 (2000).  
76. Tohyama, Y. et al. Mol Biol Cell 14, 2570-2582 (2003).  
77. Krauss, K. et al. J Biol Chem 274, 36921-36927 (1999).  
10 78. Marwali, M.R. et al. Blood 102, 215-222 (2003).  
79. Harjes, E. et al. Structure 14, 881-888 (2006).  
80. Kinashi, T.. Nat Rev Immunol 5, 546-559 (2005).  
81. Ortiz-Vega, S. et al. Oncogene 21, 1381-1390 (2002).  
82. Gerard, A. et al J Cell Biol 176, 863-875 (2007).  
15 83. Fujita, H. et al. J Biol Chem 280, 5022-5031 (2005).  
84. Raab, M. et al Immunity 15, 921-933 (2001).  
85. Prasad, K.V. et al. PNAS U S A 90, 7366-7370 (1993).  
86. Raab, M. et al. PNAS U S A 92, 8891-8895 (1995).  
87. Olsson, T.S. et al J Mol Biol (2008).  
20 88. Ladbury, J.E. Biotechniques 37, 885-887 (2004)  
89. Raab M et al Immunity (2010) In press

## Sequences

```

1  ggcgaccgcg  gctgaggtag  aggtgcctcg  cgggtgcagcc  gggtcgcctt  ccagcccgtc
61  cgctcccga  ccagggcccg  cgcccgcgtc  cgctctctc  ccgcccagcc  aaatgcaggc
121  cgccgccctc  cctgaggaga  tccgttggct  cctggaagat  gctgaagagt  ttctggcaga
5  181  aggtttgcgg  aatgagaacc  tcagcgctgt  tgcaagggat  cacagagacc  atattctacg
241  gggctttcag  caaatcaaag  ccagggtacta  ttgggatttt  cagccccaag  ggggagacat
301  tggacaggac  agctctgatg  ataatcacag  cgggactctt  ggctgtccc  tcacatccga
361  tgccccttt  ttgtcagatt  atcaggatga  gggaatggaa  gacatcgtaa  aaggagctca
421  agaacttgat  aacgtaata  agcaaggata  cttggagaag  aaaagcaaag  atcatagttt
10  481  ctttgatcg  gagtggcaga  agcgatgggt  tgttgtcagc  agaggtctct  tctactacta
541  tgctaatag  aagagcaagc  agcccaaagg  gaccttcctc  attaagggt  acgggtgtag
601  gatggcccc  cactgcgaa  gagattccaa  gaaagaatcc  tgctttgaac  tgacctccca
661  ggataggcgc  agctatgagt  ttacagctac  tagtccagca  gaagccagag  actgggtgga
721  tcaaataagt  ttcttgtaa  aggatctgag  ctcttaacc  attccatatg  aagaggatga
15  781  ggaggaagaa  gaaaaagaag  agacatatga  tgatatgat  ggttttgact  ccccaagttg
841  tggttcccg  tgcagaccca  ctatcttgcc  tgggagtgtg  gggataaaag  agcctacaga
901  ggagaaagaa  gaagaagata  tttatgaagt  cttgccagat  gaagagcatg  atctagaaga
961  ggatgagagt  ggcactcgac  gaaaaggagt  agactatgcc  agttactacc  agggctatg
1021  ggattgccat  ggtgaccagc  cagatgaact  gtccttcaa  cggggtgacc  tcatccgtat
20  1081  tctgagcaag  gagtataaca  tgtatggctg  gtgggtggga  gaactgaaca  gcctcgttgg
1141  gattgttcca  aaggagtatc  tcaccactgc  ctttgaagtg  gaagaaagat  gaaaccagg
1201  aaatatattc  ttccctctct  cctgccttta  tgaggaaact  gatcatcaaa  agttccact
1261  ccctacttct  gccacccac  caacgccttg  gactcctctc  ttgtctgaag  agaccaagt
1321  ctcttgacac  ctacagagtga  ctgtaagcta  ccagtaagac  aagtgggaag  aggcaggtt
25  1381  atcaaacctg  ttactaaacc  agcctagtca  tagctcatcc  ccactcttaa  atgtgtccac
1441  acaaccacat  ctgccttttc  cacaagcttt  tcacaaagaa  ggtgagagag  aaggaaacct
1501  tgggaggagg  acattactgg  ttgttctggc  tggtttgaag  agcacaata  aacttgggat
1561  gtggttcctt  gccatgaaaa  aaaaaaaaaa  aaaaaaaaaa  a

```

30 SEQ ID NO:1 (NM\_003726.3)

```

1  mqaaalpeei  rwlledaeef  laeglrlenl  savardhrdh  ilrgfqkika  ryywdfqpqg
61  gdigqdssdd  nhsgtlglsl  tsdapflsdy  qdegmedivk  gageldnvik  qgylekkskd
121  hsffgsewqk  rwcvsrglf  yyyanekskq  pkgtflikgy  svrmaphlrr  dskkesfcfe
35  181  tsqdrsyef  tatspaear  wvdqisflk  dlssltipye  edeeeeeeke  tyddidgfds
241  pscgsqcrpt  ilpgsvgike  pteekceedi  yevlpdeehd  leedesgtrr  kgvdyasyyy
301  glwdchgdqp  delfsqrgdl  irilskeynm  ygwwvgelns  lvgivpkeyl  ttafeveer

```

SEQ ID NO:2 (Swiss-Prot Q86WV1.2)

```

1  cgggagtagc  gcagtcgcca  aagccgccc  tgccaaagct  gccgccacta  gccgggcatg
61  gccatggcgt  ccccgcccat  cgggcagcgc  ccgtaccgc  tactattgga  ccccgagccg
121  ccgcgctatc  tacagagcct  gagcgcccc  gagctaccgc  cgccgcccc  cgaccggtcc
45  181  tcgcgcctct  gtgtcccgcc  gccctctcc  actgcgcccg  gggcgcgcg  ggggcgcagc
241  gcccgaggag  ctgcccggg  gaacctggag  ccccgcccc  gggcctccc  acccgctcgc
301  ccgctccggc  ctggtctgca  gcagagactg  cggcgcgcc  ctggagcgcc  ccgacccgcg
361  gacgtgcgga  gcatcttcga  gcagccgag  gatccagag  tcccgcgga  gcgagcgag
421  gggcactgct  tcgcccaggt  ggtgctgcc  gggcgcccc  gctggtgtga  cctgtgcgga
481  cgagaggtgc  tgcggcaggc  gctgcgctgc  actaactgta  aattcacctg  tcaccagaa
50  541  tgccgcagcc  tgatccagtt  ggactgcagt  cagcaggagg  gtttatccc  ggacagaccc
601  tctccagaaa  gcacctcac  cgtgaccttc  agccagaatg  tctgtaaacc  tgtggaggag
661  acacagcgcc  cgcccacact  gcaggagatc  aagcagaaga  tcgacagcta  caacacgga
721  gagaagaact  gcctgggcat  gaaactgagt  gaagacggca  cctacacggg  tttcatcaaa
781  gtgcatctga  aactccggcg  gcctgtgacg  gtgcctgctg  ggatccggcc  ccagtcacatc
55  841  tatgatgcc  tcaaggaggt  gaacctggcg  gctaccacgg  acaagcgga  atccttctac
901  ctgcccctag  atgccatcaa  gcagctgcac  atcagcagca  ccaccaccgt  cagtgaagtc
961  atccaggggc  tgctcaagaa  gttcatggtt  gtggacaatc  ccagaagtt  tgacttttt
1021  aagcggatag  acaaggacgg  acaagtgtc  ttccagaaac  tctccattgc  tgaccgccc
1081  ctctacctgc  gcctgcttgc  tgggcctgac  acggaggtcc  tcagctttgt  gctaaaggag

