

**The E3 Ligase TRIM32 is  
an effector of the RAS  
family GTPase RAP2**

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# Declaration

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I, Berna Demiray, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated.

London, 2014

# The E3 Ligase TRIM32 is an Effector of the RAS family GTPase RAP2

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Classical RAS oncogenes are mutated in approximately 30% of human tumours and RAP proteins are closely related to classical RAS proteins. RAP1 has an identical effector domain to RAS whereas RAP2 differs by one amino acid. RAP2 not only shares effectors with other classical RAS family members, but it also has its own specific effectors that do not bind to RAP1 or classical RAS family proteins. Thus, although closely related, RAP2 performs distinct functions, although these have been poorly characterised.

Using RAP2 as bait in Tandem Affinity Purifications, we have identified several RAP2 interacting proteins including TRIM32; a protein implicated in diverse pathological processes such as Limb-Girdle Muscular Dystrophy (LGMD2H), and Bardet-Biedl syndrome (BBS).

TRIM32 was shown to interact specifically with RAP2 in an activation- and effector domain-dependent manner; demonstrating stronger interaction with the RAP2 V12 mutant than the wild-type RAP2 and defective binding to the effector mutant RAP2 V12A38.

The interaction was mapped to the C-terminus of TRIM32 (containing the NHL domains) while mutations found in LGMD2H (R394H, D487N,  $\Delta$ 588) were found to disrupt binding to RAP2. The TRIM32 P130S mutant linked to BBS did not affect binding to RAP2, suggesting that the RAP2-TRIM32 interaction may be functionally involved in LGMD2H.

Because TRIM32 is an E3 ubiquitin ligase, the possible ubiquitination of interacting proteins by TRIM32 was assessed along with the potential for modulation by RAP2. RAP2 stimulates the ubiquitin ligase activity of TRIM32 against some substrates but not others. We propose that RAP2 uses TRIM32 to regulate the signalling properties of other RAP2 effectors.

Furthermore, our data also shows that the overexpression of TRIM32 may increase C2C12 mouse myoblast cell differentiation whereas the inhibition of RAP2 expression decreases differentiation in C2C12 cells. Further study could lead to a potential link to Limb-Girdle Muscular Dystrophy that remains to be elucidated.

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# List of Abbreviations

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<b>2T</b>	HEK 293T cells
<b>Abi-2</b>	Abl-interactor 2
<b>AF6 / MLLT4</b>	Afandin / Myeloid/Lymphoid Or Mixed-Lineage Leukemia (Trithorax (Drosophila) Homolog)
<b>AMPA</b>	$\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionate
<b>A<math>\beta</math></b>	amyloid $\beta$
<b>BBS</b>	Bardet-Biedl syndrome
<b>BMD</b>	Becker Muscular Dystrophy
<b>BSA</b>	Bovine serum albumin
<b>cAMP</b>	Cyclic adenosine monophosphate
<b>cDDP</b>	cis-diamminedichloroplatinum / Cisplatin
<b>CNH</b>	C-terminal citron homology domain
<b>CRD</b>	Cysteine-rich domain
<b>DISC1</b>	Disrupted in schizophrenia 1
<b>DMD</b>	Duchenne Muscular Dystrophy
<b>DMEM</b>	Dulbecco's Modified Eagle Medium
<b>DN</b>	D487N mutant
<b>EDMD</b>	Emery-Dreifuss muscular dystrophy
<b>EGFR</b>	Epidermal growth factor receptor
<b>EMBL</b>	European Molecular Biology Laboratory
<b>ER</b>	Endoplasmic reticulum
<b>FBS</b>	Foetal Bovine Serum
<b>GAP</b>	GTPase activating protein
<b>GCK</b>	Germinal centre kinase
<b>GCKH</b>	Germinal centre kinase homology
<b>GDP</b>	Guanosine diphosphate
<b>GEF</b>	Guanine nucleotide exchange factor
<b>GST</b>	Glutathione S-Transferase
<b>GTP</b>	Guanosine Triphosphate
<b>HCC</b>	Human Hepatocellular Carcinoma
<b>HECT</b>	Homologous to the E6-AP carboxyl terminus
<b>HEK-293</b>	Human Embryonic Kidney-293 cells
<b>HGF</b>	Hepatocyte growth factor
<b>HRAS</b>	Harvey rat sarcoma
<b>Hu</b>	Human
<b>IP</b>	Immunoprecipitation
<b>JNK</b>	c-Jun terminal kinase
<b>KRAS</b>	Kirsten rat sarcoma
<b>LB</b>	Lysogeny broth
<b>LGMD</b>	Limb Girdle muscular dystrophy
<b>LTD</b>	Long-term depression
<b>LTP</b>	Long-term potentiation
<b>MAP4K4/NIK</b>	mitogen-activated protein kinase kinase kinase kinase 4
<b>MAPK</b>	Mitogen-activated protein kinase
<b>MD</b>	Muscular Dystrophy

<b>MINK</b>	Misshapen-like kinase
<b>miRNA</b>	microRNA
<b>Mu</b>	Murine
<b>Nedd-4-1</b>	Neuronal precursor cell expressed and developmentally down-regulated protein
<b>NF-kB</b>	nuclear factor kappa-light-chain-enhancer of activated B cells
<b>NHL</b>	Ncl-1, HT2A and Lin-41 proteins
<b>NIK/MAP4K4</b>	Nck-interacting kinase
<b>NRAS</b>	neuroblastoma rat sarcoma
<b>PAK</b>	p21-activated kinase
<b>PBS</b>	Phosphate Buffered Saline
<b>PCR</b>	Polymerase chain reaction
<b>PEI</b>	Polyethylenimine
<b>PI3K</b>	Phosphoinositide 3-kinase
<b>PIAS</b>	Protein inhibitor of STATs
<b>PKA</b>	Protein kinase A
<b>pIgR</b>	polymeric immunoglobulin receptor
<b>PSD</b>	Post-synaptic density
<b>PVDF</b>	Polyvinylidene difluoride
<b>RA</b>	RAS association domain
<b>RalGEF</b>	Ral exchange factor
<b>RBD</b>	RAS Binding Domain
<b>RFG</b>	RAS Family GTPases
<b>RIN</b>	RAS and Rab interactor
<b>RING</b>	Really Interesting New Gene
<b>RT</b>	room temperature
<b>SCC</b>	Squamous cell carcinoma
<b>SH3</b>	Src-homology 3
<b>SNARE</b>	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
<b>sprLA</b>	sortillin-related receptor with A-type repeats
<b>STATs</b>	signal transducers and activators of transcription
<b>STM</b>	Sarcotubular myopathy
<b>T32KO</b>	TRIM32 knock-out mouse model
<b>TAP</b>	Tandem affinity purification
<b>TNF</b>	Tumour necrosis factor
<b>TNIK</b>	TRAF2 and NCK interacting kinase
<b>TRIM</b>	Tripartate motif
<b>Ub</b>	Ubiquitin
<b>UBD</b>	Ubiquitin Binding domain
<b>UV</b>	Ultra-violet
<b>wp</b>	well-plate
<b>wt</b>	wild-type
<b>XIAP</b>	X-linked inhibitor of apoptosis

# CHAPTER 1: RAS Proteins form Interesting Targets for Research

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## **RAS proteins**

RAS proteins are signal transducers that possess an activating mutation in around 30% of human cancers, although they could be indirectly involved in many more via aberrant signalling of their activators (Malumbres and Barbacid 2003; Repasky, Chenette et al. 2004).

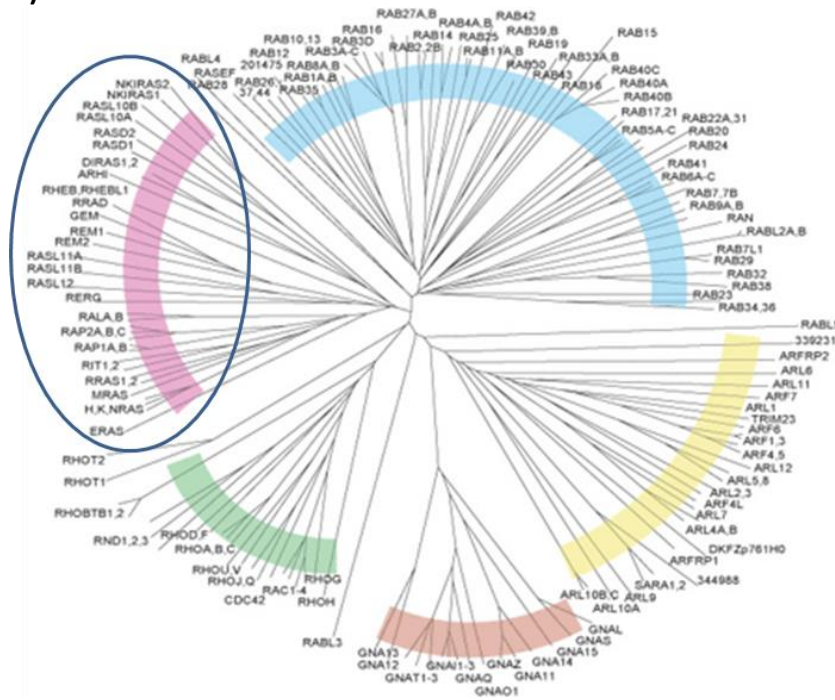
## **Significance of RAS proteins for study**

The *RAS* genes were first identified as transforming oncogenes, responsible for the carcinogenic activities of the Harvey and Kirsten sarcoma viruses, discovered by Jennifer Harvey and Werner Kirsten, respectively (Harvey 1964; Kirsten and Mayer 1967). In 1982, activated and transforming human homologues of *H-* and *KRAS* genes were discovered in human cancer cells and subsequent studies in human neuroblastoma cells identified a third human *RAS* gene, designated as *NRAS* (Shih, Padhy et al. 1981).

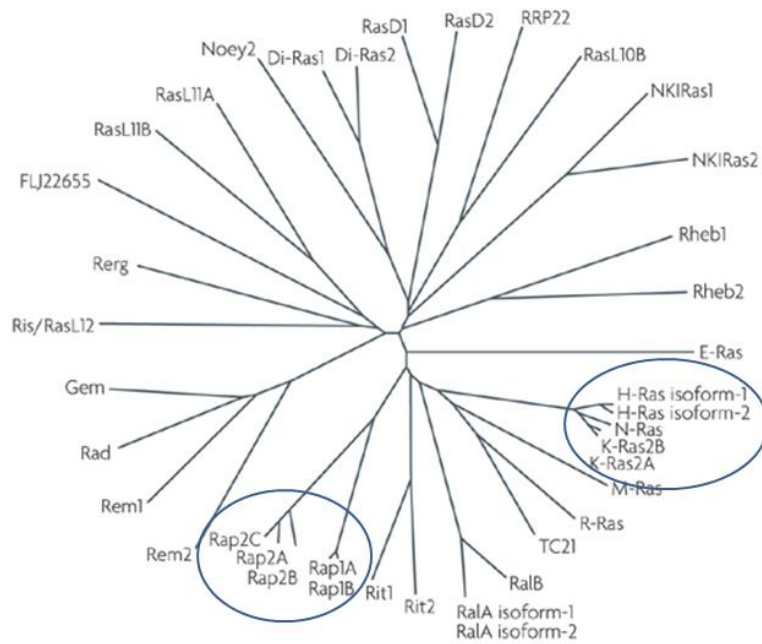
*H-*, *K-* and *NRAS* (referred to as the *classical* RAS family members) are mutated to a varying degree in specific cancers (e.g., *KRAS* in lung, colon and pancreatic tumours, *NRAS* in melanomas and haematopoietic malignancies). These differences cannot be attributed to differential expression, but suggest tissue-specific signalling properties for the three RAS proteins.

