Rab GTPases containing a CAAX motif are processed post-geranylgeranylation by proteolysis and methylation

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Running Title: Post-prenylation processing of Rab GTPases

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Post-translational modification by protein prenylation is required for membrane targeting and biological function of monomeric GTPases. Ras and Rho proteins possess a C-terminal CAAX motif, in which the cysteine is prenylated, followed by proteolytic cleavage of the AAX peptide and carboxyl methylation by the Rce1 CAAX protease and Icmt methyltransferase, respectively. Rab GTPases usually undergo double geranylgeranylation within CC or CXC motifs. However, very little is known about processing and membrane targeting of Rabs which naturally contain a CAAX motif. We show here that a variety of Rab-CAAX proteins undergo carboxyl methylation, both in vitro and in vivo, with one exception. Rab38(CAKS) is not methylated in vivo, presumably because of inhibitory action of the lysine residue within the AAX motif for cleavage by Rce1. Unlike farnesylated Ras proteins, we observed no targeting defects of overexpressed Rab-CAAX proteins in cells deficient in Rce1 or Icmt, as reported for geranylgeranylated Rho proteins. However, endogenous geranylgeranylated non-methylated Rab-CAAX and Rab-CXC proteins were significantly redistributed to the cytosol at steady-state levels and redistribution correlates with higher affinity of RabGDI for non-methylated Rabs in Icmt-deficient cells. Our data suggest a role for methylation in Rab function by regulating the cycle of Rab membrane recruitment and retrieval. Our findings also imply that those Rabs that undergo post-prenylation processing follow an indirect targeting pathway requiring initial endoplasmic reticulum membrane association prior to specific organelle targeting.

Protein prenylation involves the covalent addition of either farnesyl (15-carbon) or geranylgeranyl (20-carbon) isoprenoids onto C-terminal cysteines via thioether linkages and is necessary for proteins to associate with cellular membranes in order to carry out intracellular functions (1,2). Among the proteins that are modified by prenylation are small GTPases such as Ras, Rho and Rab family proteins, all of which require prenylation for activity. Three distinct protein prenyltransferases have been identified, all of which are heterodimeric enzymes made up of an α and β subunit (1,2). The CAAX prenyltransferases, consisting of farnesyltransferase (FT) and geranylgeranyltransferase type I (GGT-I), modify proteins with a C-terminal CAAX motif, where C is cysteine, A is usually an aliphatic residue and X is any amino acid. When X is a methionine, serine, glutamine or alanine, the substrate is farnesylated, whereas if it is a leucine or phenylalanine, the substrate is geranylgeranylated. Rab geranylgeranyltransferase (RGGT, also known as GGT-II) forms a different class
of protein prenyltransferases. RGGT specifically modifies Rab proteins but only when they are in complex with an accessory protein known as Rab Escort Protein (REP) (2). There are two proposed mechanisms for Rab protein prenylation. In the classical pathway, newly synthesised Rab binds REP, which presents the Rab to RGGT (3). Alternatively, REP can associate with RGGT and the complex can bind to unprenylated Rab (4). The enzyme then catalyses the sequential addition of geranylgeranyl groups onto two C-terminal cysteines of the Rab protein. Finally, RGGT dissociates and REP is thought to deliver the prenylated Rab protein to membranes (5).

Following prenylation, CAAX-containing Ras and Rho GTPases are targeted to the endoplasmic reticulum (ER) and undergo proteolytic cleavage of the AAX tripeptide, catalysed by the CAAX protease, Ras and α-factor converting enzyme (Rce1) (6,7). The newly exposed prenylated cysteine is then further modified by carboxyl methylation on the α-carboxyl group by isoprenylcysteine carboxyl methyltransferase (Icmt), which is also located on the ER (6,7). Carboxyl methylation enhances the hydrophobicity of the C-terminus of prenylated proteins, though this effect is more apparent in farnesylated proteins than in geranylgeranylated proteins (8,9). The importance of post-prenylation processing has been exemplified by studies using gene-targeted inactivation, where it was found that mice deficient in Rce1 (10) or Icmt (11) are embryonic lethal. Interestingly, Icmt-/- mice exhibited a more severe phenotype, which could be explained by the fact that Icmt may have more substrates than Rce1. Indeed, Rab proteins with a CXC motif are methylated on the C-terminal prenylcysteine, although the role of methylation in Rab proteins is unclear (12).

Several studies have demonstrated the importance of methylation and its role in the membrane association of many CAAX proteins, in particular Ras proteins. In cells deficient in Rce1 and Icmt, Ras proteins exhibit a significant decrease in membrane association (10). Furthermore, the absence of methylation results in mislocalisation of Ras from the plasma membrane. Consistent with its role in membrane association, methylation appears to regulate downstream signalling pathways of Ras through its localisation. The Icmt small substrate inhibitor, N-acetyl-S-farnesyl-L-cysteine (AFC), blocks EGF-stimulated Erk phosphorylation, a downstream target of EGF signalling and the activation of Mek and Raf-1 kinases (13), although AFC-mediated effects have to be interpreted carefully given that some effects are unrelated to Icmt inhibition (6). Another Icmt-specific inhibitor, cysmethynil, also leads to mislocalisation of Ras proteins and blocks EGF-induced stimulation of MAPK and Akt (6).

The majority of Rab proteins possess a di-cysteine motif such as CC, CXC or CCXX, and both cysteines are modified by geranylgeranyl lipid groups. However, a few possess a CAAX motif, such as Rab8 and Rab13, and are modified by a single geranylgeranyl moiety (14). The reason why some Rabs are mono-prenylated is not known, but the presence of a CAAX motif suggests that they have the potential to be processed by CAAX proteolysis and carboxyl methylation. In this study, we addressed the post-prenylation processing of single cysteine Rabs. We reveal for the first time that Rab-CAAX proteins are carboxyl methylated both in vitro and in vivo. In the absence of CAAX processing by Rce1 or Icmt, the localisation of Rab-CAAX proteins is unaffected. However, the cycle of membrane association and retrieval is affected with decreased levels of membrane-associated Rabs, suggesting a role for methylation in regulating Rab activity.

Experimental Procedures
**Plasmid constructs**—pEGFP-Rab11a was a kind gift of James Goldenring (Vanderbilt University School of Medicine, Nashville). pEGFP-mRab23 was a kind gift from Carol Wicking (University of Queensland, Australia) (from here on, mRab23 will be referred to as Rab23). pEGFP-Rab8aGGCC, pEGFP-Rab8aGCSC, pEGFP-Rab38CALs and pEGFP-Rab38CAVS were generated using the Stratagene Quickchange site-directed mutagenesis system (SDM), as described previously (15). pGEX-4T-1-Rab13, Rab18 and Rab23 were generated by polymerase chain reaction (PCR) amplification of the Rab cDNA of interest and cloned into pGEX-4T-1 vector using EcoRI-SalI, EcoRI-XhoI and EcoRI-XhoI, respectively. Human RabGDIβ was amplified by PCR from a cDNA library (MHS1011, Invitrogen) and cloned into pFastBac-HTB using NcoI-XbaI. The sequences of all plasmid constructs used were confirmed by DNA sequencing.