```



56

5 1141 aatgaaactg gagaggtaga gtgggatgcc ttctccatcc ctgaacttca gaacttccta  
 1201 acaatcctgg aaaaagagga gcaggacaaa atccaacaag tgcaaaagaa gtatgacaag  
 1261 tttaggcaga aactggagga ggcttaaga gaatcccagg gcaaacctgg gtaaccggtc  
 1321 ctgcttcctc tcctcctggt gcattcagat ttatttgtat tattaattat tattttgcaa  
 1381 cagacacttt ttctcaggac atctctggca ggtgcatttg tgctgcccc gcagttccag  
 1441 ctgtggcaaa agtctcttcc atggacaagt gtttgcattg gggttcagct gtgccccccc  
 1501 ccaggctgtg cccaccaca gattctgcca aggatcagaa ctcatgtgaa acaaacagct  
 1561 gacgtcctct ctcgatctgc aagcctttca ccaacaaaat agttgcctct ctcgtaacca  
 1621 aactggaacc tcacaccagc cggcaaagga aggaagaaag gttttagagc tgtgtgttct  
 1681 ttctctggct ttgattcttc ttgagttct cttacttgcc acgtacagga ccattattta  
 1741 tgagtgaaaa gttgtagcac attccttttg caggtctgag ctaagcccc gaaagcaggg  
 1801 taatgctcat aaaagactg ttcccgcgcc ccaaggtgc ctgttgttca cacttaaggg  
 1861 aagtttataa agctactggc ccagatgct cagggttaagg agcaccaaag ctgaggctgg  
 1921 ctgagagatc tcagagaag ctgcagcctg ccctggccct ggctctggcc ctggcccaca  
 1981 ttgcacatgg aaacccaaag gcataatct gcgtatgtgt ggtacttagt cacatctttg  
 2041 tcaacaaact gttcgttttt aagttacaaa ttggaattta atgttgtcat catcgtcatg  
 2101 tgtttcccca aagggaagcc agtcattgac catttaaaaa gtctcctgct aagtatggaa  
 2161 atcagacagt aagagaaagc caaaaagcaa tgcagagaaa ggtgtccaag ctgtcttcag  
 2221 ccttccccag cttaaagagc gaggagggcc tgggctactt gggttcccca tgggcctcca  
 2281 gcaactgcctc cctcctccca ctgcgactct gggatctcca ggtgctgccc aaggagtgtc  
 2341 cttgattaca gagaggggag cctccaattc ggccaacttg gattcctttc tgttttgaag  
 2401 catggggccag acccggcact gcgctcggag agccggtggg cctggcctcc ccgtcgacct  
 2461 cagtgccttt ttgttttcag agagaaatag gtagtagggc agtttgctg aagctctgct  
 2521 gctggcttct cctgccagga agtgaacaat ggcggcggtg tgggagacaa ggccaggaga  
 2581 gccgcggttc agtatgggtt gagggtcaca gacctccctc ccatctgggt gcctgagttt  
 2641 tgactccaat cagtataacc agaccacatt gacaggagg atcaaattcc tgacttacat  
 2701 ttgcaactgg ttcttgttta ggctgaatcc taaaataaat tagtcaaaaa attccaacaa  
 2761 gtagccagga ctgcagagac actccagtgc agagggagaa ggacttgtaa ttttcaaagc  
 2821 agggctggtt ttccaaccca gcctctgaga aaccatttct ttgctatcct ctgcctccc  
 2881 aagtcctctc tgggtcgggt caagcccaag cttgttcgtg tagcttcaga agttccctct  
 2941 ctgacccagg ctgagtcatt actgccccg atcccagaag gaatgctgac cctcgtcgt  
 3001 atgaactgtg catagtctcc agagcttcaa aggcaacaca agctcgcaac tctaagattt  
 3061 ttttaaacca caaaaaccct ggttagccat ctcatgctca gccttatcac ttccctccct  
 3121 ttagaaactc tctccctgct gtatattaaa gggagcaggt ggagagtcac tttcctcgt  
 3181 cctgcatgtc tctaacatta atagaaggca tggctcctgc tgcaaccgct gtgaatgctg  
 3241 ctgagaacct cctctatgg gtaggtctat tttatttttg agaaggaaaa aaaaagtcac  
 3301 gtatatatac acataaaggc atatagctat atataaagag ataagggtgt ttatgaaatg  
 3361 agaaaattat tggacaattc agactttact aaagcacagt tagaccaag gcctatgctg  
 3421 aggtctaaac ctctgaaaaa agtatagtat cgagtaccg ttccctccca gaggtgggag  
 3481 taactgctgg tagtgcttct tttggttgtg ttgctcagtg tgtaagtgtt tgtttccagg  
 3541 atattttctt tttaaatgtc ttcttatat gggttttaaa aaaaagtaat aaaagcctgt  
 3601 tgcaaaaatg actcatgtta aaaaaaaaaa aaaaaaa

SEQ ID: 3 (NM\_182663.2)

45

50 1 mamaspaigq rpypllldpe pprylqslsg pelpppppdr ssrlcvpapl stapparegr  
 61 sarraargnl eppprasrpa rplrpglqqr lrrrpgaprp rdvrsifeqp qdprvpaerg  
 121 eghcfaelvl pggpgwcdlc grevlrqlar ctncckftchp ecrsliqldc sqqeglsrdr  
 181 pspestltvt fsqnvckpve etqrptlqe ikqkidsynt rekncldgmkl sedgtytgfi  
 241 kvhlklrrpv tvpagirpqs iydaikevnl aattdkrtsf ylpldaikql hisstttvse  
 301 viqglkkkf vvdnpqkfai fkrihkdqgv lfqklsiadr plylrlilagp dtevlslfvlk  
 361 enetgevewd afsipelqnf ltilekeeqd kiqqvqkkyd kfrqkleaal resqgkpg

55

SEQ ID NO: 4 (Swiss-Prot Q8WWW0.1)

## Claims:

1. A method of identifying a compound which modulates LFA-1 mediated T-cell adhesion comprising;  
5 determining the effect of a test compound on the binding of an SKAP1 polypeptide to a RapL polypeptide,  
wherein a compound which affects said binding is a candidate modulator of LFA-1 mediated T-cell adhesion.
- 10 2. A method according to claim 1 wherein the SKAP1 polypeptide and the RapL polypeptide are contacted in the presence and absence of a test compound.
- 15 3. A method according to claim 1 or claim 2 wherein a difference in the binding of the SKAP1 polypeptide and the RapL polypeptide in the presence relative to the absence of a test compound is indicative that the compound modulates LFA-1 mediated T-cell adhesion.
- 20 4. A method according to any one of claims 1 to 3 wherein the SKAP1 polypeptide comprises an amino acid sequence having at least 70% sequence identity to the amino acid sequence of residues 1 to 104 of SEQ ID NO: 2.
- 25 5. A method according to claim 4 wherein the SKAP1 polypeptide comprises the amino acid sequence of residues 1 to 104 of SEQ ID NO: 2.
- 30 6. A method according to any one of claims 1 to 5 wherein the RapL polypeptide comprises an amino acid sequence having at least 70% sequence identity to the amino acid sequence of residues 223 to 254 of SEQ ID NO: 4.

7. A method according to any one of claims 1 to 6 wherein the RapL polypeptide comprises the amino acid sequence of residues 223 to 254 of SEQ ID NO: 4.

5

8. A method according to any one of the preceding claims comprising identifying a test compound which modulates the binding of the SKAP1 polypeptide to the RapL polypeptide.

10 9. A method according to claim 8 further comprising determining the ability of the identified compound to modulate one or more of LFA-1 activation, clustering and/or adhesion; T-cell-APC conjugation; TcR induced RapL-Rap1 complex formation; SKAP1-Rap1-RapL binding to LFA-1; and T-cell  
15 binding to immobilised ICAM1.

10. A method according to claim 8 or claim 9 further comprising determining the effect of said identified compound on T-cell mediated immune responses in vitro and/or in vivo.

20

11. A method according to any one of claims 8 to 10 comprising isolating and/or purifying the test compound.

12. A method according to any one of claims 8 to 11  
25 comprising preparing and/or synthesising the test compound

13. A method according to any one of claims 8 to 12 comprising modifying the compound to optimise its pharmaceutical properties.

30

14. A method according to any one of claims 8 to 13 comprising formulating the compound with a pharmaceutically acceptable excipient.

15. A method of detecting immunodeficiency in an individual comprising
- contacting one or more T cells obtained from an individual with a first member of a binding pair consisting of
  - 5 a SKAP1 polypeptide and RapL polypeptide, and;
  - determining binding of the first member to the other member of the binding pair in the one or more T cells.