The RAS superfamily, also referred to as the RAS family GTPases (RFG), consists of over 150 identified members (Wennerberg, Rossman et al. 2005) (Figure 1). Other members of the RFG share many of the biochemical properties of the classical RAS proteins, including the ability to behave as oncogene products and interact with a selection of known RAS effectors, though they have important functions of their own; e.g. R-RAS in adhesion, Ral in endocytosis, and Rheb in the regulation of cell growth (role in the TOR/S6K signalling pathway) (Long, Lin et al. 2005).

A)



B)



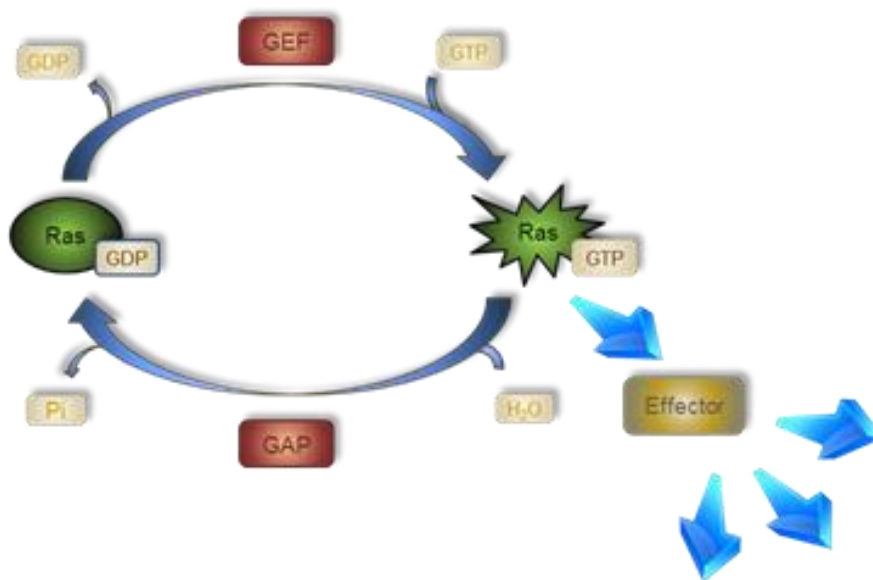
**Figure 1: RAS family GTPase members.** A) The RAS superfamily consists of over 150 members, including the Rho/Rac family and the Rab family. Circled is the RAS family sub-group, also shown below. B) There are multiple members of the RAS family with overlapping as well as distinct functions. Highlighted are the classical RAS family and RAP proteins. Figure from (Karnoub and Weinberg 2008).

The function of many RAS family members has not been elucidated. Some, such as Rerg and the DIRAS subgroup, have even demonstrated tumour suppressor properties (Yu, Xu et al. 1999; Luo, Fang et al. 2003; Key, Andres et al. 2006). How signalling specificity among the closely related RAS family members is achieved and

their role in tumourigenesis is poorly understood and still remains to be investigated.

### RAS superfamily biochemistry and regulation

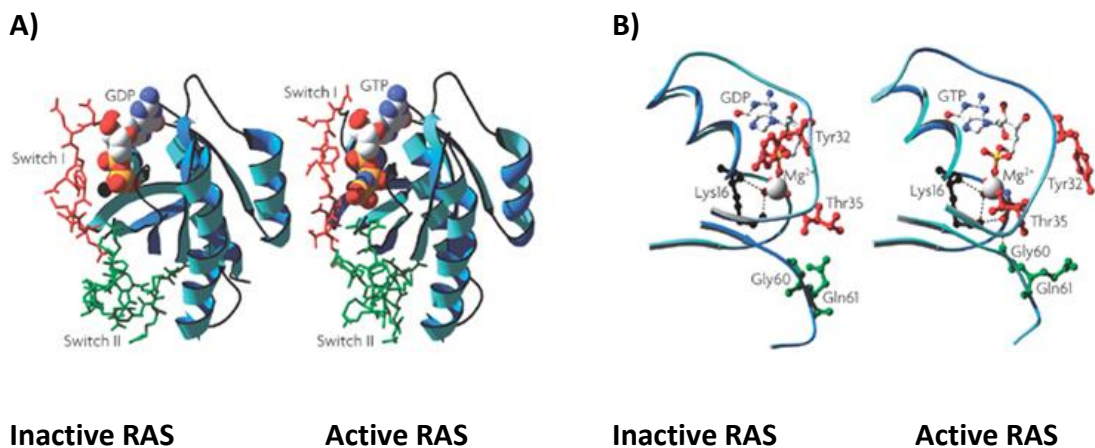
The RFG switch between active GTP and inactive GDP-bound states (see Figure 2), which enables them to function as hubs in signalling cascades (Herrmann 2003). A small number of GTPases can respond to multiple signals and also activate multiple downstream pathways. This results in diverse and specific cellular responses such as proliferation, differentiation, survival and migration (Aoki, Niihori et al. 2008).



**Figure 2: RAS functions as a molecular switch.** Guanine nucleotide exchange factors (GEFs) stimulate the GDP to GTP exchange whereas GTPase activating proteins (GAPs) stimulate the intrinsic GTPase activity that hydrolyses GTP to GDP. Figure adapted from (Malumbres and Barbacid 2003).

The exchange in activation state is enhanced by guanine nucleotide exchange factors (GEFs, which stimulate the release of GDP) and GTPase activating proteins (GAPs) which enhance the intrinsic GTPase activity that hydrolyses GTP to GDP (see Figure 2). Most upstream signals induce GEFs to act on specific GTPases and therefore GEFs serve to link activated receptors to downstream signalling cascades and provide signalling specificity (Raaijmakers and Bos 2009). As the “switch-on” and “switch-off” reactions in the cycle of RFG are intrinsically very low, the regulatory input by GEFs and GAPs determines the lifetime of the two states

(Bourne, Sanders et al. 1990; Bourne, Sanders et al. 1991; Vetter and Wittinghofer 2001). Although it is not well defined, there is some overlap in the way in which at least some of the RFGs are regulated, with both GEFs and GAPs having overlapping specificities and activating several RAS family members (Ehrhardt, Ehrhardt et al. 2002; Quilliam, Rebhun et al. 2002).



**Figure 3: The structure of RAS.** The nucleotide-sensitive switch I and II regions depicted in red and green, respectively. The GDP and GTP nucleotides are shown as balls. A) The RAS three-dimensional fold is shown to consist of six  $\alpha$ -sheets and five  $\alpha$ -helices interconnected by a series of ten loops. Crystallographic structures of inactive RASGDP99 (2.0 Å resolution; Protein Data Bank code 4q21) and active RASGppNHp97 (1.35 Å resolution; PDB code 5p21) are shown, with. B) Nucleotide-dependent structural rearrangements. Figure from (Karnoub and Weinberg 2008).

The exchange of GDP for GTP induces a conformational change in RFG that allows them to interact with their downstream effectors (introduced below) and carry out their multiple biological functions (Lowy and Willumsen 1993; Repasky, Chenette et al. 2004). Only two small regions of the protein change conformation on GTP binding: the core effector domain or switch I region (amino acids 30-40) and the switch II region (amino acids 60-76) (Figure 3). The conformational changes that result from GTP binding allow RFG to interact with its effectors via the effector domain or the RAS/RAP-binding domain (RBD); mutations in this region can disrupt this interaction. Oncogenic RFG harbour single amino-acid missense mutations in their effector domain (equivalent to residues G12V, G13V or Q61K in the classical RAS proteins) which render them insensitive to regulation by GAP. They thus remain in a constitutively active GTP bound state (Herrmann 2003).

The RAS effectors are defined as proteins with strong preferential binding to the GTP-bound form of RFG. The best characterised RAS effectors are the RAF kinases, through which RAS activates the mitogen-activated protein kinase (MAPK) cascade, the p110 catalytic subunit of class I phosphoinositide 3-kinases (PI3Ks), and a family of Ral exchange factors (RalGEFs) (Kodaki, Woscholski et al. 1994; Rodriguez-Viciana, Warne et al. 1994; Wan, Garnett et al. 2004; Wong 2009; Neel, Martin et al. 2011). The RBDs of RAF, PI3Ks, and RalGEFs have considerable structural similarities despite having little sequence homology, leading to distinct domains that are found in other proteins; e.g. RAF-type RBD, RA (RalGDS/AF6, RAS association) domains (Nassar, Horn et al. 1995).

### **Lipid modification and membrane targeting**

Posttranslational modification is a common cellular process by which proteins are modulated by intrinsic or extrinsic factors in order to potentiate or initiate a specific function. This is achieved in a variety of ways, e.g., the addition of other biochemical functional groups (such as acetate, phosphate, lipids and carbohydrates), alteration of the chemical nature of an amino acid (e.g. citrullination), or the introduction of structural changes (e.g. formation of disulfide bridges). In addition, enzymes may remove amino acids from the N-terminal end of the protein or cleave the peptide chain at specific sites. A typical example is the peptide hormone preproinsulin which is cleaved twice after disulfide bonds are formed, eventually resulting in the formation of insulin, which consists of two polypeptide chains connected by disulfide bonds. Methionine, which is present at the -N-terminus of most nascent polypeptides, is also commonly removed during post-translational modification. Other modifications, like phosphorylation, are part of common mechanisms for controlling the behaviour of a protein, for instance activating or inactivating an enzyme.