**Recombinant Proteins**—Recombinant GST-Rab5a, GST-Rab13, GST-Rab18, GST-Rab23 and GST-Rab38 were expressed in BL21 cells and purified on glutathione-agarose beads (Sigma). Recombinant RGGT and REP1 were prepared by infection of Sf9 cells with recombinant baculoviruses encoding each subunit of the desired enzyme and purified by nickel Sepharose-affinity chromatography as described previously (16,17). Recombinant human RabGDIβ was prepared by infection of Sf9 cells with recombinant baculovirus using standard procedures. Briefly, baculoviruses were generated following subcloning of RabGDIβ into pFastBac-HTB using the Bac-to-Bac® system according to manufacturer’s instructions (Invitrogen). Recombinant histidine (His)-tagged RabGDIβ was produced as follows. After 96 h infection with P4 viral stock, Sf9 cells were centrifuged at 800 x g and resuspended in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM MgCl2, 1 mM β-mercaptoethanol, 0.2% Triton X-100, Roche complete protease inhibitor cocktail). After sonication, the solution was clarified by ultracentrifugation at 100,000 x g for 1 h at 4°C. The supernatant was incubated for 90 min with Ni-NTA beads (Qiagen) at 4°C. The beads (3 ml) were first washed with 100 ml of lysis buffer and then with 100 ml of the same buffer without detergent. Recombinant His-RabGDIβ was eluted from the beads with a gradient of imidazole (0-250 mM). The eluate was dialysed overnight in buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 5 mM MgCl2, 1 mM β-mercaptoethanol and purity judged by SDS-PAGE. All recombinant proteins were more than 70% pure, snap frozen in small aliquots and stored at -80°C until use.

**Antibodies**—Anti-GFP polyclonal rabbit antibody (Ab290, Abcam) was used at 1-2 µl/tube for immunoprecipitation. Texas Red-X Phalloidin (T-7471, Molecular Probes) was used at 1:100 dilution for immunofluorescence according to manufacturer’s instructions. Anti-human Rab8 and Rab11 monoclonal antibodies (#610844 and #610656, BD Transduction Laboratories) for immunoblotting were used at 1:1000 according to the manufacturer’s instructions. Polyclonal anti-Rab7 was a gift of J. Gruenberg (U. Geneve, CH). Anti-RabGDI antibody was obtained after purification of rabbit serum raised against full length rat RabGDIβ. The antibody recognises both RabGDI isoforms with a higher potency against the β-isoform.

**Cell Culture and Transfection**—HeLa and human embryonic kidney (HEK) 293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin G, and 100 U/ml streptomycin at 37°C with 10% CO2. Wild type and mouse embryonic fibroblasts (MEFs) null for Rce1 and 1cm1 were kind gifts from...
Steve Young (UCLA) and were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin G, 100 U/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol and MEM non-essential amino acids. Rat basophilic leukaemia (RBL) cells were cultured in Iscove’s modified Dulbecco’s medium containing 10% heat-inactivated FBS, 100 U/ml penicillin G, 100 U/ml streptomycin and 2 mM L-glutamine. Cells used in immunofluorescence experiments were grown on 24-well plated coverslips for 24 h, transfected, and fixed after 24 h (HeLa and HEK 293 cells). Cells used for subcellular fractionation were grown in 10 cm dishes, transfected, and homogenized 24 h after transfection. HeLa cells and HEK 293 cells were transfected with FuGENE6 (Roche Diagnostics, Indianapolis, IN) according to manufacturers’ instructions. RBL cells were transfected by electroporation. Briefly, following trypsinisation, cells were resuspended at 10^7 cells in 250 µl and placed in an electro-cuvette with 10 µg of plasmid DNA. After incubation for 10 min at 4°C, cells were electroporated at 250 mV, 960 μF using a Bio-Rad Gene-Pulser and returned to 4°C for a further 10 min. Electroporated cells were then cultured on coverslips in a 10 cm dish containing 10 ml of medium. Cells were then fixed 24-48 h later using 3% (w/v) paraformaldehyde.

**In vitro methylation assay - In vitro**

In vitro prenylation of GST-Rab proteins was performed in 25 μl reaction volumes in buffer containing 50 μM HEPES pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, and 0.5 μl of cold geranylgeranyl pyrophosphate (GGpp) to a final concentration of 20 μM. Each reaction contained 10 μM GST-Rab protein substrate and prenylation was initiated by the addition of 2 μM REP1 and 50 nM RGGT recombinant and incubated for 30 min at 37°C. Following prenylation, 62.5 μg of Rce1 and/or 15 μg of Icmt Sf9 membranes (kind gifts from Patrick Casey, Duke Univ.) were added to each condition, together with 0.5 μl of the methyl donor [³H]-S-adenosyl-L-methionine (AdoMet) (700 cpm/pmol) and cold AdoMet to a final concentration of 10 μM, in a final volume of 30 μl. Reactions were incubated at 37°C for 40 min and terminated by the addition of 50 μl of 10% Triton X-100 in PBS. 15 μl of glutathione beads in 500 μl of PBS were added to each condition and allowed to bind on a rotator for 1 h at room temperature. Next, the samples were centrifuged at 10,000 x g for 15 seconds, the supernatant was discarded and the beads were washed three times in PBS. Finally, the beads were resuspended in 100 μl of PBS, transferred directly into a scintillation vial containing 4 ml of scintillation fluid and the disintegrations per minute (dpm) were counted using a scintillation counter.