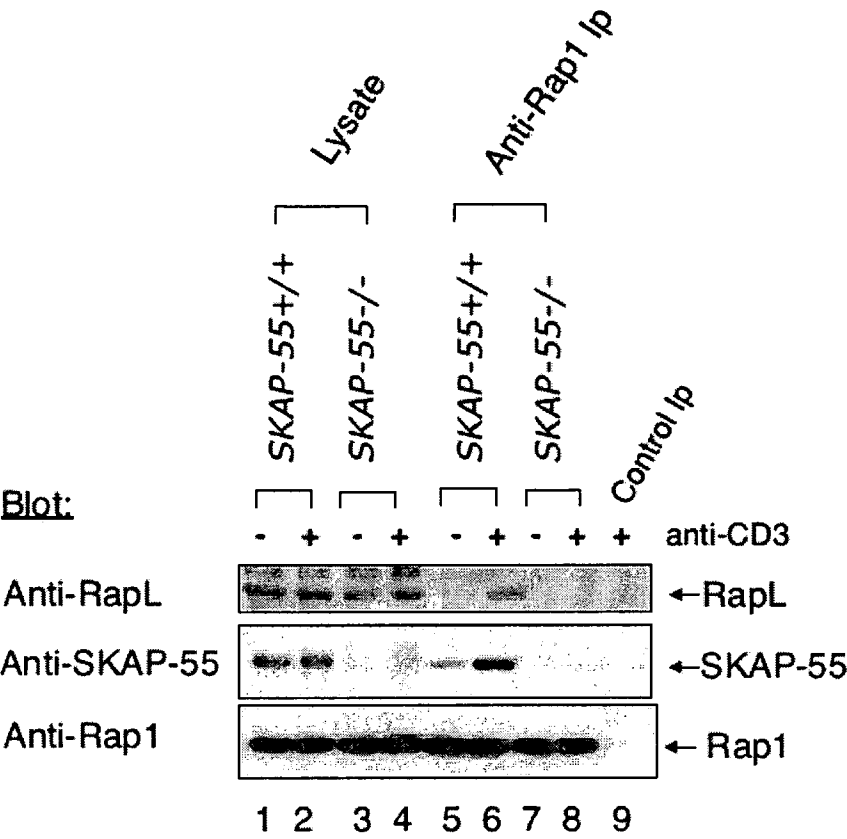


Figure 1

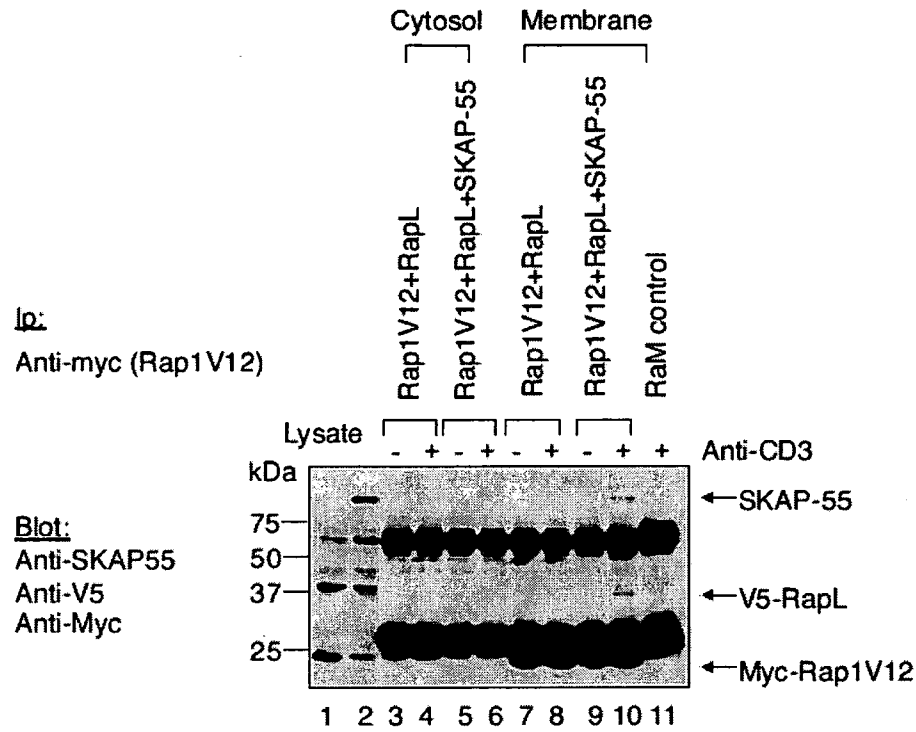


Figure 2

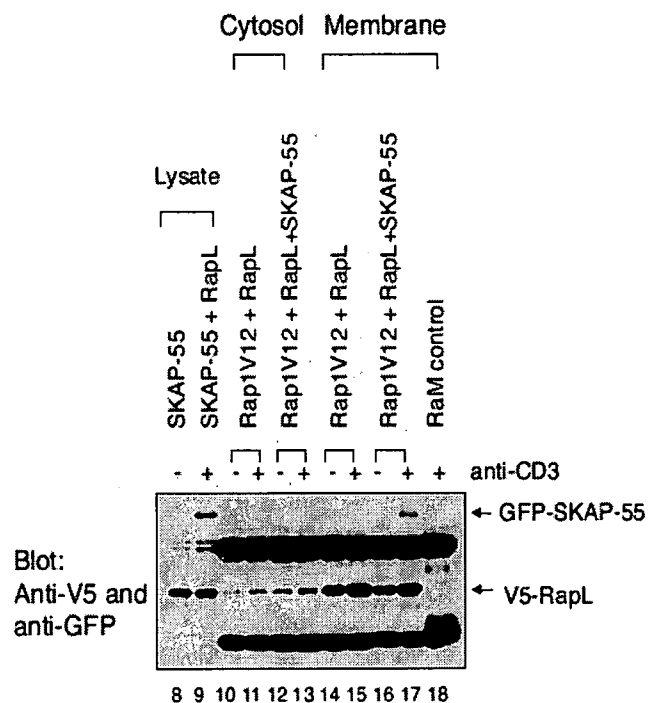
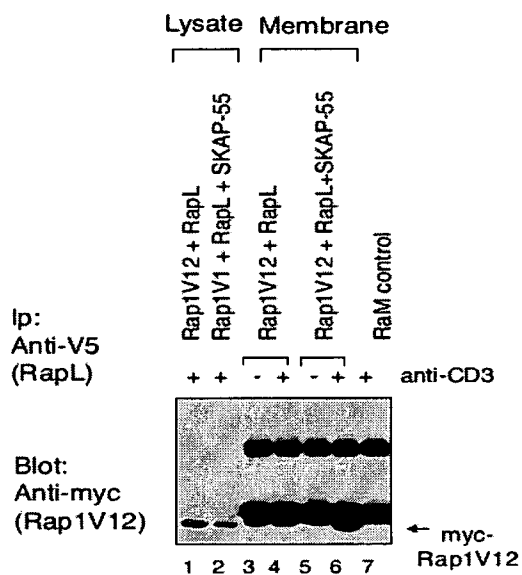