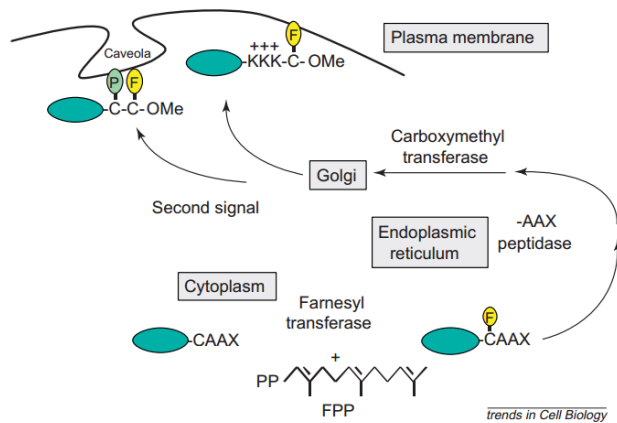
Proteins can be covalently modified by a variety of lipids such as octanoic acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid, a farnesyl or geranylgeranyl group and cholesterol. Most of these modifications take place in the cytoplasm or in the cytoplasmic face of membranes. The processing of RAS family proteins is essential for effector activation and membrane localisation (Takai, Sasaki et al. 2001). RAS proteins are located on the inner layer of the plasma membrane

and are switched on in response to the activation of cell surface receptors. Active RFGs function as adaptor proteins which recruit effectors to the membrane and allow their interaction with other proteins or lipids in order to generate intracellular signals.

RAS family proteins undergo two types of C-terminal post-translational processing (Takai, Sasaki et al. 2001; Rocks, Peyker et al. 2006) primarily associated with the CAAX motif ("C", cysteine; "A", aliphatic; "X", any residue). Proteins synthesised in the cytosol are prenylated at the CAAX cysteine (farnesylated or geranylgeranylated, depending on "X" (Takai, Sasaki et al. 2001)), whereupon the prenylcysteine targets the proteins to the endoplasmic reticulum (ER). Here, proteolytic removal of "AAX", and carboxyl-methylation of the prenylcysteine takes place. Following CAAX-processing, some RAS family proteins undergo a second type of processing either in the ER or the Golgi, where cysteine(s) upstream of the prenylcysteine is/are palmitoylated. For instance, all RAS proteins are farnesylated during the CAAX-processing. Subsequently, NRAS and HRAS are palmitoylated at one and two sites, respectively (Takai, Sasaki et al. 2001).

RAF proteins interact with RAS proteins at two sites: the RBD and the cysteine-rich domain (CRD) (Hu, Kariya et al. 1995; Morrison and Cutler 1997; Takai, Sasaki et al. 2001). The RBD-RAS interaction is involved in initial complex formation and the CRD-RAS interactions are involved in activation. Unprocessed RAS proteins interact with but do not activate RAF1 and BRAF because the processing is a prerequisite for the CRD-RAS interaction (Hu, Kariya et al. 1995; Morrison and Cutler 1997; Takai, Sasaki et al. 2001). Therefore, a fully processed HRAS cannot activate RAF1 properly if the CRD-binding interface is mutated (Tamada, Hu et al. 1997). Mutations in palmitoylation sites impair the HRAS-induced transformation of NIH-3T3 cells (Hancock, Paterson et al. 1990), demonstrating the significance of these posttranslational modifications.





**Figure 4: RAS proteins are post-translationally modified.** RAS proteins differ in their trafficking to and association with the plasma membrane. RAS proteins are synthesised initially as cytosolic proteins in which the C-terminal CAAX motif is the target of post-translational modification that involves the addition of a farnesyl isoprenoid lipid, catalysed by the enzyme farnesyl transferase. Endoplasmic reticulum-associated enzymes then catalyse the proteolytic cleavage of the AAX residues and carboxyl methylation of the now C-terminal farnesylated cysteine residue by isoprenylcysteine carboxymethyltransferase-1 enzyme. Different targeting mechanisms might result in their localisation to functionally distinct microdomains of the plasma membrane. Figure from (Shields, Pruitt et al. 2000)

## Prenylation

Prenylation involves the covalent addition of either farnesyl (15-carbon) or more commonly geranylgeranyl (20-carbon) isoprenoids via thioether linkages to cysteine residues at or near the C-terminus of intracellular proteins. The attached lipid is required for proper function of the modified protein, either as a mediator of membrane association or a determinant for specific protein-protein interactions (Novelli and D'Apice 2012).

There are three enzymes that carry out prenylation in the cell, farnesyl transferase (Maltese 1990; Clarke 1992), geranylgeranyl transferase I and Rab geranylgeranyltransferase/protein geranylgeranyltransferase type II (Casey and Seabra 1996). Farnesyl transferase and geranylgeranyl transferase I are designated CAAX prenyltransferases since they recognise the CAAX box at the C-terminus of the target protein (Clarke 1992; Zhang and Casey 1996). Substrates for farnesyl transferase include RAS GTPases, lamin B and several proteins involved in visual signal transduction (Clarke 1992; Omer and Gibbs 1994; Caldwell, Naider et al. 1995). Known targets of geranylgeranyl transferase I include most subunits of heterotrimeric G proteins and RAS-related GTPases such as members of the RAS

and Rac/Rho families (Clarke 1992; Glomset and Farnsworth 1994). Geranylgeranyltransferase type II attaches geranylgeranyl groups to two C-terminal cysteines in the Rab family that terminates in CC or CXC motifs (Seabra, Goldstein et al. 1992; Glomset and Farnsworth 1994).

### **Palmitoylation**

Palmitoylation is the covalent attachment of fatty acids, such as palmitic acid, to cysteine and less frequently to serine and threonine residues of proteins. The exact function of palmitoylation depends on the particular protein being considered. For example, it can enhance the hydrophobicity of proteins and contribute to their membrane association, can also play a role in the subcellular trafficking of proteins between membrane compartments (Rocks, Peyker et al. 2005), or modulate protein-protein interactions. Some of these proteins are modified sequentially with different lipids but others are exclusively S-palmitoylated. As the bond between palmitic acid and the protein is often a thioester bond, palmitoylation is usually reversible and the reverse reaction is catalysed by palmitoyl protein thioesterases.

### **Other common posttranslational modifications**

#### ***Myristoylation***

Myristoylation is an irreversible, co-translational protein modification found in animals, plants, fungi, protozoans and viruses. Myristoylation is crucial for the cellular proliferation process and is required for the growth and development in a number of organisms including many human pathogens and viruses.

In this protein modification, a myristoyl group (derived from myristic acid) is covalently attached via an amide bond to the alpha-amino group of an N-terminal amino acid of a nascent polypeptide. It is more common on glycine residues exposed during co-translational N-terminal methionine removal but also occurs on other amino acids (Farazi, Waksman et al. 2001). This protein modification is catalysed by the enzyme N-Myristoyl Transferase (NMT) that uses myristoyl-CoA and the peptide N-terminus as co-substrates after recognition of a GXXX(S/T/C) N-terminal consensus sequence (Maurer-Stroh, Eisenhaber et al. 2002; Maurer-Stroh, Eisenhaber et al. 2002).

It has been estimated that approximately 0.5% of eukaryotic proteins are myristoylated (Maurer-Stroh and Eisenhaber 2004). In many cases, protein N-myristoylation is required (though not sufficient) for stable and permanent membrane anchoring; (Peitzsch and McLaughlin 1993; Shahinian and Silvius 1995; Navarro-Lerida, Alvarez-Barrientos et al. 2002) it often occurs together with S-acylation of proximal cysteine residues or a polybasic amino acid domain next to the N-terminus. N-myristoylation can also occur post-translationally when an internal glycine becomes exposed by caspase-mediated proteolytic cleavage (Zha, Weiler et al. 2000).

### *Glycosylation*

Glycosylation is a form of posttranslational modification which consists of the enzymatic addition of a glycosyl group to either arginine, asparagine, cysteine, hydroxylysine, serine, threonine, tyrosine, or tryptophan resulting in a glycoprotein (Freeze and Sharma 2010; Reis, Osorio et al. 2010). Glycosylation mostly occurs in the ER or Golgi apparatus and can affect protein folding and stability, influence protein trafficking and interfere with protein function (Reis, Osorio et al. 2010; Roth, Zuber et al. 2010; Pinho, Seruca et al. 2011).

### *Phosphorylation*

Phosphorylation is the addition of a phosphate group, usually to serine, threonine, and tyrosine (O-linked), or histidine (N-linked). Posttranslational modification through phosphorylation acts as a major regulatory pathway in normal protein life cycles and can be further affected in pathological states by the upregulation of kinase pathways. These pathways regulate protein functions through addition of a phosphate group by kinases, which may lead to alterations in the hydrophobicity, charge and potentially structural organization of proteins, either promoting or inhibiting normal functions (Huttlin, Jedrychowski et al. 2010; Davis 2011; Nishi, Hashimoto et al. 2011).

### *S-nitrosylation*

Posttranslational modification can also occur through S-nitrosylation, in which nitric oxide can bind to a reactive cysteine thiol, producing an S-nitrosothiol (Stamler, Simon et al. 1992). While proteins often contain multiple cysteine residues, the

majority of the observed biological effects imparted by nitric oxide occur on single or only a few cysteine residues within a protein. In addition to direct modification of proteins by S-nitrosylation, this posttranslational modification can indirectly regulate other protein modifications including acetylation, phosphorylation and ubiquitination (Park, Yu et al. 2004; Yasukawa, Tokunaga et al. 2005; Whalen, Foster et al. 2007; Hess and Stamler 2012).

### *Others*

Other existing posttranslational modifications are either introduced elsewhere, or not discussed as they are beyond the scope of this project. These include acylation, alkylation, amide bond formation, butyrylation, Vitamin K-dependent gamma-carboxylation, malonylation, hydroxylation, iodination, nucleotide addition (such as ADP-ribosylation), oxidation, propionylation, pyroglutamate formation, S-glutathionylation, succinylation, sulfation, and selenoylation.

Posttranslational modifications involving the addition of cofactors which enhance enzymatic activity include lipoylation, attachment of a lipoate (C8) functional group, flavin moiety (FMN or FAD) covalent attachment, heme C attachment via thioether bonds with cysteins, phosphopantetheinylation, the addition of a 4'-phosphopantetheinyl moiety from coenzyme A, polyketide, non-ribosomal peptide and leucine biosynthesis, and retinylidene Schiff base formation.

Posttranslational modifications involving unique modifications of translation factors consist of diphthamide formation (on a histidine found in eEF2), ethanolamine phosphoglycerol attachment (on glutamate found in eEF1 $\alpha$ ), and hypusine formation (on conserved lysine of eIF5A (eukaryotic) and aIF5A (archaeal)).

Posttranslational modifications involving non-enzymatic additions *in vivo* and *in vitro* include glycation, the addition of a sugar molecule to a protein without the controlling action of an enzyme, biotinylation, acylation of conserved lysine residues with a biotin appendage, and pegylation.

Posttranslational modifications involving changing the chemical nature of amino acids are citrullination, or deimination, the conversion of arginine to citrulline, deamidation, the conversion of glutamine to glutamic acid or asparagine to aspartic

acid, eliminylation, the conversion to an alkene by beta-elimination of phosphothreonine and phosphoserine, or dehydration of threonine and serine, as well as by decarboxylation of cysteine, and carbamylation, the conversion of lysine to homocitrulline.