**In vivo carboxyl methylation of proteins in cultured cells - In vivo**

Methylation assay was performed as described previously (18). HEK 293 cells transfected with pEGFP plasmids for 8 h were first incubated in methionine-free medium (MFM, R7513, Sigma) for 1 h. They were then incubated in 1:9 complete medium:MFM made up to 5% FBS, along with 200 μCi of [³H-methyl]-L-methionine (Amersham) overnight. The following day, cells were harvested mechanically, transferred to eppendorf tubes and washed twice with ice cold PBS. The cells were resuspended in 200 μl of RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.5% sodium deoxycholate) containing complete protease inhibitor cocktail.
(Roche) for 10 min to allow lysis to occur. This was followed by centrifugation at 10,000 x g and the post-nuclear supernatant (PNS) was transferred to a fresh tube. 2 µl of anti-GFP antibody (Abcam) was added to the PNS and the solution was mixed on a rotator at 4°C for 1 h. Then, 10 µl of Protein G Sepharose beads (in a 1:1 slurry of in RIPA buffer) were added to the PNS at 4°C for 1 h. The beads were washed three times with RIPA buffer, and once with 50 mM Tris-HCl pH 6.8 to remove excess detergents. The protein-antibody complexes were subjected to electrophoresis on 12.5% SDS-PAGE gels. Gels were then immersed in a solution containing 10% acetic acid and 45% methanol for 5 min to ensure fixation of the proteins, and then re-hydrated in water. This was followed by immersion in 1 M sodium salicylate for 20 min, after which the gels were immediately vacuum-dried on blotting paper. Detection of radiolabelled proteins was performed using autoradiographic film and exposed after 3-4 days. The radioactive bands of interest were excised and methyl-esterified proteins were detected by an alkali hydrolysis/diffusion assay as described previously (19). Open tubes containing the gel piece were lowered into scintillation vials containing scintillation fluid. 1 M NaOH was added to each tube to immerse the gel piece and the vial was capped immediately, leaving the tube open inside. The vials were incubated at 37°C overnight. Ester-linked methyl groups are hydrolytically cleaved by alkali, releasing [3H]methanol which is distilled into the scintillation fluid. Following treatment with alkali, each eppendorf tube was carefully removed and the vial containing alkali-labile methanol was capped. An equal volume of 1 M HCl was added to the eppendorf tube to neutralise the alkali and the contents were transferred to a fresh vial containing scintillation fluid. This latter vial contained alkali-stable [3H]methionine that was incorporated into the peptide backbone. The amount of radioactivity in the alkali-labile and alkali-stable samples was measured using a scintillation counter with a tritium channel. To determine whether a protein was methylated in vivo, the methylation stoichiometry was calculated by the following equation: (alkali-labile dpm x number of methionine residues in the protein)/alkali-stable dpm.

**Immunofluorescence and Confocal Microscopy**

After transfection with pEGFP plasmids, cells were washed with PBS and then incubated in permeabilisation buffer (80 mM KPIPES pH 6.8, 5 mM EGTA, 1 mM MgCl2, 0.05% (w/v) saponin) for 5 min and were then fixed in 3% (w/v) paraformaldehyde in PBS for 15 min. Excess fixative was removed by repeated washing in PBS. When Texas-Red phalloidin was used, cells were further incubated for 15 min in PBS containing 0.5% bovine serum albumin and 0.05% saponin. The subsequent steps were performed in this solution. The cells were incubated with Texas-Red phalloidin for 30 min and washed three times. The coverslips were mounted in ImmunoFluor medium (ICN, Basingstoke, Hants, United Kingdom) and the fluorescence was visualized using a DM-IRBE Leica confocal microscope. Images were processed using TCS-NT software associated with the microscope and Adobe Photoshop 5.5 software. All images presented are single sections in the z-plane and are representative of at least 80% of the transfected cells in the coverslip.

**Temperature Block Experiments**

Temperature block experiments were performed as described previously (15). HeLa cells were seeded in 24-
well plates and grown overnight as described above. The following day, cells were transfected and 4 h later, the medium was replaced with complete medium supplemented with 20 mM HEPES buffer (15630-056; Invitrogen, Carlsbad, CA) and cells were placed at 20°C for 3 h to block exit of proteins from the Golgi apparatus. Cells were fixed at this point or further incubated at 37°C for 1 h. All cells were permeabilised and fixed as described above.

**Subcellular Fractionation-** After transfection, HEK 293 cells were harvested mechanically, transferred to 15 ml tubes and centrifuged at 1000 x g for 5 min at 4°C. Cells were washed with phosphate-buffered saline (PBS) and centrifuged once more. Cells were then resuspended in hypotonic lysis buffer (20 mM Tris pH 7.5, 5 mM MgCl₂, 1 mM DTT, Roche complete protease inhibitor cocktail) and lysed by sonication, followed by centrifugation at 800 x g for 10 min at 4°C. The post-nuclear supernatant (PNS) was transferred to a Beckman Centrifuge Tube and subjected to ultracentrifugation at 100,000 x g for 1 h at 4°C using a TLA45 Beckman rotor. The supernatant (S100) containing the cytosolic fraction was transferred to a fresh tube and the pellet (P100) containing the membrane fraction was resuspended in an equivalent volume of lysis buffer. The fractions were subjected to electrophoresis on 12.5% SDS-PAGE gels, transferred to polyvinylidene difluoride (PVDF) membranes and proteins were detected by Western immunoblotting. Densitometry quantification was achieved using Fuji Film Intelligent Dark Box LAS-3000 and Aida Image Analyse 3.52 Software.

**RabGDI extraction assay-** Membrane proteins (30 µg) prepared from MEFs in buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTE, 1 mM GDP and Roche complete protease inhibitor cocktail, were incubated with increasing amounts of purified His-RabGDIβ (0-8 µM) for 20 min at 37°C. The extracted Rab proteins in complex with RabGDI were separated from membrane proteins by ultracentrifugation at 100,000 x g for 1 h at 4°C. The soluble fraction (S100) and the membrane fraction (P100) were resolved on 12.5% SDS-PAGE.

**Gel filtration chromatography-** S100 (100 µg) fractions prepared as above were loaded onto a Superdex 200 3.2/30 column using a SMART system (Pharmacia). The column was equilibrated in buffer containing 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 8 mM MgCl₂, 2 mM EDTA, 1 mM DTT and 10 µM GDP, at a flow rate of 40 µl/min. The samples (50 µl) were injected, and the material eluting between 0.8 ml and 2 ml was collected in 50µl fractions.

**Results**

**In vitro carboxyl methylation of Rab-CAAX proteins**
To determine whether Rab proteins which naturally possess a CAAX motif are substrates for Rce1 and Icmt in vitro, recombinant GST-Rab fusion proteins were produced after expression in E. coli. Since prenylation and proteolysis are required for Icmt-dependent methylation, the in vitro assay was designed as a coupled prenylation/proteolysis/methylation assay. Geranylgeranylation reactions of GST-Rab substrates were performed in the presence of recombinant RGGT and recombinant REP1, followed by the addition of Rce1 and Icmt-enriched membrane fractions to initiate AAX proteolysis and carboxyl methylation, respectively. Subsequently, proteins
were isolated using glutathione beads and the amount of $[^3\text{H}]$-S-adenosyl-L-methionine ($[^3\text{H}]$AdoMet) incorporated was quantified by liquid scintillation counting.