Figure 3

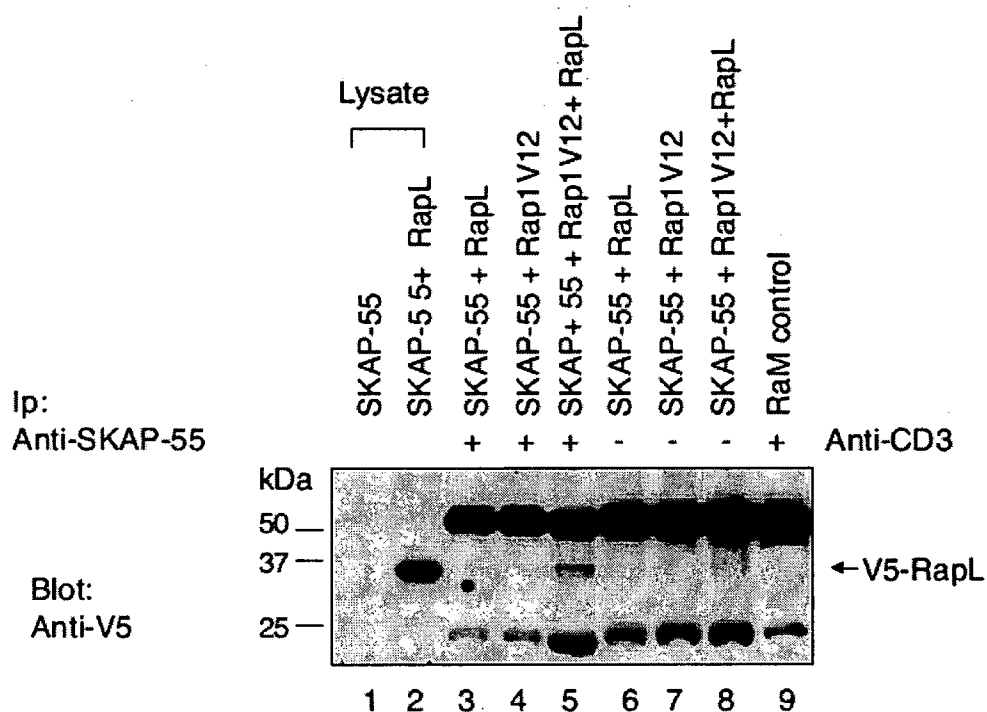


Figure 4

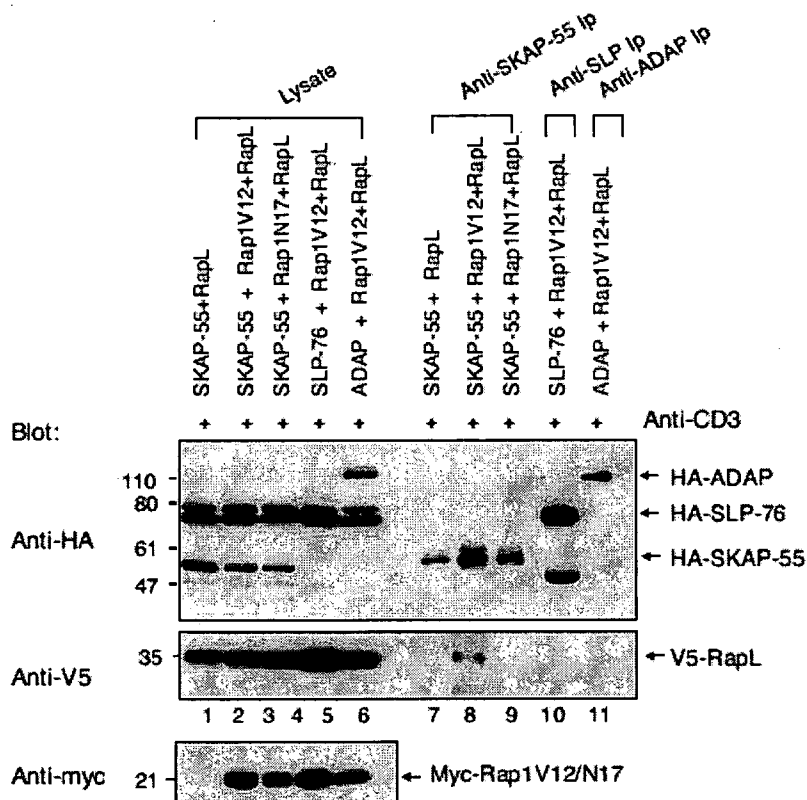


Figure 5



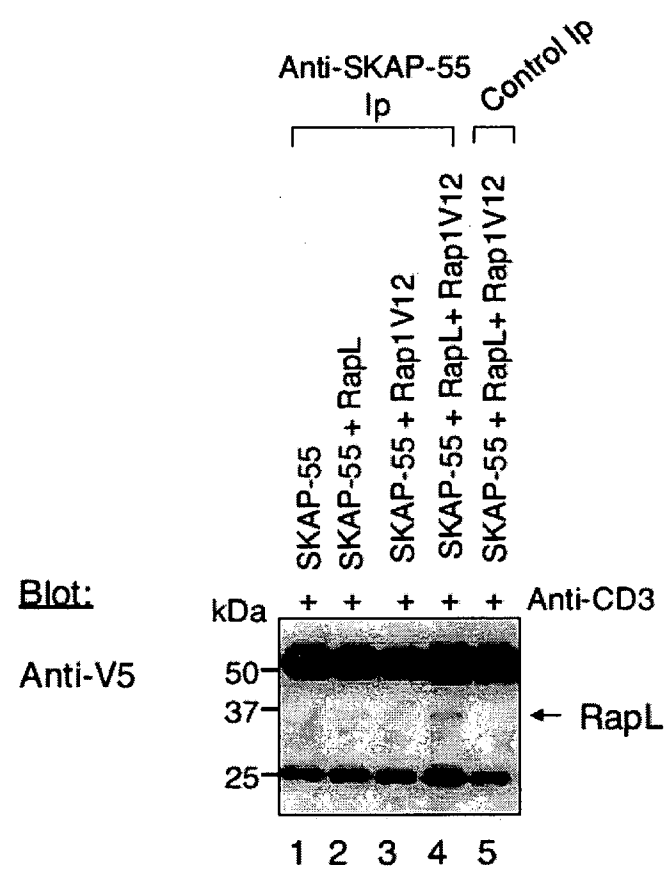


Figure 6

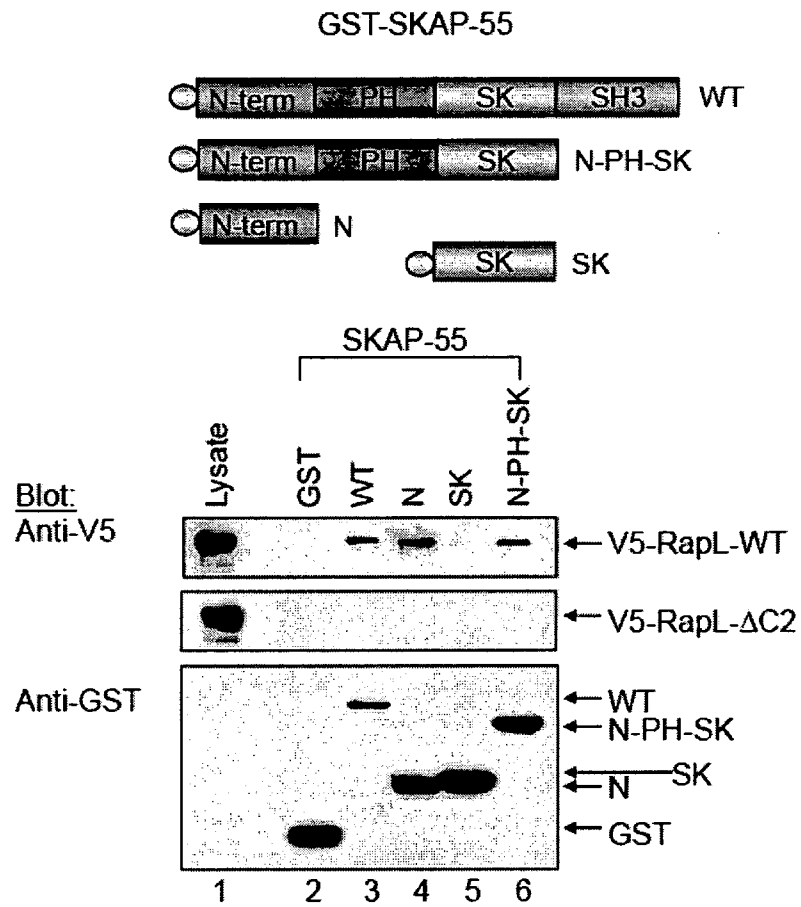