Finally, the posttranslational modifications involving structural changes are the formation of disulfide bridges, the covalent linkage of two cysteine amino acids, proteolytic cleavage, cleavage of a protein at a peptide bond, and racemisation of proline by prolyl isomerase (Wang, Pattison et al. 2013).

## **RAP proteins**

The RAP proteins are members of the RFG, and contain similar effector binding domains as RAS. Effector proteins for RAP also typically contain an RBD or an RA domain (Taira, Umikawa et al. 2004; Kukimoto-Niino, Takagi et al. 2006). Mammals express two RAP1 (RAP1A and RAP1B) and three RAP2 (RAP2A, RAP2B, RAP2C) proteins. RAP1 and RAP2 differ in their effector domain by one amino acid and share 60% sequence homology.

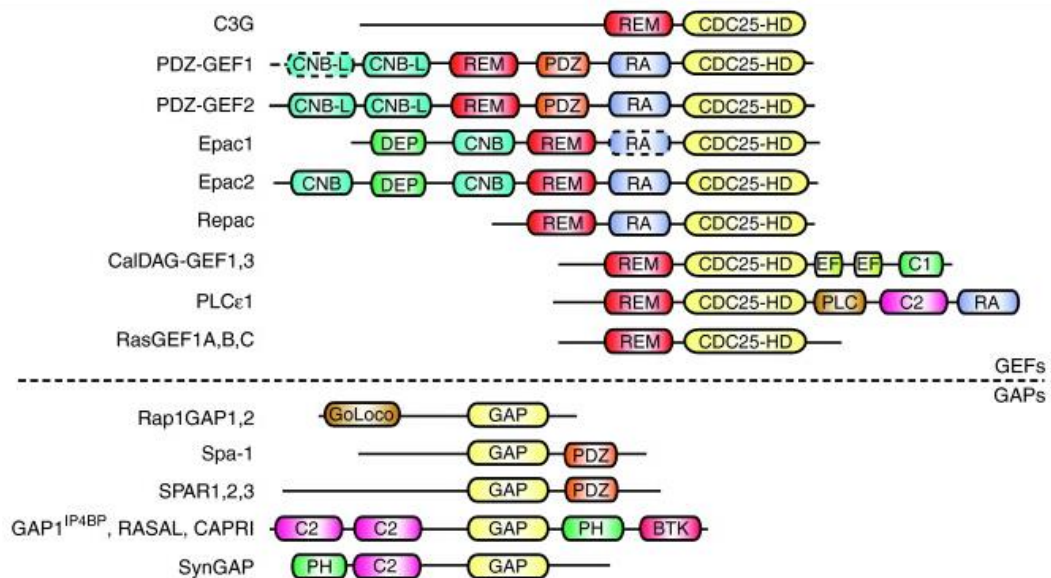
## **Localisation**

As with RAS proteins, RAP membrane anchoring is mediated by lipid modifications within the C-terminal (CAAX) tail which results in their differential modification due to tail sequence variations. RAP proteins were previously predicted to localise to the Golgi apparatus (Pizon, Desjardins et al. 1994), although they have since been shown to localise at recycling endosomes (Uechi, Bayarjargal et al. 2009).

Several signalling pathways modulate RAP by altering its cellular distribution by post-translational modifications; RAP1 is phosphorylated in its C-terminus by the cAMP-effector protein Protein Kinase-A (PKA), which has been proposed to alter substrate binding as well as its localisation (Rundell, Repellin et al. 2004; Edreira, Li et al. 2009). Two enzymes, NEDD4-1 and Cullin-5, have been shown to ubiquitinate RAP2 and thereby control its membrane targeting and effector binding respectively (Lee, Iioka et al. 2007; Kawabe, Neeb et al. 2010).

## Activation

The diverse functions of RAP are partially cell type-specific. The existence of distinct cellular pools of RAP as well as an exclusive sub-set of activator and effector proteins might further explain the multiple biological responses triggered by RAP, even in a single cell.



**Figure 5: Domain architecture of mammalian RAPGEFs and RAPGAPs.** GEFs and GAPs for which a direct effect on nucleotide binding of RAP has been demonstrated *in vitro* are shown. Binding of GEFs modulates the nucleotide-binding site, resulting in the release and subsequent replacement of the bound nucleotide by the cellularly abundant GTP. Broken lines indicate the putative extra CNB-L domain in PDZ-GEF1 and a putative RA domain in Epac1.

Abbreviations: BTK, Bruton's tyrosine kinase motif; CDC25-HD, CDC25 homology domain; CNB, cyclic nucleotide binding; CNB-L, cyclic nucleotide binding-like; DEP, dishevelled; Egl-10, pleckstrin; EF, EF-hands; GAP, GTPase-activating protein; PH, pleckstrin homology; PLC, phospholipase C catalytic domain; RA, RAS/RAP association; REM, RAS exchange motif; PDZ, PSD95, DlgA, Zo-1. Figure from (Gloerich and Bos 2011).

GEFs and GAPs which have a direct effect on RAP are referred to as RAPGEFs and RAPGAPs respectively. These individual RAPGEFs and RAPGAPs are regulated by distinct upstream signals that affect their activity, cellular distribution or stability (for review see (Spilker and Kreutz 2010; Vigil, Cherfils et al. 2010; Gloerich and Bos 2011)). Numerous mammalian RAPGEFs have been identified (Figure 5) that act primarily on both RAP1 and RAP2. Some show preference for distinct RAP family members; for example, RASGEF1 selectively activates RAP2 (Yaman, Gasper et al. 2009), whereas C3G displays higher exchange activity towards RAP1 (van den

Berghe, Cool et al. 1997). Moreover, some of the RAPGEFs also act on other members of the RAS family, which provides overlap between these signalling pathways. Additionally, several mammalian RAPGAPs that either act selectively on RAP or display broader specificity have been described (Gloerich and Bos 2011).

Although closely related to RAS, the RAP proteins are implicated in a variety of distinct biological processes.

## **RAP1**

RAP1 has a role in the regulation of integrin-mediated adhesion, cell-cell junction formation, exocytosis, cell proliferation, and establishment of cell polarity (Bos, de Bruyn et al. 2003; Rangarajan, Enserink et al. 2003; Fukuhara, Sakurai et al. 2005; Frische and Zwartkruis 2010).

RAP1 also regulates cadherins, adhesion molecules that are components of adherens junctions. These form calcium-dependent, homotypic interactions which stabilise cell-cell contacts. The cytoplasmic tails of cadherins bind to several proteins, including  $\beta$ -catenin and p120ctn, to form a connection with the actin cytoskeleton (Aberle, Butz et al. 1994; Davis, Ireton et al. 2003).

RAP1 activity has also been shown to promote metastasis in human breast and prostate cancer cells (Itoh, Nelson et al. 2007; Bailey, Kelly et al. 2009) and is required for RET/PTC1-induced BRAF activation, mitogenesis and cytoskeletal reorganisation in thyroid cells (De Falco, Castellone et al. 2007).

## **RAP2**

### **Structure**

The three RAP2 isoforms, RAP2A, RAP2B, and RAP2C are differentially post-translationally modified at the C-terminus due to variations in their C-terminal membrane targeting (CAAX) region (Figure 4, Figure 6). After farnesylation during the CAAX-processing, RAP2A and RAP2B are farnesylated and geranylgeranylated, respectively (Farrell, Yamamoto et al. 1993), and RAP2C is assumed to be farnesylated, as its "X" is identical to that in RAP2A (Paganini, Guidetti et al. 2006).

The two cysteines (C176 and C177) upstream of the CAAX cysteine (C180) are the sites of palmitoylation in RAP2 proteins. Once fully processed, these are targeted to the recycling endosomes in a palmitoylation-dependent manner. As palmitoylation is necessary for membrane-association of RAP2A and RAP2C but not RAP2B, this has possible implications for differential localisation, e.g. different membrane compartments (Uechi, Bayarjargal et al. 2009).

hHa-Ras	KLNPPDESGPGCMSCKCYLS	189	C, palmitoylated
hRap2A	MNYAAQPKDDPCCSACNIO	183	C, farnesylated
mRap2A	MNYAAQPKDDPCCSACNIO	183	C, geranylgeranylated
mRap2B	MNYAAQPNGDEGCCSACVIL	183	
mRap2C	MNYSSLPEKQDQCCTT CVVO	183	
	CAAX		

**Figure 6: High degree of homology in the C-termini between RAP2 isoforms.** Variations in the CAAX domain lead to specific post-translational modifications (Uechi, Bayarjargal et al. 2009). RAP2A is farnesylated, RAP2B geranylgeranylated, and RAP2C is predicted to be farnesylated (Takai, Sasaki et al. 2001; Paganini, Guidetti et al. 2006). Figure from (Uechi, Bayarjargal et al. 2009)

### Biological functions of RAP2: Neural function

In hippocampal pyramidal neurons RAP2 opposes RAS-ERK signalling by inhibiting dendritic spine development/maintenance; shortening of dendrites and axons, loss of dendritic spines and spiny synapses in neurons, as well as an increase in filopodia-like protrusion and shaft synapses. These RAP2 morphological effects are absent in interneurons, or when RAP1 is overexpressed (Fu, Lee et al. 2007).

RAP2 impairs learning and promotes synaptic depression rather than long-term potentiation (LTP) (Ryu, Futai et al. 2008), whereas RAP1 has been shown to be critical for long-term depression (LTD) of synaptic transmission in hippocampal synapses (Zhu, Qin et al. 2002; Huang, You et al. 2004). The findings also implicate RAP2 signalling in fear extinction mechanisms, which are thought to be aberrant in anxiety disorders and posttraumatic stress disorder (Fischer, Radulovic et al. 2007; Myers and Davis 2007; Ryu, Futai et al. 2008).

Nedd-4-1 is a “neuronal precursor cell expressed and developmentally down-regulated protein” and among the most abundant E3 ubiquitin ligases in



mammalian neurons, playing a critical role in dendrite formation. Nedd4-1, TNIK and RAP2A form a complex that controls Nedd4-1-mediated ubiquitination of RAP2A. Mono- or di-ubiquitination of RAP2 has been shown to inhibit its function. By perturbing the RAP2 interaction with the RAP2 effector TNIK, activity of the TNIK kinases is inhibited, leading to promoted dendrite growth and branching (Kawabe, Neeb et al. 2010).

### **Biological functions of RAP2: Receptor trafficking**

RAP2 is a key player in the regulation of receptor trafficking that might affect cell responsiveness to extracellular ligands and signalling duration (Choi, Kim et al. 2008). RAP2 is required to remove  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors during synaptic depotentiation (Zhu, Qin et al. 2002; Zhu, Pak et al. 2005). RAP2 also positively regulates the trafficking of Activin/Nodal receptors to modulate signalling activity by sorting internalised Activin/Nodal receptors into a recycling pathway in the absence of ligand activation and thus maintaining their proper levels at the cell surface (Fu, Lee et al. 2007; Choi, Kim et al. 2008).