Rab18 was used initially as a model Rab protein with a CAAX motif for the in vitro methylation assays in order to determine optimal conditions. We found that GST-Rab18 methylation was strictly dependent on REP1 and RGTT (Figure 1A). This is consistent with the fact that both Rce1 and Icmt only modify prenylated substrates. The incorporation of $[^3\text{H}]$AdoMet increased with time to over 14 pmol after 60 min following the addition of Rce1, Icmt and $[^3\text{H}]$AdoMet. The yield was calculated to be ~5%, taking into account the estimate that 10-30% of the total protein was prenylated (unpublished observations). GST-Rab18 showed an attenuated level of methylation in the presence of either Rce1 or Icmt alone. The reduced methylation is most likely due to the endogenous levels of both proteins present in the enriched membrane preparations used in the assay.

The methylation status of GST-Rab18 was compared with that of other candidate Rab proteins possessing a CAAX motif (GST-Rab13, GST-Rab23 and GST-Rab38) (Figure 1B). The results suggest that GST-Rab13, GST-Rab18, GST-Rab23 and GST-Rab38 were all carboxyl methylated to the same extent, suggesting that they are all substrates for Rce1 and Icmt alone. The reduced methylation is most likely due to the endogenous levels of both proteins present in the enriched membrane preparations used in the assay.

The methylation status of GST-Rab18 was compared with that of other candidate Rab proteins possessing a CAAX motif (GST-Rab13, GST-Rab23 and GST-Rab38) (Figure 1B). The results suggest that GST-Rab13, GST-Rab18, GST-Rab23 and GST-Rab38 were all carboxyl methylated to the same extent, suggesting that they are all substrates for Rce1 and Icmt (Figure 1B). As a negative control, GST-Rab18 was mixed with Triton X-100 prior to addition of Rce1 and Icmt, which solubilises these integral membrane proteins and abolishes their activity. As expected, no methylation was observed for GST-Rab18 when Triton X-100 was included prior to addition of Rce1 and Icmt membranes. GST-Rab5a was used as a control since it is doubly geranylgeranylated on adjacent cysteines, and therefore expected not to be a substrate for either Rce1 or Icmt (12). As expected, GST-Rab5a showed no significant methylation under the same conditions in vitro. We conclude that GST-Rab13, GST-Rab18, GST-Rab23 and GST-Rab38 are carboxyl methylated in vitro.

**In vivo carboxyl methylation of Rab proteins**

To investigate whether carboxyl methylation of CAAX-containing Rab proteins occurred in vivo, HEK 293 cells were transfected with EGFP-Rab constructs and labelled with $[^3\text{H}]$-L-methionine. The EGFP-tagged proteins were immunoprecipitated and subjected to SDS-PAGE and autoradiography. Since $[^3\text{H}]$-L-methionine is used to synthesise both the AdoMet pool and the tRNA-methionine pool in cells, an alkali hydrolysis assay was used to differentiate between methylated versus non-methylated proteins (18). Methyl esters are subject to hydrolysis in the presence of alkali, releasing vapour phase $[^3\text{H}]$methanol. Labelled methionines in the protein backbone are insensitive to this treatment and thus are differentiated from the labelled methyl groups. As a positive control, we used EGFP-Rac1 (C-terminal sequence, CLLL) which is known to be methylated (20). We then tested Rab1a(CC), Rab4a(CGC), Rab7a(CSC) and Rab8a(CVLL), Rab11a(CCQI), Rab13(CSLG), Rab18(CSVL), Rab23(CSVP), Rab27a(CGC), Rab38(CAKS) and finally RalA(CCIL), a Ras-like protein which possesses a double-cysteine motif and thus could potentially undergo double-prenylation.

The results showed that the CAAX-containing Rabs, EGFP-Rab8a, EGFP-Rab13, EGFP-Rab18 and EGFP-Rab23, were carboxyl methylated in vivo consistent with the
in vitro studies (Figure 2). Furthermore, EGFP-Rab4a, EGFP-Rab7a and EGFP-Rab27a (the latter expressed in neuroendocrine AtT20 cells) were also carboxyl methylated consistent with previous studies which demonstrated that CXC-containing Rab proteins are carboxyl methylated (11,12). In contrast, EGFP-Rab1a was not carboxyl methylated as previously demonstrated in vitro, due to the vicinity of the geranygeranylated cysteines (12). Also, EGFP-Rab11a(CCQNI) and EGFP-Rab5a(CCSN) did not show carboxyl methylation, further suggesting that these proteins undergo geranylgeranylation on consecutive cysteines, despite the context of a potential CAAX motif. Interestingly, RalA was carboxyl methylated in vivo despite the fact that it exhibits a similar double-cysteine motif as Rab5a. This result suggests that RalA is indeed singly prenylated and processed by a CAAX prenyltransferase.

Surprisingly, EGFP-Rab38 was not carboxyl methylated in contrast with the in vitro observations described above. We hypothesised that HEK 293 cells may not represent a physiological cell type for Rab38 and therefore may not undergo correct processing in these cells. To test this hypothesis, the experiment was repeated using a melanoma-derived cell line, MNT1, which normally expresses Rab38 and had been stably transfected with EGFP-Rab38. Consistent with the results observed in HEK 293 cells, EGFP-Rab38 was not carboxyl methylated in MNT1 cells (Figure 2). An alternative possibility could be that the CAAX motif of Rab38 may not be a good substrate for processing by Rce1 in vivo. Since Rab8a was found to be carboxyl methylated in vivo, the CAKS motif of Rab38 was substituted for the CVLL motif of Rab8a. Surprisingly, the EGFP-Rab38CVLL mutant was carboxyl methylated in vivo in HEK 293 cells. In previous work, the amino acid at the A2 position was shown to be critical for post-prenylation processing (21). Since V, L, and I residues are favoured at that position, EGFP-Rab38CALS and EGFP-Rab38CAVS mutants were generated by site-directed mutagenesis and assayed for carboxyl methylation. As predicted, a single substitution at the A2 position of lysine for leucine or valine enabled carboxyl methylation, presumably because the mutants were efficiently processed by Rce1. Our results highlight further the importance of the A2 residue in CAAX proteolysis.

The calculated stoichiometries from the carboxyl methylation assays were variable. These were calculated from the disintegrations per minute (dpm) obtained for the alkali-labile and alkali-stable counts, as well as the number of methionines present in each EGFP-tagged protein (Supplementary Table 1). For some proteins, the values were sometimes more than 1.0. Several possibilities exist to explain these discrepancies. It is possible that there is a difference in the specific activity of the Met-tRNA pool and the S-AdoMet pool, depending on how cells utilise the radiolabel intracellularly. Also, the N-terminal methionine in a protein is often cleaved off, such that the estimate of number of methionines based on primary sequence is only tentative (22).