Figure 7

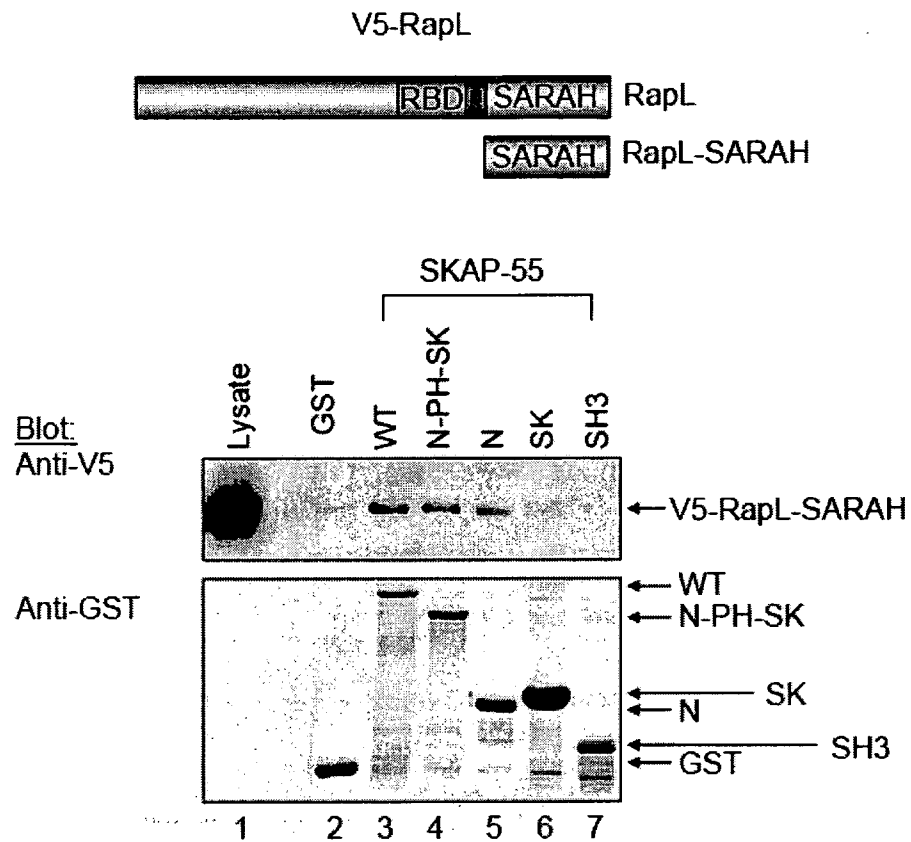


Figure 8

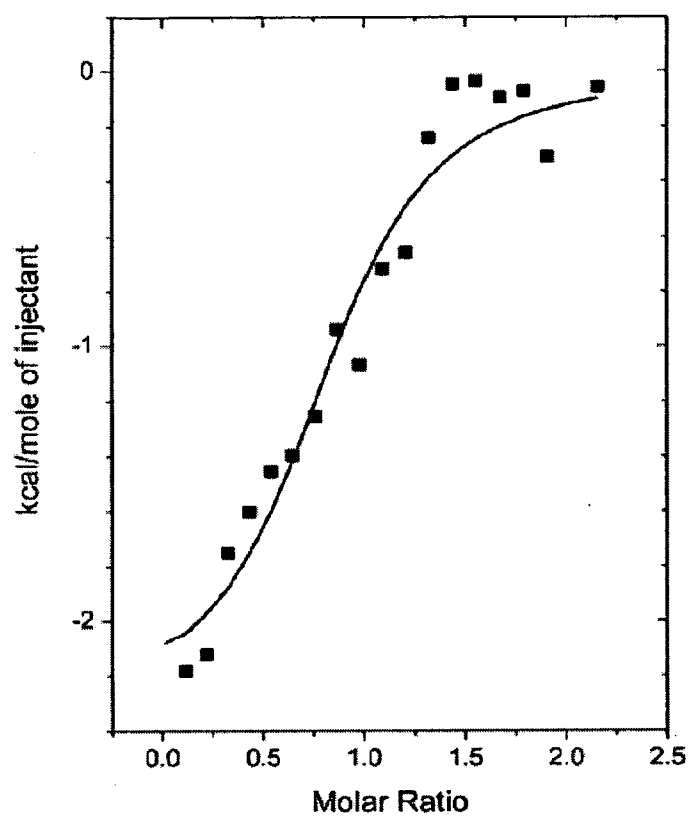


Figure 9

## V5-RapL

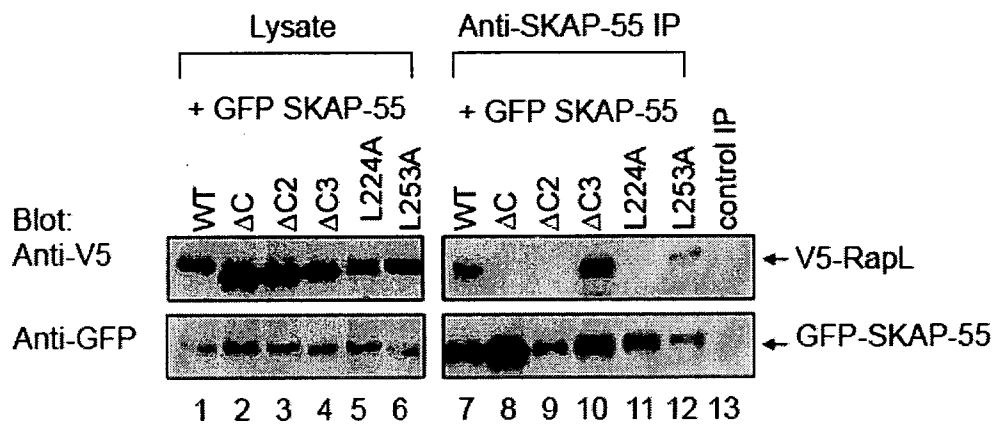
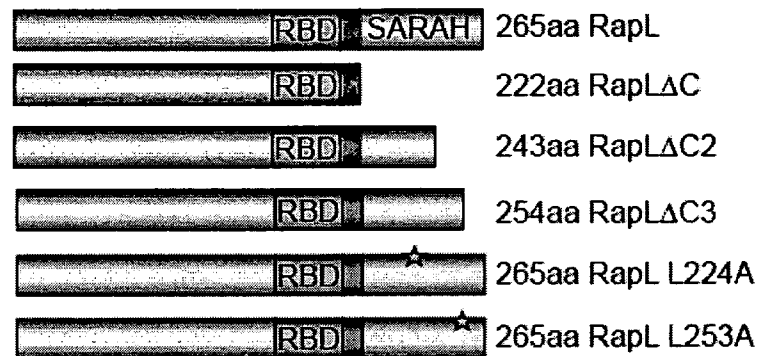