RAP2A is required for intestinal epithelial brush border formation, whereas overexpression of RAP2C inhibits brush border formation. RAP2A requires its effector TNIK, but not MINK or NIK, to mediate brush border formation. RAP2A directly links polarisation of intestinal epithelial cells to the formation of an apical brush border (Gloerich, ten Klooster et al. 2012).

### **RAP effectors**

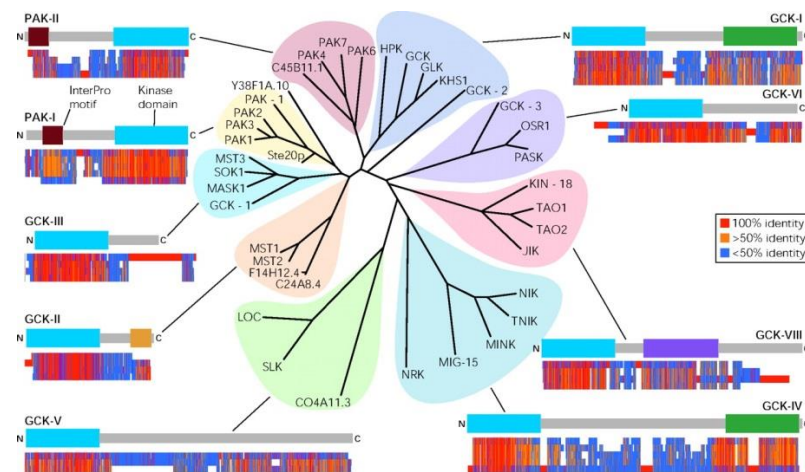
#### **Ste20 kinases – TNIK, MINK and MAP4K4/NIK**

TRAF2- and *Nck*-Interacting Kinase (TNIK) (Taira, Umikawa et al. 2004) and Mitogen-activated protein kinases (MAPK) kinase kinase kinase 4 (MAP4K4)/*Nck*-Interacting Kinase (NIK) (Machida, Umikawa et al. 2004) and the *Misshapen*/NIK-related Kinase (MINK) (Nonaka, Takei et al. 2008) are a subgroup of MAPKs that have been identified as specific downstream effectors of RAP2, but not RAS or RAP1 (Fu, Shen et al. 1999; Dan, Watanabe et al. 2000; Dan, Watanabe et al. 2001; Machida, Umikawa et al. 2004; Taira, Umikawa et al. 2004; Bos 2005; Nonaka, Takei et al. 2008).

## Background

MAPK are a family of conserved serine/threonine protein kinases that transmit extracellular signals into the cytoplasm. They are commonly activated through phosphorylation cascades, such as by the Ste20 kinases (Sells and Chernoff 1997; Bagrodia and Cerione 1999; Kyriakis 1999), and subsequently phosphorylate a number of effectors that contribute to the regulation of diverse cellular events, including differentiation (Eriksson and Leppa 2002; Aouadi, Bost et al. 2006), proliferation (Barr and Bogoyevitch 2001; Roux and Blenis 2004), survival (Lee, Sartor et al. 2004), migration (Su, Lu et al. 2009), and invasion (Ip and Davis 1998; Widmann, Gibson et al. 1999; Wendt and Schiemann 2009).

## Phylogenetic grouping



**Figure 7: Phylogenetic tree, domain structure, and multiple sequence alignments of the GCK and PAK subfamilies of STE20-type kinases from humans and *C. elegans*.** p21-activated kinases (PAKs) fall into two structurally similar subfamilies, PAK-I and PAK-II, whereas germinal center kinases (GCKs) fall into eight subfamilies, one of which (GCK-VII) is not represented in *C. elegans*. The human kinases are designated in the phylogram with their common names. STE20-related kinases grouped by ClustalW protein alignment, and distance matrices and trees were subsequently calculated using Phylip.

The *C. elegans* kinases are shown as cosmid open reading frames for predicted genes or official gene names where designated. Y38F1A.10 does not fall into any of the subfamilies, but it is most closely related to the PAKs. For reference, yeast kinase Ste20p, the founding member of this family, is also shown. Representative domain structures are shown for each subfamily. Protein kinase domains (IPR000719) are indicated by blue boxes. Citron-like domains (IPR001180, green box) may be involved in macromolecular interactions, particularly with small GTPases. The SARAH domain (IPR0011524, orange box) facilitates dimerisation and is unique to the GCK-II family. PAK domains (IPR000095, brown box) allow the PAK family kinases to bind to members of the p21 and Rho families. Finally, the DUF334 domain (IPR005602, purple box) is of unknown function. Motifs that are functionally conserved generally show up in multiple sequence alignments. In the alignments for each subfamily shown immediately below the domain structures, red indicates 100%, orange >50%, and blue <50% identity. Figure from (Strange, Denton et al. 2006).

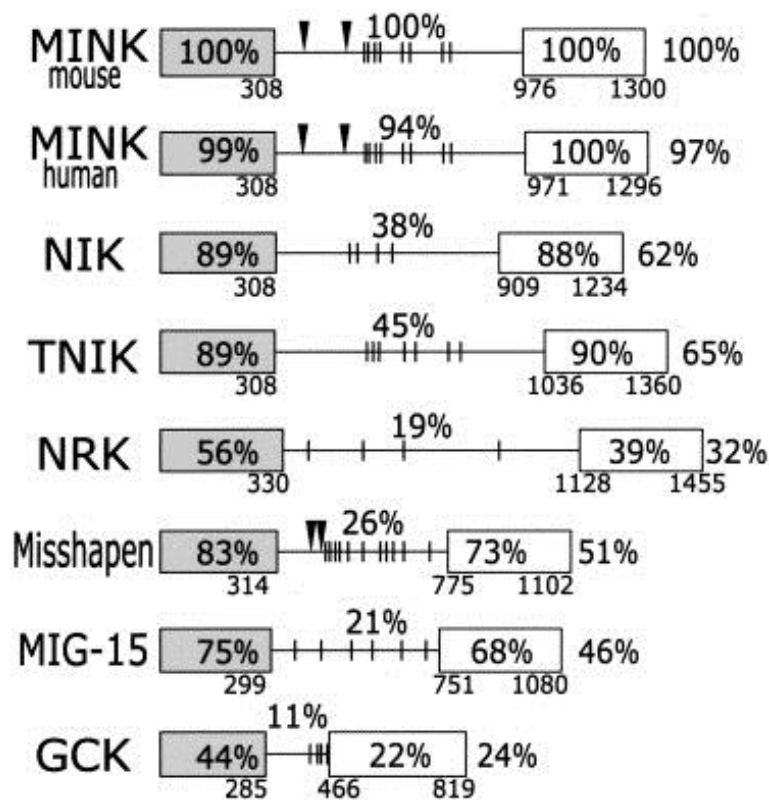
The Ste20 group of protein kinases are a large group of protein kinases comprising approximately 30 members ([www.UniProt.org](http://www.UniProt.org)) (see Figure 7). They are further divided into the p21-Activated Kinase (PAK) and Germinal Centre Kinase (GCK) families, which have various intracellular regulatory effects including the regulation of apoptosis, rearrangement of the cytoskeleton leading to cell-shape change and cell motility (Sells and Chernoff 1997; Bagrodia and Cerione 1999; Kyriakis 1999).

TNIK, MINK and NIK belong to the GCK IV subgroup (also referred to as the NIK-related kinases or the NIK subfamily kinases) of the Ste20 family of protein kinases (Figure 7) and show overlapping as well as distinct functions.

### *Structure*

Their structure (see Figure 8) is characterised by an N-terminal kinase domain and a non-catalytic C-terminal citron homology (CNH)/GCK homology (GCKH) domain. NIK-related kinases share around 90% amino acid identity in the CNH/GCKH region (Su, Han et al. 1997; Su, Treisman et al. 1998; Fu, Shen et al. 1999; Kanai-Azuma, Kanai et al. 1999), which is also the RAP2 binding site for TNIK, MINK and NIK (Taira, Umikawa et al. 2004; Nonaka, Takei et al. 2008). In the intermediate region the sequence homology is less striking and MINK displays less than 45% amino acid identity with other NIK-related kinases (Fu, Shen et al. 1999; Dan, Watanabe et al. 2001), leading to activation of diverse signalling pathways.

TNIK, MINK and NIK contain eight proline-rich motifs (PXXP) that are the Src homology 3 (SH3) domain binding regions, where they interact with Nck (Su, Han et al. 1997; Fu, Shen et al. 1999). Nck is implicated in the regulation of the actin cytoskeleton by receptor or non-receptor tyrosine kinases (McCarty 1998; Buday, Wunderlich et al. 2002; Rivera, Briceno et al. 2004). Although regions of high homology lead to overlapping functions, sequence deviation of MINK in its intermediate region suggests that it may also be involved in different signalling cascades (Dan, Watanabe et al. 2000).



**Figure 8: Homologies of MINK to other NIK subfamily kinases and GCK.** The kinase domain is represented by gray boxes, the intermediate region by horizontal lines, and the GCKH region by white boxes. Locations of proline-rich motifs (PXXP) are indicated by vertical bars and glutamine-rich motifs are shown by vertical arrowheads. Amino acid identities relative to muMINK within each domain and in the whole molecule (on the right) are shown. The huMINK sequence was predicted from a genomic sequence. Figure from (Dan, Watanabe et al. 2000).

### Expression

MINK is ubiquitously expressed; however it is most abundant in the brain (Dan, Watanabe et al. 2000) and moderately expressed in kidney and spleen, whereas NIK and TNIK are expressed at low levels. NIK and TNIK are abundant in adult skeletal muscle and the heart while MINK is not (Dan, Watanabe et al. 2000).

### Function

TNIK, MINK and NIK activate c-Jun N-terminal kinase (JNK), a stress-activated MAPK (Dan, Watanabe et al. 2000), via different mechanisms. TNIK and MINK have also been shown to differentially impinge upon RAP2 signalling in hippocampal dendrites/neuronal cells as MINK, but not TNIK, overexpression is sufficient to disrupt RAP2-mediated removal of AMPA-Rs (Zhu, Pak et al. 2005; Fu, Lee et al. 2007; Hussain, Hsin et al. 2010).

### TNIK

TNIK was originally identified as “TRAF2- and Nck-interacting kinase” (Fu, Shen et al. 1999). TRAF2 belongs to a family of adaptor proteins that shares a common structural domain (the TRAF domain) and is implicated in the regulation of JNK and

the transcription factor NF- $\kappa$ B by the tumour necrosis factor (TNF) receptor or related receptors (Bradley and Pober 2001).