In conclusion, these results suggest that Rab proteins containing a C-terminal CAAX motif (Rab8a, Rab13, Rab18 and Rab23) are carboxyl methylated in vivo. Rab5a and Rab11a, which have a CAAX-like motif, are not carboxyl methylated in vivo, most likely due to the double geranylgeranylation of adjacent cysteines, as with Rab1a. Furthermore, Rab4a, Rab7a and Rab27a were also substrates for methylation as expected for CXC motif-containing Rabs. Finally, Rab38 is methylated in vitro
but not in vivo. Remarkably, introduction of a valine or a leucine residue at the A2 position of EGFP-Rab38 resulted in the protein being carboxyl methylated in vivo, strengthening the proposal that this position plays a critical role for processing by Rce1.

**Localisation of Rab-CAAX proteins in Rce1- and Icmt-deficient cells**

To examine whether the membrane targeting of Rab-CAAX proteins was affected in the absence of post-prenylation processing, we used cultured cells derived from Rce1 or Icmt knock-out mice. EGFP-Rab8a, EGFP-Rab18 and EGFP-Rab23 were transiently expressed in wild type, Rce1-/- or Icmt-/- MEFs, and their localisation was observed by confocal microscopy. The localisation of the EGFP-Rab fusion proteins was compared with that of EGFP-HRas. Consistent with previous observations, EGFP-HRas was localised to the plasma membrane with some Golgi staining in wild type cells, but in Rce1-/- cells the plasma membrane staining was significantly reduced with a concomitant increase in cytosolic staining (Figures 3A-B). In contrast, EGFP-Rab8a showed staining in the perinuclear region as well as at the tips of dendrites in both wild type and Rce1 null cells (Figures 3C-D). Incidentally, we observed that Rce1-/- cells expressing EGFP-Rab8a showed an increase in surface area of the cell body, although the reason for this was unclear. EGFP-Rab18 stained vesicular structures in both wild type and Rce1-/- cells (Figures 3E-F), whilst EGFP-Rab23 localised predominantly to the plasma membrane in both Rce1+/+ and Rce1-/- cells (Figures 3G-H).

Similarly, EGFP-HRas exhibited plasma membrane staining in wild type cells but was mislocalised in Icmt-/- cells (Figures 4A-B). No significant changes in subcellular localisation of the Rab-CAAX proteins were observed in Icmt-deficient cells. EGFP-Rab8a was prominently localised to the perinuclear area (Figures 4C-D). EGFP-Rab18 showed a vesicular staining pattern (Figures 4E-F), while EGFP-Rab23 was mostly localised to the plasma membrane (Figure 4G-H) in wild type and Icmt-/- cells.

**Rab-CAAX proteins do not traffic via the secretory pathway**

Ras proteins undergo post-prenylation processing and are then targeted to the plasma membrane via the classical secretory pathway. Since Rab8a and Rab23 also undergo post-prenylation processing, we hypothesised that they may also be targeted to membranes via the same pathway. To test this hypothesis, we used a temperature block assay (15). Upon 20°C incubation, EGFP-HRas accumulated in the Golgi, indicative of a protein that moves through the secretory pathway (Figure 5). Returning the cells to 37°C removes the trafficking block, allowing the protein to exit the Golgi and target to the plasma membrane (Figure 5). In contrast, there was no change in the localisation of EGFP-Rab8a (unpublished observations) or EGFP-Rab23 in the presence or absence of the temperature block (Figure 5). This suggests that Rab8a and Rab23 do not traffic through the secretory pathway. We also analysed the di-cysteine mutants to determine whether di-geranylgeranylation influenced the mechanism of membrane targeting, which could explain the transient Golgi association observed. However, temperature block experiments showed that the di-cysteine mutants displayed the same staining at 20°C or 37°C as the wild type CAAX-containing Rab23 (unpublished observations), suggesting that they do not travel along the secretory pathway as shown for H-Ras.
Functional consequences of prenylation and post-prenylation processing

The functional activities of the Rab-CAAX proteins are mostly unknown and thus assessing their functionality was not possible. However, it has been reported that overexpression of Rab8 leads to a dendritic morphology due to rearrangements of the cytoskeleton (23). The dramatic morphological changes upon overexpression of Rab8 involve the reorganisation of actin, with attenuation of stress fibres and the formation of a more prominent cortical concentration of actin (23). To test the functional consequences of post-prenylation processing, we used two approaches. One was to create a double-cysteine mutant of Rab8 which cannot be methylated (EGFP-Rab8aCC) and as control a double-cysteine mutant which can be methylated (EGFP-Rab8aCSC) (12). HeLa cells were transiently transfected with the EGFP-Rab8a mutants, fixed and their intracellular localisation was observed by confocal microscopy. In addition, the cells were stained with Texas-Red phalloidin which binds filamentous actin and labels the actin network. Cells transfected with wild type EGFP-Rab8a showed significant changes in cell morphology, and were often found to have a large number of cellular protrusions (Figure 6). In addition, these cells showed dramatic redistribution of actin, mostly to newly formed cell processes in the cell periphery with attenuation of stress fibres. The EGFP-Rab8a di-cysteine mutants, EGFP-Rab8aCC and EGFP-Rab8aCSC, produced a similar staining pattern to the wild type Rab8a (Figure 6). Similar results were obtained when BHK or RBL cells were transfected with the same constructs (unpublished observations). The observed cytoskeletal changes and cell morphological effects appeared specific to Rab8a because EGFP-Rab5a overexpression did not lead to either formation of cell protrusions or actin reorganisation. These results suggest that the EGFP-Rab8a di-cysteine proteins are functional. In addition, Rab8 was reported to induce cellular processes (24). Quantitative analysis in RBL cells, in which over 70% of cells overexpressing wild type EGFP-Rab8a or the di-cysteine mutants had 2 or more processes, while in non-transfected cells less than 20% exhibited 2 or more processes, suggest again that the Rab8 mutants were functional (unpublished observations). Finally, we noted that formation of cell protrusions was observed upon EGFP-Rab8a overexpression in MEFs, including the Rce1 and Icmt mutant cell lines analysed, suggesting that Rab8a was able to function properly in the absence of post-prenylation processing (Figures 3C-D and 4C-D).