Figure 10

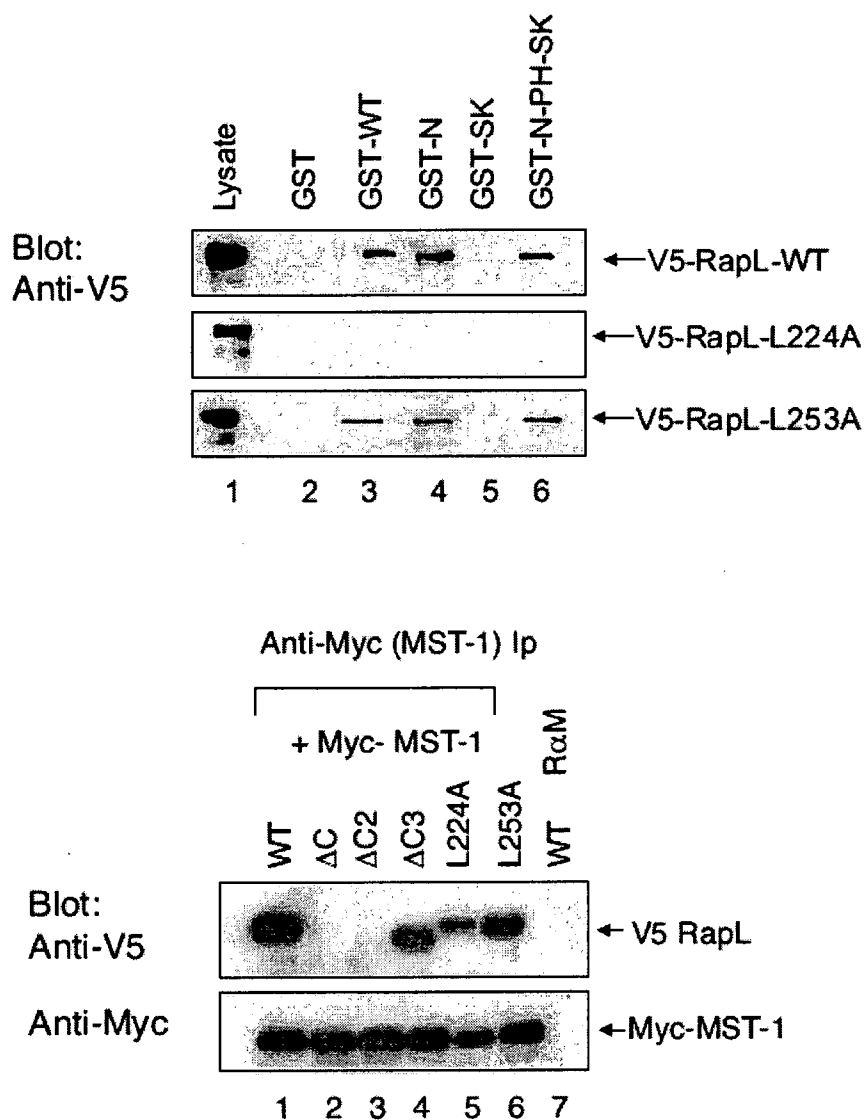


Figure 11

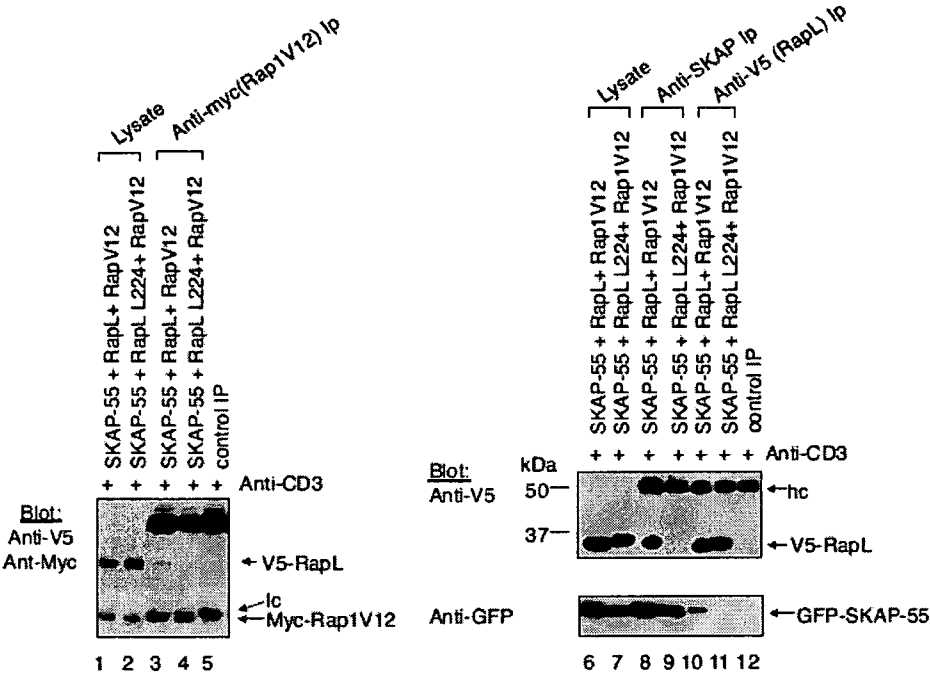


Figure 12

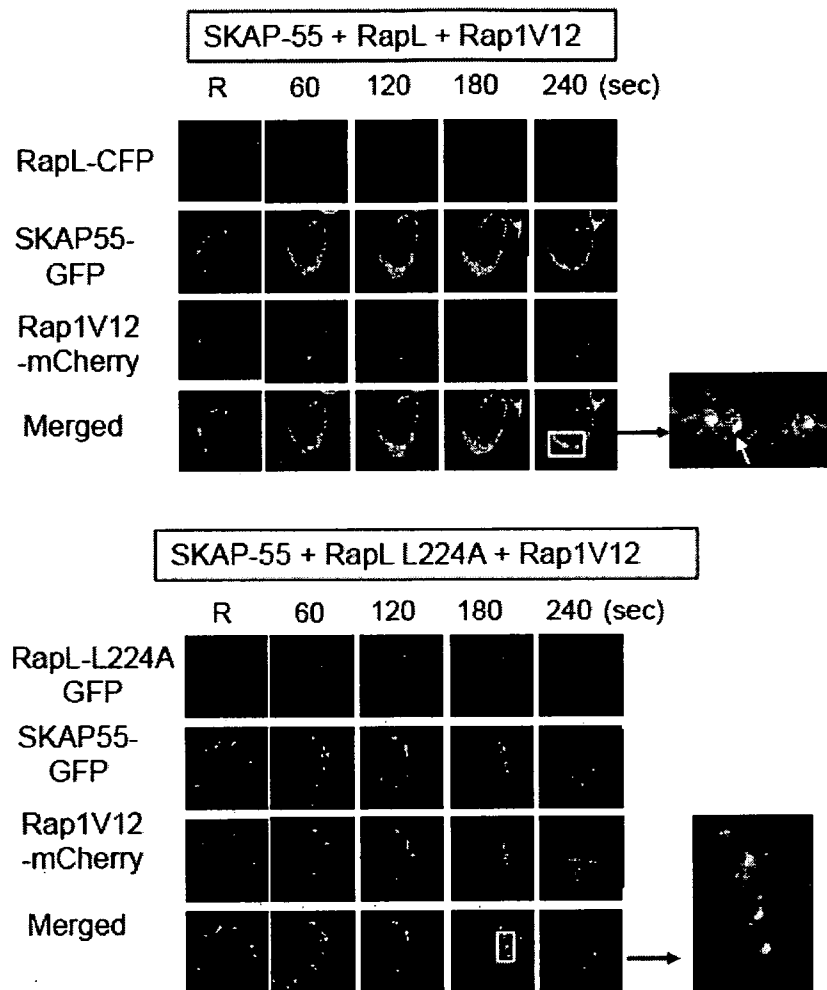


Figure 13



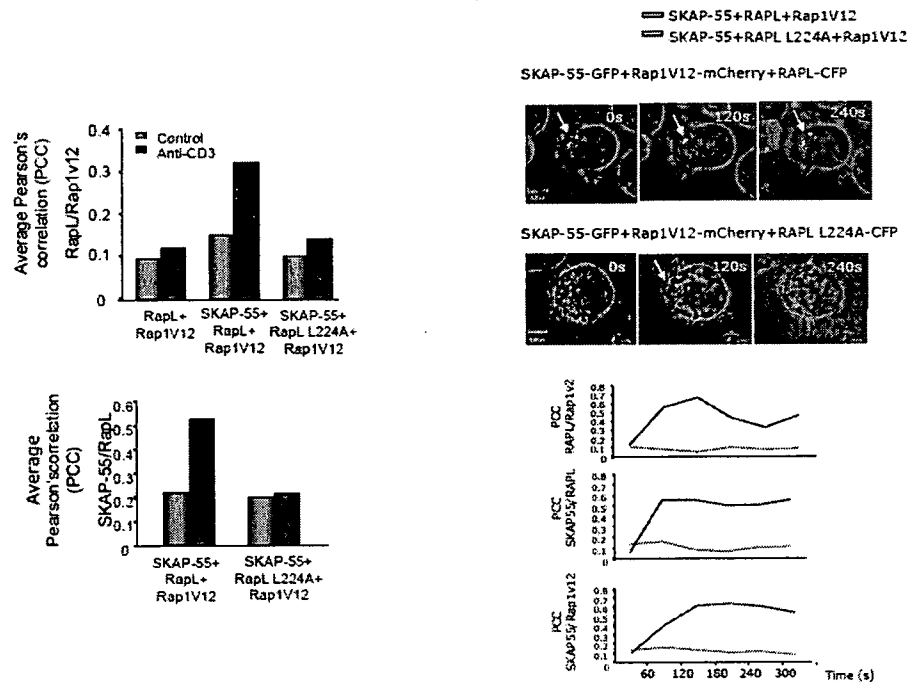


Figure 14

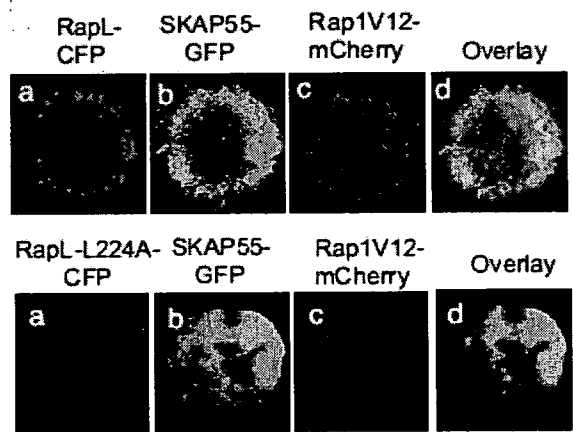


Figure 15

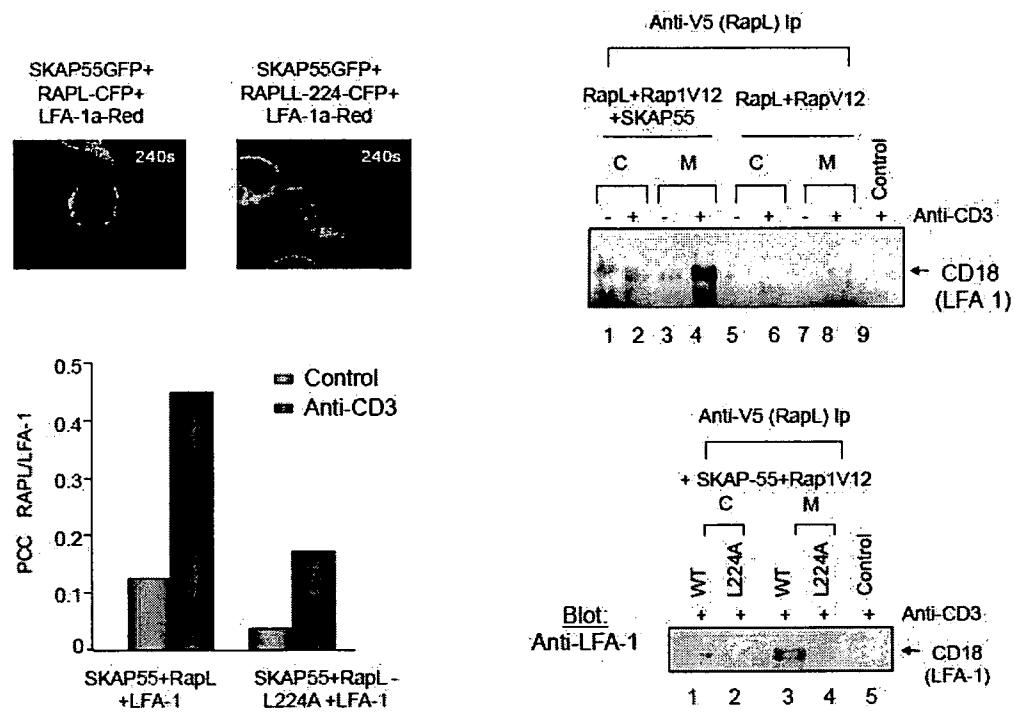