TNIK functions as an essential activator of Wnt target genes (Mahmoudi, Li et al. 2009; Gui, Yang et al. 2011) by interacting directly with  $\beta$ -catenin and TCF4 (a key transcription factor of Wnt signalling) in a kinase dependent manner (Shitashige, Satow et al. 2008; Mahmoudi, Li et al. 2009). TNIK phosphorylates TCF4 at S154 and activates the transcriptional activity of the  $\beta$ -catenin-TCF4 complex which is indispensable for colon cancer cell growth. Colorectal cancer cells have been shown to be highly dependent on the expression levels and kinase activity of TNIK for proliferation (Mahmoudi, Li et al. 2009; Shitashige, Satow et al. 2010).

In the brain, TNIK functions as a susceptibility gene for schizophrenia and related diseases (Glatt, Everall et al. 2005; Matigian, Windus et al. 2007; Potkin, Turner et al. 2009; Shi, Levinson et al. 2009). TNIK mRNA is upregulated in the dorsolateral prefrontal cortex of schizophrenia patients (Glatt, Everall et al. 2005) and in lymphoblastoid cell lines from bipolar disorder patients (Matigian, Windus et al. 2007). TNIK is a regulatory kinase at the postsynaptic density (PSD) (Peng, Kim et al. 2004; Collins, Yu et al. 2005) and is inhibited by a physical interaction with Disrupted in schizophrenia 1 (DISC1) (Wang, Charych et al. 2011), a strong candidate gene for schizophrenia and other psychiatric disorders (Chubb, Bradshaw et al. 2008; Brandon, Millar et al. 2009).

TNIK also regulates the actin cytoskeleton by inducing actin fibre disassembly through its kinase domain (Fu, Shen et al. 1999; Taira, Umikawa et al. 2004). Therefore, with the expression of TNIK, adherent cells round up and lose attachment to culture dishes, but remain viable and do not undergo apoptosis (Fu, Shen et al. 1999). GTP-bound RAP2 interacts with TNIK in an effector domain dependent manner (Taira, Umikawa et al. 2004) and enhances the inhibitory function of TNIK against cell spreading by promoting auto-phosphorylation and translocation of TNIK to the cytoskeleton. This suggests that TNIK is a specific effector of RAP2 and regulates the actin cytoskeleton (Fu, Shen et al. 1999; Taira, Umikawa et al. 2004). In HEK-293T cells RAP2 proteins localise and recruit TNIK to the recycling endosomes (Uechi, Bayarjargal et al. 2009).

### *MINK*

MINK is involved in the p38 pathway, interacts with Nck and activates the JNK pathway independently of its kinase activity. MINK is also involved in the regulation of the actin cytoskeleton, cell-matrix adhesion and cell-cell adhesion, leading to changes in cell morphology, migration and invasion (Hu, Leo et al. 2004).

MINK is a distal target of RAS signalling in the induction of a growth-arrested, senescent-like phenotype that may act to oppose oncogenic transformation in human ovarian surface epithelial (HOSE) cells (Nicke, Bastien et al. 2005).

The expression of MINK has been found to be up-regulated during the postnatal development of the mouse brain (Dan, Watanabe et al. 2000). As with RAP2, expression of MINK (and TNIK) in neurons is required for normal dendritic arborisation and surface expression of AMPA receptors. This overlapping function suggests a potential role of the RAP2 interaction with MINK and TNIK in neuronal development and cellular differentiation (Zhu, Pak et al. 2005; Fu, Lee et al. 2007).

### *MAP4K4/NIK*

MAP4K4 consists of multiple isoforms resulting from alternative splicing, (Wright, Wang et al. 2003) and is known in humans and mice as hematopoietic progenitor kinase (HPK)/GCK-like kinase (HGK) (Yao, Zhou et al. 1999) and NIK (Su, Han et al. 1997) respectively.

NIK activates the JNK/SAPK pathway (Machida, Umikawa et al. 2004; Collins, Hong et al. 2006) and RAP2 interacts with the CNH domain of NIK (Machida, Umikawa et al. 2004), enhancing the JNK activation by NIK (Machida, Umikawa et al. 2004). Conversely, expression of TNIK alone substantially activates JNK whilst co-expression of RAP2 does not enhance this activation (Fu, Shen et al. 1999).

NIK is overexpressed in many types of human cancer (Collins, Hong et al. 2006; Liang, Wang et al. 2008). NIK over-expression is prognostically significant in patients with stage II pancreatic ductal adenocarcinoma (Liang, Wang et al. 2008). NIK is also frequently overexpressed in human hepatocellular carcinoma (HCC) tissues as well as several HCC cells lines including HepG2 and Hep3B, again associated with worse prognosis of HCC patients (Liu, Cai et al. 2011). Experimentally, silencing of NIK

causes reduced tumour cell motility in SKOV-3 ovarian carcinoma-derived cell lines (Collins, Hong et al. 2006) and in HepG2 and Hep3B liver cells (Liu, Cai et al. 2011).

## RIN

The RAS and Rab interactor (RIN) family consists of three members, RIN1, RIN2 and RIN3. These function as GEFs for Rab5 (Bliss, Venkatesh et al. 2006) and are associated with endosomal trafficking (Saito, Murai et al. 2002; Grosshans, Ortiz et al. 2006).

Rab proteins constitute a subfamily of over 40 small GTPases that localise to distinct intracellular compartments and regulate the transport between specific organelles (Olikkonen and Stenmark 1997; Zerial and McBride 2001). Rab5 is primarily localised to early endosomes (Gorvel, Chavier et al. 1991) and plays a role in membrane budding and trafficking in the early endocytic pathways (Kajiho, Saito et al. 2003; Saito, Kajiho et al. 2005; Kajiho, Sakurai et al. 2011; Woller, Luiskandl et al. 2011).

RIN1, RIN2 and RIN3 contain an N-terminal SH2 domain, intermediate proline-rich, RH and Vps9 domains and a C-terminal RA domain (Han, Wong et al. 1997) (Colicelli, Nicolette et al. 1991; Saito, Murai et al. 2002). RIN2 and RIN3 preferentially interact with the GTP-bound form of Rab5, while RIN1 preferentially interacts with GDP-bound Rab5 (Tall, Barbieri et al. 2001; Saito, Murai et al. 2002).

The RIN1 RA domain was initially identified as a region interacting with HRAS (Hofer, Fields et al. 1994; Ponting and Benjamin 1996), suggesting that RINs are capable of binding not only to Rab5 but also to the RAS-family GTPases. The GEF activity of RIN1 for Rab5 is enhanced by its interaction with HRAS (Han and Colicelli 1995; Tall, Barbieri et al. 2001). However, HRAS does not interact with RIN3 or RIN2 to stimulate their GEF activities in conditions under which it certainly binds to RIN1 (Rodriguez-Viciano, Sabatier et al. 2004; Wohlgemuth, Kiel et al. 2005).

RIN1, RIN2 and RIN3 mRNA distribution is different; RIN1 is highly expressed in neuronal tissues in the mature forebrain neurons and moderately expressed in epithelial and hematopoietic cells (Hu, Bliss et al. 2005; Deininger, Eder et al. 2008), RIN2 is abundant in the heart, kidney and lung (Saito, Murai et al. 2002), and RIN3 in peripheral blood cells.

The intracellular localisations of the three RIN members are not identical. In HeLa cells RIN3 and RIN2 localise with Rab5 to endocytic vesicles, whereas RIN1 exhibits a cytoplasmic distribution. RIN1 partially co-localises with Rab5-positive vesicles upon co-expression, and stimulates epidermal-growth-factor-receptor (EGFR)-mediated endocytosis (Tall, Barbieri et al. 2001).

RIN1 was originally identified as a RAS effector that interferes with RAS-induced phenotypes in yeast *Saccharomyces cerevisiae* (Colicelli, Nicolette et al. 1991; Han, Wong et al. 1997; Bliss, Venkatesh et al. 2006). RIN1 has also been shown to positively regulate endocytosis and cytoskeletal remodelling, epithelial-cell adhesion and migration (Hu, Bliss et al. 2005; Cao, Tanis et al. 2008; Ziegler, Eiseler et al. 2011) by activating downstream Rab5 GTPases and the Abl tyrosine kinase (Han, Wong et al. 1997; Tall, Barbieri et al. 2001; Barbieri, Kong et al. 2003; Hu, Bliss et al. 2005).

RIN1 was also reported to be involved in regulating insulin receptor and IL3 receptor signal transduction pathways (Hunker, Galvis et al. 2006; Hunker, Giambini et al. 2006). It directly interacts with activated EGFR through its SH2 domain (Afar, Han et al. 1997; Han, Wong et al. 1997; Barbieri, Kong et al. 2003; Hu, Bliss et al. 2005; Cao, Tanis et al. 2008) playing an important role in mediating EGFR trafficking and degradation (Tall, Barbieri et al. 2001; Barbieri, Fernandez-Pol et al. 2004; Chen, Kong et al. 2009; Tomshine, Severson et al. 2009).

RIN1 gene expression is closely associated with mitosis, neoplastic transformation (Samant, Debies et al. 2002; Bliss, Venkatesh et al. 2006; Fujioka, Goi et al. 2009; Bliss, Gray et al. 2010; Chetcuti, Aktas et al. 2011; Thai, Ting et al. 2011) and, through duplications or aberrant expression, with several types of cancers including squamous cell, cervical, colorectal, gastric, breast and non-small cell lung cancer, Wilms' tumour and acute myeloid leukaemia (Shuster, Han et al. 2000; Zainabadi, Benyamini et al. 2005; Milstein, Mooser et al. 2007; Senda, Goi et al. 2007; Fujioka, Goi et al. 2009; Tomshine, Severson et al. 2009; Wang, Gao et al. 2012). RIN1 knockout mice demonstrated a major physiological role for RIN1 in mature neurons (Dhaka, Costa et al. 2003).



RIN2 has also been shown to regulate E-cadherin internalisation (Kimura, Sakisaka et al. 2006) in hepatocyte growth factor (HGF)-induced endocytosis of E-cadherins in rat liver cells. It is a direct target of RAS and transduces signal activation from activated RAS to Rab5 (Kimura, Sakisaka et al. 2006).

### **RalGEF family**

RalGDS is a known RAS effector protein. It is a guanine exchange factor (GEF) that contains a RAS-GEF domain in its N-terminal domain. RalGDS is one of the GEFs, along with Rlf, Rgl, Rgl3, that are downstream effectors of RAS proteins. They aid the dissociation of GDP for the small GTPase proteins RalA and RalB, thus allowing GTP binding and subsequent activation. In addition to binding to and activating the Ral G-proteins, it also enables PDK1 to interact with Akt thus allowing its phosphorylation on T308 (Hao, Wong et al. 2008); this contributes to the activation of Akt by growth factors.

### **RGL/Rlf**

Ral guanine nucleotide dissociation stimulator-like 2 is encoded by RGL2 in humans (Isomura, Okui et al. 1996) and has been shown to interact with HRAS (Peterson, Trabalzini et al. 1996; Mitin, Ramocki et al. 2004).