Membrane-associated non-methylated Rab proteins are more efficiently extracted by RabGDI

We then investigated the membrane/cytosol partitioning of endogenous Rab proteins in Icmt−/− MEFs. Steady-state levels of Rab8 (CVLL), Rab7 (CSC) and Rab11 (CCQNI) proteins were analysed after subcellular fractionation (Figure 7). Rab11, which served as a non-methylated control, did not show any apparent difference in partitioning in Icmt+/+ versus Icmt−/− MEFs. Approximately 55% of the total protein was present in the cytosolic fraction regardless of the host cell. In contrast, we observed a significant shift in Rab7 and Rab8 partitioning from the membrane to the cytosol fraction in Icmt−/− cells. This shift was more pronounced for the singly geranylgeranylated Rab8 proteins (from ~20% to ~70% soluble) than for the doubly geranylgeranylated Rab7

membrane/cytosol partitioning of endogenous Rab proteins in Icmt−/− MEFs. Steady-state levels of Rab8 (CVLL), Rab7 (CSC) and Rab11 (CCQNI) proteins were analysed after subcellular fractionation (Figure 7). Rab11, which served as a non-methylated control, did not show any apparent difference in partitioning in Icmt+/+ versus Icmt−/− MEFs. Approximately 55% of the total protein was present in the cytosolic fraction regardless of the host cell. In contrast, we observed a significant shift in Rab7 and Rab8 partitioning from the membrane to the cytosol fraction in Icmt−/− cells. This shift was more pronounced for the singly geranylgeranylated Rab8 proteins (from ~20% to ~70% soluble) than for the doubly geranylgeranylated Rab7
protein (from ~20% to ~50% soluble) (Figure 7).

We hypothesised that lack of methylation disrupted the RabGDI-mediated membrane/cytosol cycle of Rab proteins by altering the ability of RabGDI to extract membrane-associated Rabs (5). Thus, we measured the extraction of endogenous Rab7, Rab8 and Rab11 by recombinant RabGDIβ from Icmt+/+ and Icmt-/- MEF membranes (Figure 8A). Increasing concentrations of RabGDIβ led to increased amounts of Rab proteins extracted. We did not observe a significant difference when comparing Rab11 extraction from Icmt+/+ and Icmt-/- MEF membranes (Kd = 515 ± 63 and 445 ± 23 nM, respectively) (Figure 8A). In contrast, the Kd values for Rab8 and Rab7 protein extraction were significantly decreased in Icmt-/- cells compared with wild type cells (Figure 8A). We observed a 6-fold decrease in Kd for non-methylated Rab8 (320 ± 106 nM versus 55 ± 17 nM for wt and Icmt-/-, respectively) and a 4-fold decrease for Rab7 (700 ± 13 nM and 170 ± 20 nM for wt and Icmt-/-, respectively). To address the possibility that non-methylated Rab proteins are subject to non-specific solubilisation, we incubated membranes from Icmt+/+ and Icmt-/- MEFs without RabGDI for different time points (0-90 min). We did not observe any differences in partitioning between membrane preparations. The levels of cytosolic Rab proteins were very low (~5%) (data not shown), suggesting that the effects observed were RabGDI dependent.

Finally, we verified that the non-methylated Rab proteins were complexed with RabGDI in cells by subjecting the cytosolic fractions of Icmt+/+ and Icmt-/- MEFs to gel filtration chromatography (Figure 8B). We observed that Rab8 was co-eluted with RabGDIα and RabGDIβ in the extracts. We did not observe any trace of Rab8 in the low molecular weight fractions as expected from the monomeric protein (fractions 20-23). These results suggest that as expected, RabGDIIs form a stable complex with the solubilised Rab proteins and thus are probably responsible for the increased soluble steady-state levels of non-methylated Rab proteins in Icmt-/- cells.

Discussion

The present study reveals that Rab proteins which undergo single geranylgeranyl modification are carboxyl methylated both in vitro and in vivo, as observed in Ras-like proteins containing a CAAX-motif. The post-prenylation processing occurring in Rabs does not affect their membrane targeting. However, geranylgeranylated non-methylated Rab-CAAX and Rab-CXC proteins are affected in their membrane/cytosol partitioning at steady-state levels, suggesting a role for methylation in Rab function by regulating the cycle of Rab membrane recruitment and retrieval.

We show here that Rab proteins with CAAX motifs, such as Rab8a, Rab13, Rab18 and Rab23 are methylated in vitro and in vivo, as are Rab4a, Rab7 and Rab27a, which all possess CXC motifs, as previously suggested (12,25). In contrast, Rab1a, Rab5a and Rab11a are not methylated, again consistent with previous studies (12,25) and presumably because the proximity of the two geranylgeranyl groups prevents processing by Icmt.

We also tested RaLA, a Ras family protein which contains a CCIL motif very similar to Rab5a, and found it to be carboxyl methylated unlike Rab5a. This observation strengthens our previous suggestion that only Rab proteins are di-prenylated and that non-Rab proteins are not substrates of the REP:RGGT pathway.
As for Ras and Rho proteins, prenylation is an absolute requirement for further processing since absence of either REP1 or RGGT abolishes carboxyl methylation in vitro. Similarly, cleavage by Rce1 is essential for carboxyl methylation since no methylation was detected when Rce1 was absent. An interesting finding was that Rab38 was able to be methylated in vitro but not in vivo. Our experiments suggest that this is due to the critical A2 position in the CAAX motif since substitution of the K for either V or L at this site allowed Rab38 methylation in vivo. Thus, not all single cysteine motif-containing Rabs can be automatically assumed to undergo post-prenylation processing. Conversely, methylation of all Rab proteins is likely to be catalysed by Icmt rather than a specific methyltransferase for Rab proteins, since deletion of Icmt abolishes the carboxyl methylation of Rab proteins with a CXC motif (11).

The best studied function of carboxyl methylation is that of increasing the hydrophobicity of prenylated proteins and thus increasing membrane affinity, particularly for farnesylated proteins (9). Furthermore, carboxyl methylation contributes to farnesylated protein targeting since absence of methylation reduces association of Ras proteins with cellular membranes and mislocalisation from the plasma membrane (10,11). However, the effect on geranylgeranylated Rho proteins is more subtle (8). Our present results confirm and extend these findings by suggesting that post-prenylation processing does not appear to affect membrane targeting of geranylgeranylated Rab proteins. However, the cycle of membrane/cytosol partitioning of methylated Rab proteins is significantly affected in Icmt-/- MEFs. This effect is likely to be due to the decreased hydrophobicity of non-methylated Rabs and is more apparent for singly prenylated Rab8 than for doubly prenylated Rab7.

The increased cytosolic pool of non-methylated Rabs in Icmt-/- appears to be related to increased RabGDI extraction. We observed a decrease in the Kd values for RabGDI extraction in Icmt-/- cells for Rabs that are normally methylated (Figure 8A). Furthermore, the increased soluble pool of non-methylated Rabs in Icmt-/- cells is retained in 1:1 RabGDI:Rab complexes in the cytosol (Figure 8B). These observations are consistent with studies on Rho/Rac proteins where an increased affinity of RhoGDI for non-methylated Rac1 in Icmt-/- MEFs was observed (8).