Figure 16

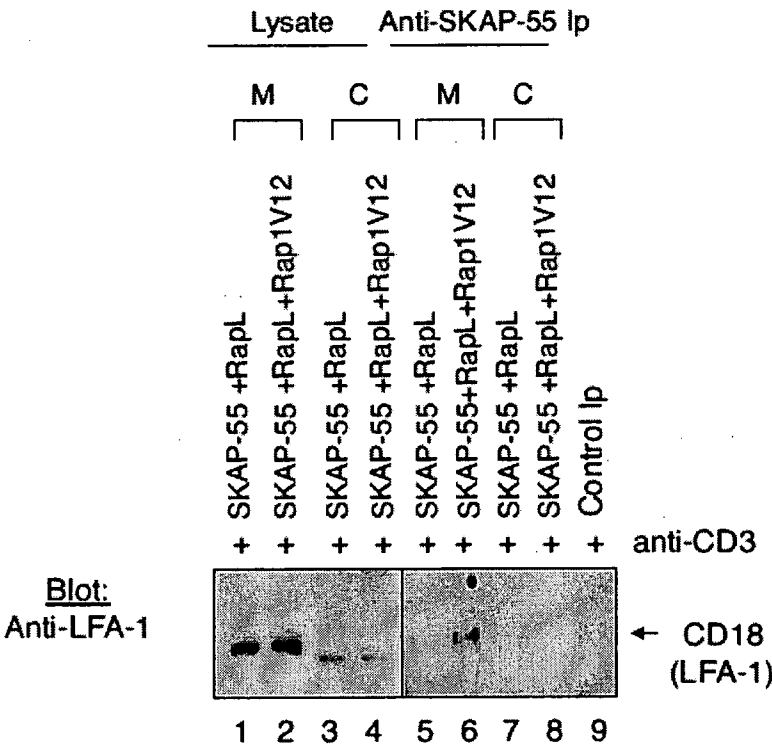


Figure 17

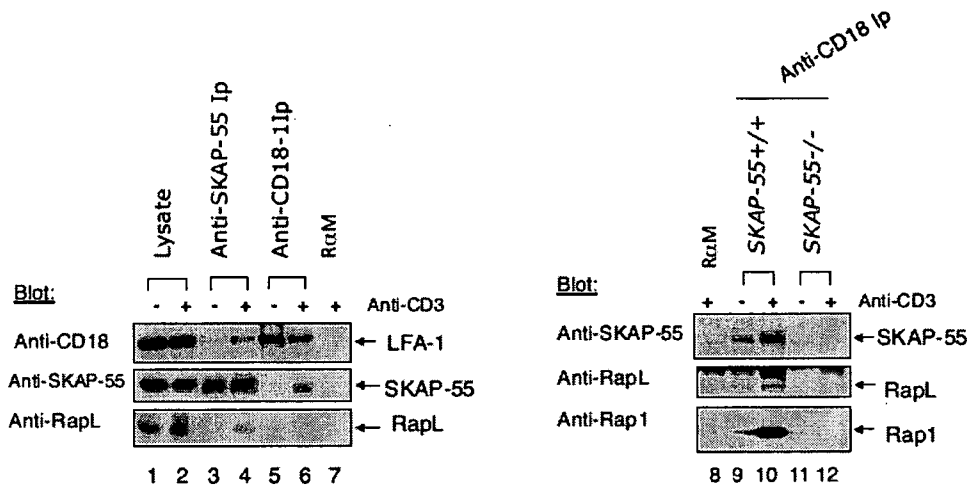


Figure 18

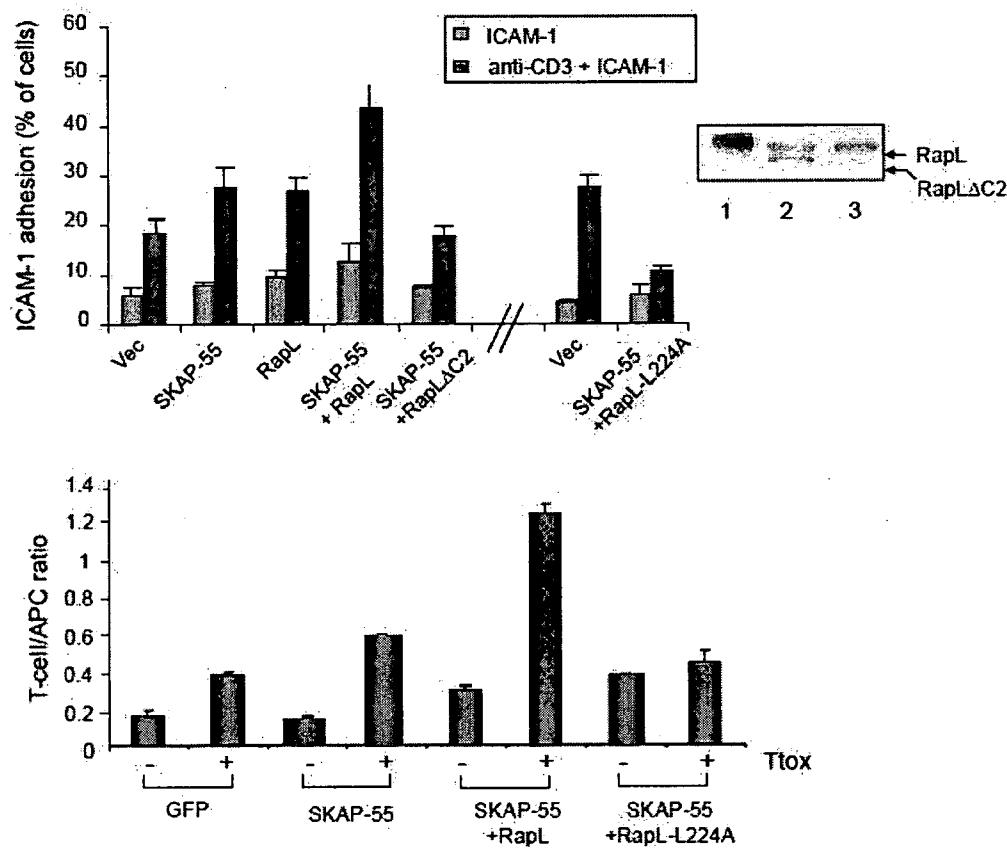


Figure 19

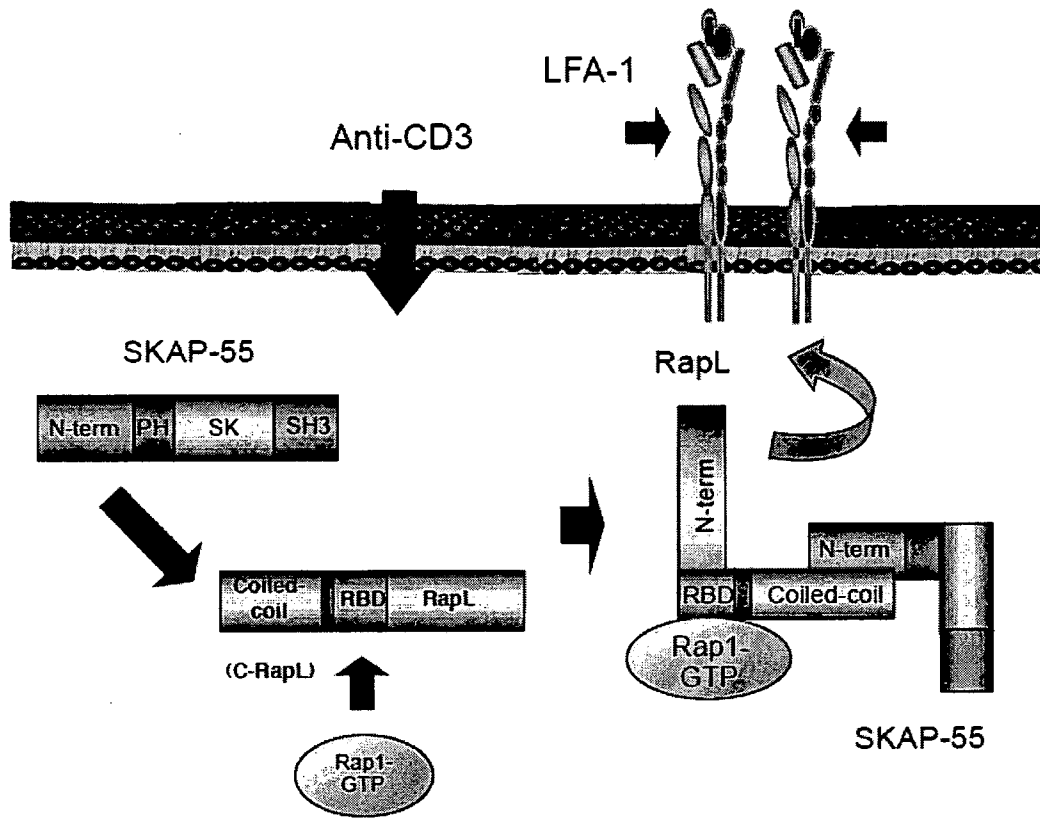


Figure 20

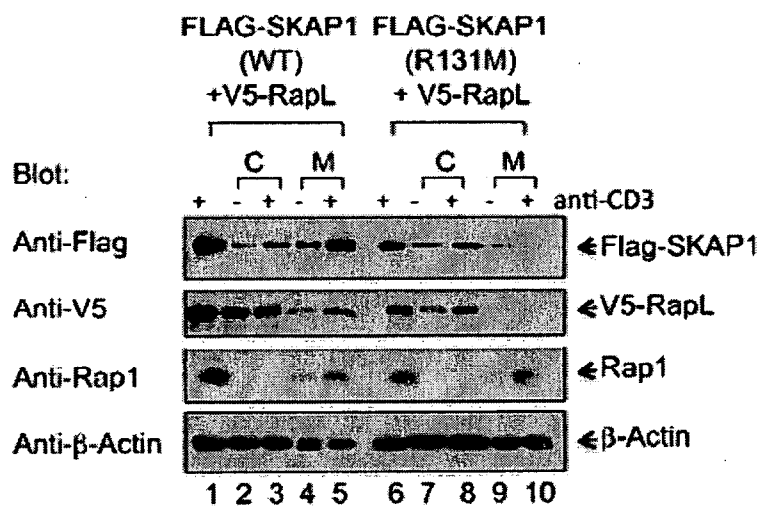
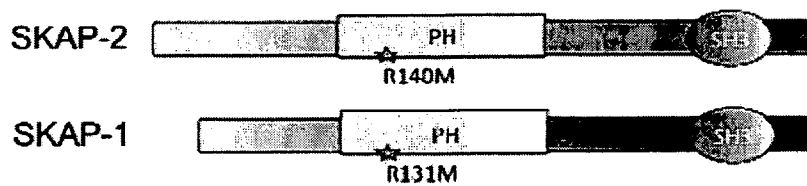