RGL2 is phosphorylated both *in vitro* and in mammalian cells by PKA at S737 located at the C-terminus of its RBD. Phosphorylation significantly reduces the ability of RGL2 to bind HRAS and RAP1B (Ferro, Magrini et al. 2008). This phosphorylation site is conserved in the RGL2 murine ortholog RLF but not in RalGDS, RGL and RGL3. Thus, while all RalGDS proteins are able to bind RAS proteins, only in the case of RGL2/RLF, this interaction may be regulated by PKA phosphorylation. This suggests that RAS proteins may distinguish between the different RalGDS family members through their phosphorylation by PKA and pointing towards a general mechanism used by RAS to distinguish the different effectors (Ferro, Magrini et al. 2008).

### **AF6/Afadin**

AF6 is a multidomain F-actin-binding protein that is expressed in almost all epithelial tissues, and in a variety of other cell types including neurons, fibroblasts and endothelial cells (Mandai, Nakanishi et al. 1997; Takai, Ikeda et al. 2008).

AF6 is an adaptor protein that localises to cell-cell junctions. AF6 is described as a regulator of integrin-mediated cell adhesion (Su, Hattori et al. 2003) and cell migration (Lorger and Moelling 2006; Miyata, Ogita et al. 2009; Severson, Lee et al. 2009). AF6 has been implicated in RAS-induced junction breakdown (Yamamoto, Harada et al. 1997), and in binding p120 catenin in a RAP-dependent manner to prevent internalisation of E-cadherin (Hoshino, Sakisaka et al. 2005).

The long isoform of AF6 comprises (from the N- to C-terminus) two RA domains (RAS/RAP1 association); the N-terminal RA domain binds both RAS and RAP1 with high affinity, a Forkhead, a dilute domain, a PDZ domain and an actin-binding domain (Hofmann and Bucher 1995; Ponting 1995; Mandai, Nakanishi et al. 1997; Yamamoto, Harada et al. 1997; Linnemann, Geyer et al. 1999). The longer isoform of AF6 regulates E-cadherin in a RAP-independent manner (Lorger and Moelling 2006) whereas the short isoform lacks the actin-binding domain (Saito, Matsushima et al. 1998).

In T-cells, AF6 is a negative regulator of RAP-induced integrin mediated cell adhesion (Zhang, Rehmann et al. 2005). Additionally, the role of AF6 in oncogenesis has been documented in some acute lymphoid and myeloid leukaemias. The *MLLT4* gene is a fusion partner of the mixed lineage leukaemia gene *MLL* (Prasad, Gu et al. 1993). Self-association of AF6 activates the oncogenic potential of MLL-AF6 fusion protein (Liedtke, Ayton et al. 2010).

In mice, AF6 knockout results in embryonic lethality with defects starting at gastrulation. This includes disorganisation of the ectoderm, impaired migration of the mesoderm and loss of somites (Ikeda, Nakanishi et al. 1999; Zhadanov, Provance et al. 1999).

## Potential RAP2 Interactors

### VPS35/VPS29

The retromer complex is a key mediator of trafficking from endosomes to the Golgi complex (Bonifacino and Hurley 2008; McGough and Cullen 2011; Temkin, Lauffer et al. 2011). These complexes are involved in the recycling of endosomal SNAREs (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor), trafficking

of Wnt receptors, retrograde transport of shiga toxins, the delivery of polymeric immunoglobulin receptor (pIgR) and the regulation of amyloid  $\beta$  (A $\beta$ )-peptide processing by its sorting receptor, the sortilin-related receptor with A-type repeats (sorLA) (Hettema, Lewis et al. 2003; Verges, Luton et al. 2004; Andersen, Reiche et al. 2005; Bujny, Popoff et al. 2007; Belenkaya, Wu et al. 2008).

The retromer complex can be functionally dissected into two subcomplexes; the cargo-selective subcomplex is a trimer of the VPS26, VPS29 and VPS35 proteins, whilst the SNX-BAR dimer of the VPS5 and VPS17 proteins acts to form membrane tubules at the endosome (Seaman, McCaffery et al. 1998).

VPS29 is a rigid scaffold that interacts with various proteins that function with retromer in endosomal protein sorting (Swarbrick, Shaw et al. 2011). VPS29 links the two subcomplexes together with a conserved hydrophobic patch on the surface of VPS29 that is critical for the assembly of the heteropentamer.

VPS35 selects cargo proteins for retrieval (Nothwehr, Ha et al. 2000; Collins, Norwood et al. 2008) and can be mutated in autosomal dominant late-onset Parkinson disease (Vilarino-Guell, Wider et al. 2011; Zimprich, Benet-Pages et al. 2011) at D620N, P316S and R524W.

Although the RAP2-VPS35/VPS29 interaction was validated by overexpressing tagged proteins (see later), we were not able to validate the endogenous interaction with our antibodies and it was not pursued further as not many tools or reagents were available at the time.

### **TBC1D10B**

TBC1D10B functions as a GAP for several proteins of the Rab family and is involved in exocytosis (Ishibashi, Kanno et al. 2009; Hsu, Morohashi et al. 2010). The TBC (Tre2, Bub2, and Cdc16) domain regulates the activity of small Rab GTPases via a dual-finger mechanism (Pan, Eathiraj et al. 2006). Rab GAPs are essentially implicated in the spatial and temporal dynamics of the cellular endomembrane system (Barr and Lambright 2010).

Using tagged constructs in co-transfection experiments we were not able to reproducibly validate this interaction. Furthermore, because there are no

commercial antibodies available against TBC1D10B, this interaction was not further pursued and remains to be validated.

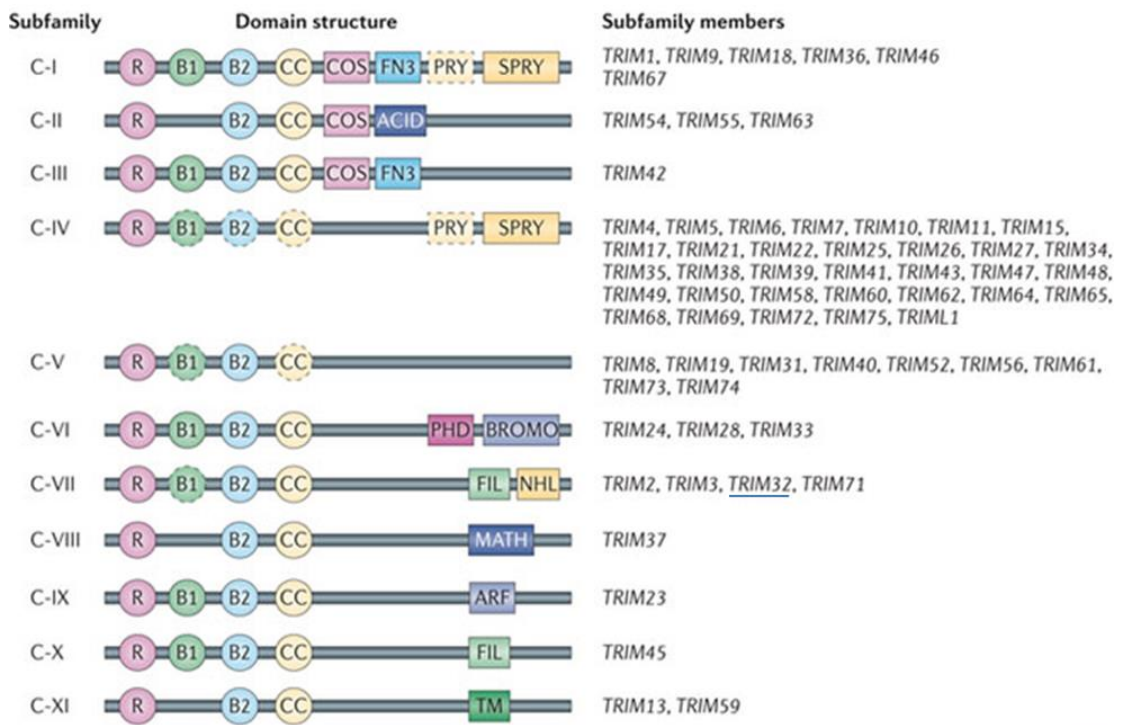
## **TRIM32**

The TRIM32 interaction is the main focus of this project and is part of the tripartite motif (TRIM) family of proteins.

## **TRIM**

TRIM family proteins are involved in a broad range of biological processes and their mutations are associated with diverse pathological conditions, such as neurodegenerative diseases, developmental disorders, viral infections and cancer (Meroni and Diez-Roux 2005; Ozato, Shin et al. 2008). Most of the TRIM proteins function as E3 ubiquitin ligases; proteins that carry out post-translational modification of targets via a cascade of ubiquitin-modifying reactions (Pickart and Eddins 2004) (described in more detail later).

There are more than 70 known TRIM proteins in humans and mice, which are encoded by approximately 71 genes in humans, several of which are clustered together. TRIM proteins are classified into subfamilies I to XI on the bases of their domain structure (Figure 9) (Short and Cox 2006; Ozato, Shin et al. 2008; McNab, Rajsbaum et al. 2011). They contain three linked motifs namely a RING finger, 1-2 zinc binding B-box domains, and a coiled coil domain, together with a variable C-terminal region (Reymond, Meroni et al. 2001). They are often defined as E3 ubiquitin ligases due to the presence of a RING-finger domain, although not all proteins contacting a RING-finger domain function as E3 ubiquitin ligases (Reymond, Meroni et al. 2001).



**Figure 9: The structural classification of TRIM subfamily (C-I to C-XI).** Almost all TRIM proteins have a RING-finger domain (R), one or two B-box domains (B) and a coiled-coil domain (CC). Some members of the subfamily lack one or more amino-terminal domains (shown as dashed outline domains). ACID, acid-rich region; ARF, ADP-ribosylation factor family domain; BROMO, bromodomain; COS, cos-box; FIL, filamin-type I G domain; FN3, fibronectin type III repeat; MATH, meprin and TRAF-homology domain; NHL, NCL1, HT2A and LIN41 domain; PHD, PHD domain; PRY, PRY domain; SPRY, SPRY domain; TM, transmembrane region; Ub, ubiquitin. Figure from (Hatakeyama 2011).

### TRIM32

TRIM32 is classified as a member of the C-VII sub-family, and its variable region consists of 6 NHL motifs; [NHL repeats are conserved domains defined by amino acid sequence homologies among Ncl-1, HT2A and Lin-41 proteins (InterPro database)], likely to mediate protein-protein interactions (Figure 10)(Slack and Ruvkun 1998).



**Figure 10: Structure of Trim32.** RING finger, one or two B-Box zinc binding domains, a coiled coil domain and six NHL repeats. Figure adapted from (Saccone, Palmieri et al. 2008).

RING-type zinc finger domains are 40 to 60 residues long, bind two atoms of zinc and are involved in mediating protein-protein interactions (Freemont 1993). The RING domain of TRIM32 has E3 ubiquitin ligase activity (Kudryashova, Kudryashov et al. 2005; Albor, El-Hizawi et al. 2006; Kano, Miyajima et al. 2008).