Our data thus suggest that methylation plays a role in regulating the interaction between Rab proteins and RabGDI. Whilst not all Rab proteins are methylated, such a mechanism may regulate the cycling of methylatable Rab proteins on and off membranes, in conjunction with factors that affect the activation state of the GTPase at the membrane surface, such as GEFs and GAPs. Carboxyl methylation is potentially reversible, suggesting that methylation-dependent fine tuning of the membrane in/out cycle of Rab proteins may be functionally important. Future studies will require the identification of one or more methyl-esterases for prenylated proteins in order to further study the importance of this regulatory mechanism.

Another possible function of carboxyl methylation is to affect protein turn-over. Carboxyl methylation prolongs the half-life of RhoA in mammals (26) and α-factor in S. cerevisiae (27,28). However, many other Rho/Rac members are unaffected and thus the general significance of these findings remains unclear.
Nevertheless, a possible selective role of carboxyl methylation on Rab turnover is a distinct possibility.

The mechanisms underlying Rab membrane targeting remain unclear. Since Ras and Rho proteins are postulated to first associate with membranes at the surface of the ER where Rce1 and Icmt are localised, we hypothesise that Rab-CAAX proteins would use the same mechanism. Following the initial membrane encounter where prenylated proteins are processed by proteolysis and carboxyl methylation, some proteins travel the secretory pathway (such as H-Ras), while other (such as K-Ras) do not (29). Our results suggest that Rab proteins behave like the latter. The trafficking of Rab8a and Rab23 are not perturbed by a temperature block (20°C), which affects H-Ras trafficking. We have previously observed similar results using Rab5a-CAAX mutants (15); these results are consistent with the hypothesis that membrane association of newly-prenylated Rabs is mediated by REP or alternatively RabGDI or both (2,5).

In light of these data, we propose two pathways in Rab membrane targeting, one direct and one indirect (Figure 9). After prenylation by RGGT, the prenylated Rab bound to REP in a stable complex is available for membrane delivery. The prenylation motif then dictates whether the proteins require further processing or targeting to specific compartments. Rab proteins containing two adjacent prenylated cysteines, such as CC or CCXX, do not require further processing and may thus be delivered directly to their cognate membrane compartment. Monogeranylgeranylated Rab-CAAX proteins need to first come into contact with ER membranes and be recognised by Rce1 and Icmt, sequentially. Then, the fully processed proteins are re-directed to their target membrane, in a step likely to be mediated by a cytosolic transfer mediated by REP and/or RabGDI. Di-geranylgeranylated Rab proteins containing a CXC motif do not need processing by Rce1; however, they require carboxyl methylation catalysed by Icmt within ER membranes. Following methylation, Rab-CXC proteins may also be delivered to the correct membrane organelle by REP and/or RabGDI, as Rab-CAAX proteins. An alternative possibility contrary to current view is that the post-prenylation enzymes are widely distributed among endomembranes, in which case all Rab delivery could be direct. Future studies should address these hypotheses, which will hopefully lead to a better understanding of the intracellular membrane organisation of eukaryotic cells.

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Foot Notes

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Figure Legends

Figure 1: In vitro carboxyl methylation assay of recombinant GST-Rab proteins. (Panel A) Coupled in vitro prenylation and methylation of GST-Rab18 was performed for the indicated times and methylation was measured by the incorporation of [3H]AdoMet as described under MATERIALS AND METHODS. Control reactions were performed in the absence of either REP1, RGGT, Rce1-enriched fraction or Icmt-enriched fraction. Error bars were calculated by dividing the average deviation in dpm by the total amount of radioactivity in dpm/mol. All reactions were performed in duplicate and the results are representative of at least 3 experiments. (Panel B) Coupled in vitro prenylation and methylation of GST-Rab proteins was performed for 40 min and methylation was measured by the incorporation of [3H]AdoMet as described under MATERIALS AND METHODS. As a negative control, 10% Triton X-100 was added prior to addition of Rce1 and Icmt. All reactions were performed in duplicate and the results are representative of at least 3 experiments.

Figure 2: In vivo carboxyl methylation assay of Rab proteins. HEK 293 (or MNT1 where indicated) cells were transiently transfected with constructs leading to the expression of the indicated EGFP-Rab fusion proteins. The transfected cells were then labelled with [3H-methyl]-L-methionine for 13-16 h. Methylation was measured by an alkali hydrolysis assay, as described under MATERIALS AND METHODS. The stoichiometry represented in the graph was determined by a ratio of the alkali-labile counts to the alkali-stable counts. The values were corrected for background and for the number of methionine residues in each protein. All reactions were performed in duplicate and the results are representative of at least 3 experiments.

Figure 3: Intracellular localisation of Rab-CAAX proteins in Rce1-/- MEFs. Rce1+/+ or Rce1-/- MEFs were transiently transfected with EGFP-HRas (A, B), EGFP-Rab8a (C, D), EGFP-Rab18 (E, F) or EGFP-Rab23 (G, H). After 24 h, cells were fixed and subjected to confocal immunofluorescence microscopy as described under MATERIALS AND METHODS. Bar, 20 µm.

Figure 4: Intracellular localisation of Rab-CAAX proteins in Icmt-/- MEFs. Icmt+/+ or Icmt-/- MEFs were transiently transfected with EGFP-HRas (A, B), EGFP-Rab8a (C, D), EGFP-Rab18 (E, F) or EGFP-Rab23 (G, H). After 24 h, cells were fixed and subjected to confocal immunofluorescence microscopy as described under MATERIALS AND METHODS. Bar, 20 µm.

Figure 5: Temperature block assay and intracellular localisation of EGFP fusion proteins in HeLa cells. HeLa cells were transiently transfected with EGFP-tagged fusion protein constructs and incubated at 37°C for 4 h. The temperature block was induced by incubating the cells at 20°C for 3 h and the block was removed by a further incubation at 37°C for 1 h. Cells were then fixed and subjected to confocal
immunofluorescence microscopy as described under MATERIALS AND METHODS. Bar, 20 µm.

**Figure 6:** Transient overexpression and intracellular localisation of EGFP-Rab8a fusion proteins and their effect on actin cytoskeleton and cell morphology. HeLa cells were transiently transfected with either EGFP-Rab5a (A, B), wild type EGFP-Rab8a (C, D), EGFP-Rab8aCC (E, F) or EGFP-Rab8aCSC (G, H). After 24 h, cells were fixed, stained with Texas-Red phalloidin and subjected to confocal immunofluorescence microscopy as described under MATERIALS AND METHODS. Arrowheads indicate non-transfected cells with abundant stress fibres. Arrows indicate transfected cells with redistribution of actin. Bar, 20 µm.

**Figure 7:** Subcellular fractionation of Rab proteins from *Icmt*+/+ and *Icmt*-/− MEF. (Panel A) Equivalent volumes of S100 and P100 fractions were resolved by SDS-PAGE and proteins detected by immunoblotting. (Panel B) The signals corresponding to Rab proteins were quantified by densitometry using Fuji Film Intelligent Dark Box LAS-3000 and Aida Image Analyse 3.52 Software. The graph data shown represents the mean of duplicate determinations from a single experiment, which is representative of three such experiments.