Figure 21

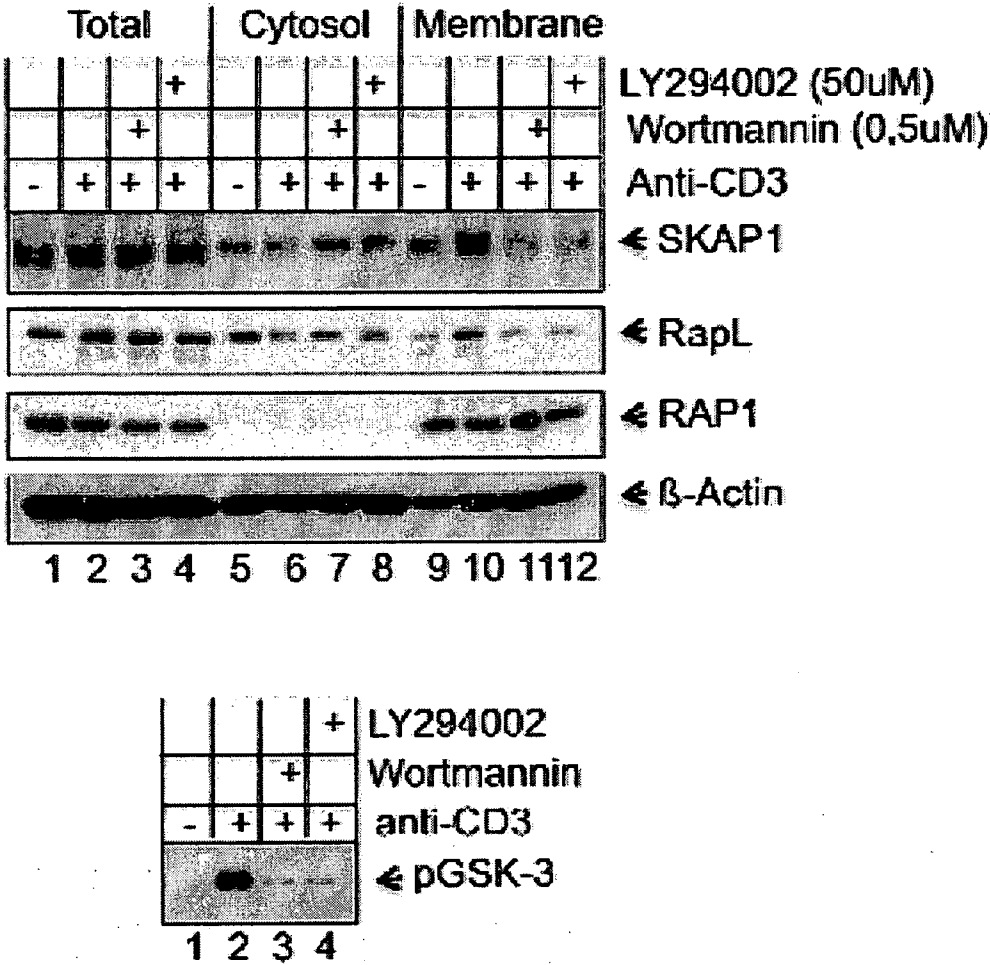


Figure 22

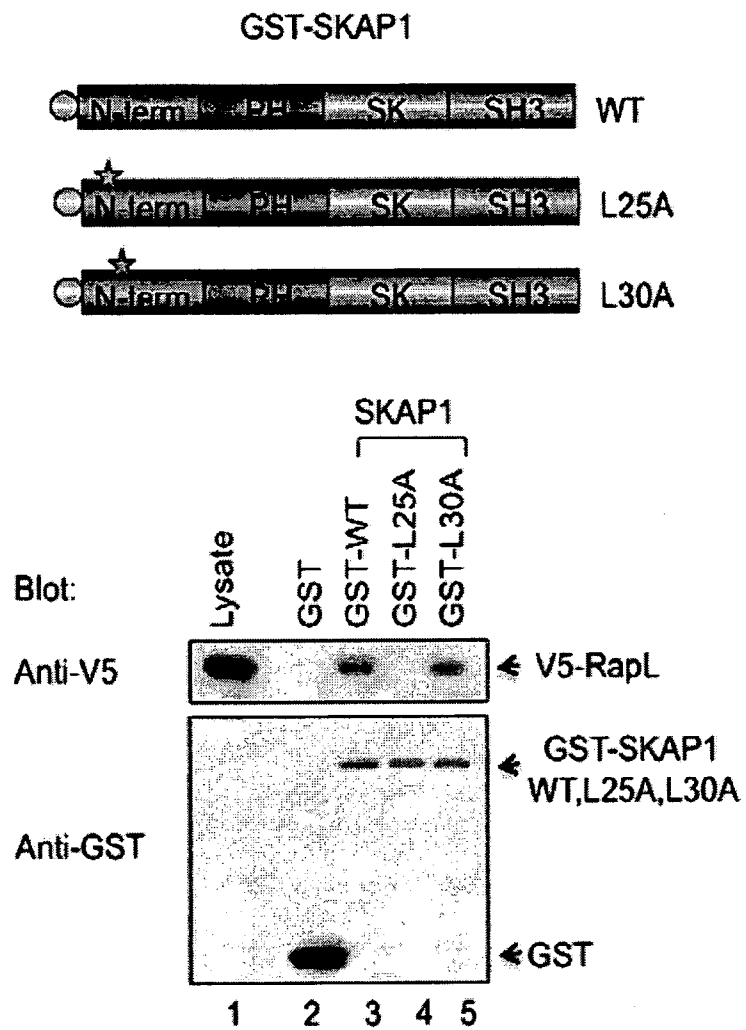


Figure 23

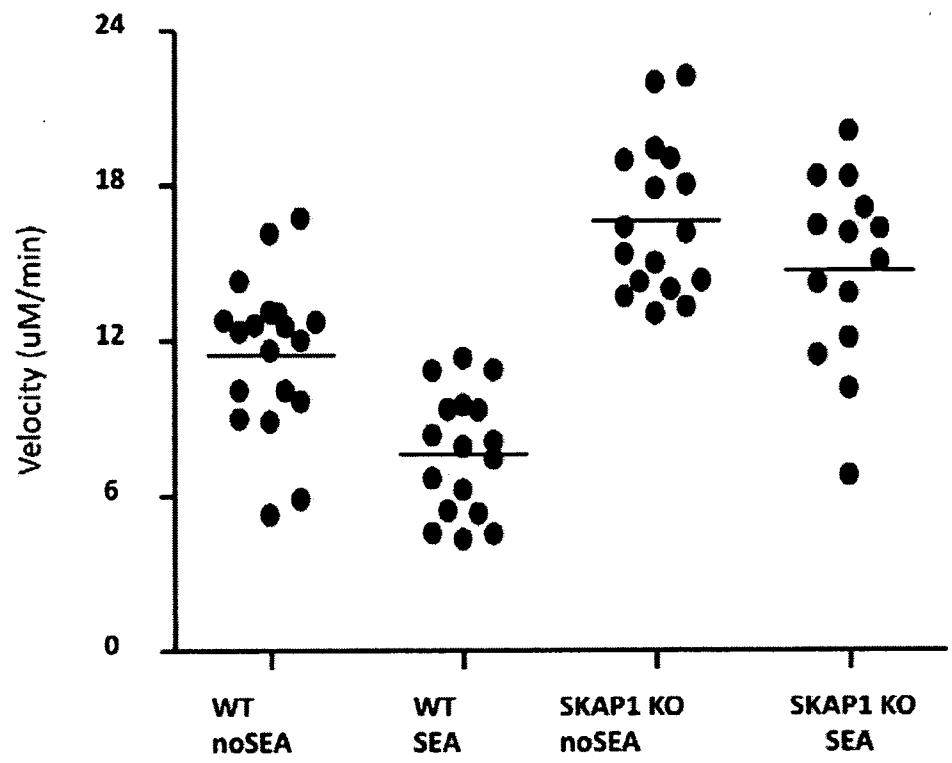


Figure 24



# INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2010/000468

## A. CLASSIFICATION OF SUBJECT MATTER

INV. G01N33/50

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, EMBASE, BIOSIS, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                 | Relevant to claim No. |
|-----------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| A         | US 2005/267098 A1 (SHEN WANG [US] ET AL)<br>1 December 2005 (2005-12-01)<br>p 36, col 1, para 0280;<br>* abstract                                                                                                                                  | 1-15                  |
| A         | MOR ADAM ET AL: "Small GTPases and LFA-1<br>reciprocally modulate adhesion and<br>signaling"<br>IMMUNOLOGICAL REVIEWS,<br>vol. 218, August 2007 (2007-08), pages<br>114-125, XP002592285<br>ISSN: 0105-2896<br>p 118, col 2, para 3;<br>* abstract | 1-15                  |



Further documents are listed in the continuation of Box C.



See patent family annex.

\* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*8\* document member of the same patent family

Date of the actual completion of the international search

16 July 2010

Date of mailing of the international search report

03/08/2010

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040,  
Fax: (+31-70) 340-3016

Authorized officer

Bigot-Maucher, Cora

## INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2010/000468

## C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                               | Relevant to claim No. |
|-----------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| A         | <p>WANG H ET AL: "SKAP-55, SKAP-55-related and ADAP adaptors modulate integrin-mediated immune-cell adhesion" TRENDS IN CELL BIOLOGY, ELSEVIER SCIENCE LTD, XX LNKD-DOI:10.1016/J.TCB.2008.07.005, vol. 18, no. 10, 1 October 2008 (2008-10-01), pages 486-493, XP025468975 ISSN: 0962-8924 [retrieved on 2008-08-28] * abstract; figure 2</p>                   | 1-15                  |
| X,P       | <p>WANG HONGYAN ET AL: "SLP-76-ADAP adaptor module regulates LFA-1 mediated costimulation and T cell motility." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 28 JUL 2009 LNKD- PUBMED:19617540, vol. 106, no. 30, 28 July 2009 (2009-07-28), pages 12436-12441, XP002592286 ISSN: 1091-6490 * abstract</p>                    | 1-15                  |
| T         | <p>RAAB M ET AL: "T Cell Receptor "Inside-Out" Pathway via Signaling Module SKAP1-RapL Regulates T Cell Motility and Interactions in Lymph Nodes" IMMUNITY 2010 CELL PRESS USA LNKD-DOI:10.1016/J.IMMUNI.2010.03.007, vol. 32, no. 4, April 2010 (2010-04), pages 541-556, XP002592287 ISSN: 1074-7613 p 36, col 1, para 0280; * abstract the whole document</p> | 1-15                  |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2010/000468

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|-------------------------------------------|---------------------|----------------------------|---------------------|
| US 2005267098 A1                          | 01-12-2005          | US 2010093693 A1           | 15-04-2010          |
|                                           |                     | US 2008182839 A1           | 31-07-2008          |
|                                           |                     | US 2008176896 A1           | 24-07-2008          |
| <hr/>                                     |                     |                            |                     |