**Figure 8:** RabGDI-mediated extraction of Rab proteins. (Panel A) Membrane proteins (30 µg) from wild type and *Icmt*-/− MEFs were incubated with the indicated increasing amounts of human RabGDIβ. After 20 min at 37°C, the soluble complex proteins were separated from membrane bound Rab proteins by ultracentrifugation for 1 h at 100,000 x g. The S100 and P100 fractions were quantified by densitometry using Fuji Film Intelligent Dark Box LAS-3000 and Aida Image Analyse 3.52 Software. The Kd values were calculated and fitted using Microsoft Excel and GOSA software. (Panel B) Cytosolic extracts (100 µg) from wild type and *Icmt*-/− MEFs were resolved by gel filtration chromatography on Superdex 200 and analysed by immunoblotting using Rab8 and RabGDI antibodies.

**Figure 9:** Post-prenylation processing pathways of Rab proteins. Following prenylation, Rab proteins with a CAAX motif are delivered to the ER by REP, where they are first processed by Rce1 and then by Icmt. Rab proteins with a CXC motif bypass the Rce1 step and are targeted to Icmt for methylation. Fully processed Rab proteins are then delivered to their target membrane. Rab proteins with adjacent prenylated cysteines are not processed by either Rce1 or Icmt and are targeted directly to their specific membrane compartment.

**Supplementary Table 1: In vivo methylation assay.** The dpm for the alkali-labile counts represents labelled methyl groups and the dpm for the alkali-stable counts represents labelled methionines incorporated into the peptide backbone. The prenylation motif and the number of methionines in each GFP-tagged fusion protein are also indicated. The stoichiometric values were calculated by multiplying the alkali-labile dpms by the number of methionines and dividing this value by the alkali-stable dpms. The table is a representative of typical values for each construct obtained using this assay and results were reproducible. (Y) denotes methylation and (N) denotes no methylation.
Figure 1

A

pmol of AdoMet transferred to Rab18

REP1  RGTT  Rce1  Lcmt
-     +     +     +     +     +
+     -     +     +     +     +
+     +     -     +     +     +
+     +     +     +     +     +

B

pmol of AdoMet transferred to Rab

Rab18 + Triton X-100  Rab6a  Rab13  Rab18  Rab23  Rab38
Figure 2
Figure 3

Rce1+/+  Rce1-/-

A. EGFP-HRas  B. EGFP-HRas
C. EGFP-Rab8a  D. EGFP-Rab8a
E. EGFP-Rab18  F. EGFP-Rab18
G. EGFP-Rab23  H. EGFP-Rab23
Figure 4

Icmt+/+

A. EGFP-HRas

C. EGFP-Rab8a

E. EGFP-Rab18

G. EGFP-Rab23

Icmt-/-

B. EGFP-HRas

D. EGFP-Rab8a

F. EGFP-Rab18

H. EGFP-Rab23
Figure 5

| 37°C | 20°C | 20°C + 37°C |
|------|------|------------|
| EGFP-HRas |    |            |
| EGFP-Rab23 |  |    |
Figure 6
Figure 7

A

|        | lcmt +/+ | lcmt -/- |
|--------|----------|----------|
| S      |          |          |
| P      |          |          |

Rab7

Rab8

Rab11

B

% soluble Rab protein

Rab7

Rab8

Rab11
**Figure 8**

**A**

| RabGDIβ | S | P | S | P | S | P | S | P | Kd (nM) |
|---------|---|---|---|---|---|---|---|---|---------|
| lcmt +/+ |   |   |   |   |   |   |   |   | 700 ± 13 |
| lcmt -/- |   |   |   |   |   |   |   |   | 170 ± 20 |

| Rab7    | S | P | S | P | S | P | S | P | Kd (nM) |
|---------|---|---|---|---|---|---|---|---|---------|
| lcmt +/+ |   |   |   |   |   |   |   |   | 320 ± 106 |
| lcmt -/- |   |   |   |   |   |   |   |   | 55 ± 17 |

| Rab8    | S | P | S | P | S | P | S | P | Kd (nM) |
|---------|---|---|---|---|---|---|---|---|---------|
| lcmt +/+ |   |   |   |   |   |   |   |   | 515 ± 63 |
| lcmt -/- |   |   |   |   |   |   |   |   | 445 ± 23 |

**B**

[Immunoblot images and molecular weight markers indicating void, 670 kDa, 158 kDa, 44 kDa, and 17 kDa.]
Supplementary Table 1

|                | dpm (Alkali labile) | dpm (Alkali stable) | Methionine | Stoichiometry | Methylation |
|----------------|---------------------|----------------------|-------------|----------------|-------------|
| Background     | 14                  | 735                  | -           | -              | -           |
| Hicas (CVL.S)  | 199                 | 2447                 | 11          | 0.88           | Y           |
| Rac1 (CLLL)    | 66                  | 1076                 | 9           | 0.55           | Y           |
| RabA (CCIL)    | 685                 | 3850                 | 12          | 0.88           | Y           |
| Rab9a (CC)     | 81                  | 24507                | 10          | 0.03           | N           |
| Rab4a (GGC)    | 429                 | 5861                 | 10          | 0.78           | Y           |
| Rab5a (CCSN)   | 31                  | 19884                | 11          | 0.62           | N           |
| Rab7a (CSC)    | 1161                | 11959                | 10          | 0.97           | Y           |
| Rab9a (CVLL)   | 761                 | 6937                 | 12          | 1.32           | Y           |
| Rab11a (CCONH) | 29                  | 17387                | 9           | 0.02           | N           |
| Rab13 (CSL.G)  | 682                 | 7061                 | 11          | 1.03           | Y           |
| Rab18 (CS01)   | 1455                | 9099                 | 9           | 1.35           | Y           |
| Rab23 (CSIP)   | 4085                | 27436                | 11          | 1.64           | Y           |
| Rab27a (CGC)   | 794                 | 14142                | 12          | 0.66           | Y           |
| Rab38 (CAKS)   | 33                  | 5752                 | 10          | 0.03           | N           |
| MN1 Rab38 (CAKS) | 698               | 82156                | 10          | 0.01           | N           |
| Rab38CVLL      | 2057                | 16851                | 10          | 1.12           | Y           |
| Rab38CALS      | 1244                | 23534                | 10          | 0.53           | Y           |
| Rab38CAVS      | 1038                | 20718                | 10          | 0.33           | Y           |
Rab GTPases containing a CAAX motif are processed post-geranylgeranylation by proteolysis and methylation

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