B-box-type zinc finger domains are around 40 residues in length. B-box zinc fingers can be divided into two groups, where types 1 and 2 B-box domains differ in their consensus sequence and in the spacing of the 7-8 zinc-binding residues. TRIM proteins contain a type 2 B-box domain, and may also contain a type 1 B-box (Reymond, Meroni et al. 2001).

The NHL repeat of TRIM32 folds into a six-bladed  $\beta$ -propeller structure, with the blades arrayed in a radial fashion around a central axis, and each blade composed of a highly twisted four stranded antiparallel beta-sheet (Edwards, Wilkinson et al. 2003). These mediate protein-protein interactions (Slack and Ruvkun 1998) (Gentry, Worby et al. 2005).

TRIM32 (OMIM 602290) has been implicated in diverse pathological processes such as head and neck cancer (Horn, Albor et al. 2004; Kano, Miyajima et al. 2008), squamous cell carcinogenesis (Albor, El-Hizawi et al. 2006; Kano, Miyajima et al. 2008), Bardet-Biedl syndrome (BBS) (Chiang, Beck et al. 2006), Alzheimer's disease (Yokota, Mishra et al. 2006), psoriasis lesions (Liu, Lagowski et al. 2010) and Limb-Girdle Muscular Dystrophy (LGMD2H) (Frosk, Weiler et al. 2002; Schoser, Frosk et al. 2005; Saccone, Palmieri et al. 2008) which is discussed in more detail below. Several muscle-specific substrates and interacting partners of TRIM32 have been identified, including myosin, actin (Kudryashova, Kudryashov et al. 2005), and dysbindin (Locke, Tinsley et al. 2009), yet the biological role of these interactions is not understood.

### Role in Cancer

It has been reported that the expression of TRIM32 is increased in transformed keratinocytes, ultraviolet (UV) irradiation-induced squamous cell carcinoma (SCC), chemically induced papillomas, and head and neck SCCs (Horn, Albor et al. 2004). In

addition, TRIM32 overexpression protects keratinocytes from TNF and UV irradiation-induced apoptosis, suggesting that TRIM32 can function as an oncogene (Horn, Albor et al. 2004).

In NIH-3T3 and Hep-2 (epithelial) cells, TRIM32 binds to and ubiquitinates Abl-interactor 2 (ABI2), resulting in its degradation (Kano, Miyajima et al. 2008); ABI2 is a tumour suppressor gene that inhibits cell migration and overexpression of TRIM32-promoted cell proliferation, motility and transformation. This suggests that TRIM32 can promote tumour growth and metastasis through degradation of ABI2 (Kano, Miyajima et al. 2008).

Conversely, in HEK-293T cells TRIM32 induces tumour necrosis factor (TNF)-mediated apoptosis through its direct interaction and ubiquitination of X-linked inhibitor of apoptosis (XIAP). The degradation of XIAP prevents its inhibition of pro-apoptotic caspases (Ryu, Lee et al. 2011). These findings suggest that TRIM32 could function as a tumour suppressor. TRIM32 suppresses apoptosis induced by *cis*-diamminedichloroplatinum (II)(cDDP) in Hep2 cell lines (Kano, Miyajima et al. 2008).

The *Drosophila melanogaster* homologues of TRIM32; *Brat* and *mei-26*, cause tumour formation when mutated in flies (Neumuller, Betschinger et al. 2008; Schwamborn, Berezikov et al. 2009) suggesting they may have tumour suppressor properties.

#### Role in Bardet-Biedl Syndrome (BBS)

BBS (OMIM 209900) is a pleiotropic autosomal recessive human genetic disorder that affects many body systems and has variable expressivity and a wide range of clinical variability observed both within and between families. Symptoms include polydactyly, truncal obesity and learning difficulties (Beales, Elcioglu et al. 1999; Abd-El-Barr, Sykoudis et al. 2007)<sup>1</sup>.

Many of the gene products encoded by these BBS genes are assembled into a multiple protein complex, called the “BBSome” that is proposed to be responsible for transporting intracellular vesicles to the base of the cilia and to play an important role in ciliary function. Since abnormalities of cilia are known to be related to a wide range of disease symptoms including those commonly seen in BBS

patients, it is now widely accepted that mutated BBS genes affect normal cilia functions. Fourteen different genetic mutations have been currently identified in BBS (OMIM 209900), one of which consists of a homozygous P130S mutation in the B-box domain of TRIM32 (BBS11) (Chiang, Beck et al. 2006).

#### TRIM32 is required for neuronal differentiation

The *Drosophila* orthologues of TRIM32, Brat and Brat-like protein Mei-P26, control stem cell proliferation in the *Drosophila* nervous system and ovaries respectively (Bello, Reichert et al. 2006; Betschinger, Mechtler et al. 2006; Lee, Robinson et al. 2006; Neumuller, Betschinger et al. 2008; Harris, Pargett et al. 2011). Thus, deregulation of muscle stem cell activity upon loss of TRIM32 could contribute to the formation of LGMD2H. TRIM32 has been shown to be necessary for the regulation of differentiation and self-renewal in neural progenitor cells during mouse embryonic brain development. In cultured cortical progenitors TRIM32 regulates protein degradation and microRNA (miRNA) activity to control the balance of two daughter cell types in neural progenitor cells. PKC $\zeta$  binds TRIM32 and by mediating its localisation in the cytoplasm inhibits the poly-ubiquitination of nuclear c-Myc. PKC $\zeta$  and probably TRIM32 itself are targets for TRIM32-dependent ubiquitination. Upon neuronal differentiation, PKC $\zeta$  levels decrease, allowing TRIM32 to become polarised and concentrated in one of the daughter cells, where it ubiquitinates and degrades c-Myc inducing neuronal differentiation (Schwamborn, Berezikov et al. 2009; Hillje, Worlitzer et al. 2011). Additionally, in two different lines of TRIM32 null mice, loss of TRIM32 function caused a LGMD2H-like phenotype which is associated with dysfunctional muscle satellite cells. Moreover, skeletal muscle regeneration is greatly impaired in these mice, indicating an important role for TRIM32 in the regulation of skeletal muscle stem cells.

TRIM32 functions as one of the co-activators for RAR $\alpha$ -mediated transcription; overexpression of TRIM32 in mouse neuroblastoma cells and embryonal carcinoma cells promotes the stability of RAR $\alpha$ , resulting in increased neural differentiation (Sato, Okumura et al. 2011). These findings suggest that TRIM32 is a potential therapeutic target for developmental disorders and RAR $\alpha$ -dependent leukaemias.



## Role in Muscular Dystrophy

The Muscular Dystrophies (MD) are an inherited group of genetic muscle disorders characterised by progressive muscle wasting and weakness. They share common histological features of “dystrophic” muscle biopsy changes (including variation in muscle fibre size, muscle fibre degeneration and regeneration) and replacement of muscle by connective tissue and fat (Motlagh, MacDonald et al. 2005). More than 30 genetically distinct types of muscular dystrophy have been identified. The prognosis for people with muscular dystrophy varies according to the type and progression of the disorder. Some have prenatal onset, while others affect only adults; some are rapidly progressive, while others are associated with prolonged periods of stability. Many are associated with multisystem involvement including the heart, eyes and, central nervous, gastrointestinal, and endocrine systems (Emery 2002; Bushby, Lochmuller et al. 2009; Kaplan 2009)<sup>ii</sup>.

The major variants of MD are Duchenne (DMD), Becker (BMD), Limb-Girdle (LGMD), congenital, facioscapulohumeral, myotonic, oculopharyngeal, distal, and Emery-Dreifuss muscular dystrophy (EDMD)<sup>ii</sup>. These diseases predominately affect males, although females may be carriers.

Although a curative treatment is not yet available, many individuals can now survive into adulthood.

MD	OMIM	GENE	ONSET	SYMPTOMS	PROGNOSIS
Duchenne Muscular Dystrophy	310200	dystrophin at Xp21 (Muntoni, Torelli et al. 2003).	2-5 years	Abnormal gait, scoliosis, respiratory insufficiency, cardiomyopathy, calf hypertrophy and difficulty in rising from the floor. Speech delay in minority of cases <sup>iii</sup> (Essex and Roper 2001). Feeding difficulties and weight loss common in the late stages of disease.	Poor; death in late teens or early twenties
Becker muscular dystrophy	300376	truncated, but partially functional form of dystrophin		Cognitive and behavioural deficits, autistic spectrum disorders (Young, Barton et al. 2008); typical hypertrophic, proximal weakness, quadriceps myopathy, myoglobinuria (Bushby, Cleghorn et al. 1991) and isolated cardiomyopathy in up to 72% of patients (Piccolo, Azan et al. 1994).	Variable; most patients survive past 40.
Limb-girdle muscular dystrophy	Multiple	Multiple	childhood or adult-onset	Skeletal muscle typically affected. Proximal weakness and wasting with features of muscle degeneration/regeneration.	Variable
Congenital muscular dystrophy	Multiple	Multiple	At birth	Mild or severe muscle degeneration; Severe brain malformations (e.g. lissencephaly, hydrocephalus). Generalised muscle weakness and joint deformities; slow progression.	Variable but generally poor
Distal muscular dystrophy	254130	DYSF	20 to 60 years	Weakness and wasting of muscles of the hands, forearms, and lower legs; slow progression	Good prognosis.
Miyoshi myopathy (sub-type of Distal muscular dystrophy)					
Emery-Dreifuss muscular dystrophy	310300, 181350	EMD, LMNA	childhood and the early teenage years	Distal limb muscle weakness and wasting, progressing to limb-girdle muscles. Cardiac conduction defects and arrhythmias in most patients.	Increased risk of stroke and sudden death.
Facioscapulothoracic muscular dystrophy	158900	DUX4	teenage years	Muscles of the face, shoulders, and upper arms affected (progressive weakness.).	Severe disability
Myotonic muscular dystrophy	160900, 602668	DMPK, ZNF9	adult	Myotonia (delayed relaxation of muscles), muscle wasting and weakness. Other body systems can be affected including the heart, endocrine organs, eyes, and gastrointestinal tract.	Variable

**Table 1: Clinical overview of muscular dystrophy disorders**

## Limb-Girdle Muscular Dystrophy

Limb-girdle muscular dystrophy (LGMD) refers to childhood- or adult-onset muscular dystrophies that are distinct from the much more common X-linked dystrophinopathies, DMD and BMD. LGMDs are much rarer than DMD and BMD, and in addition there is a significant genetic and clinical heterogeneity. Individuals with LGMD generally show weakness and wasting restricted to the limb musculature, as well as muscle degeneration/regeneration on muscle biopsy. Onset, progression, and distribution of the weakness and wasting vary considerably among individuals and genetic subtypes.

LGMD affects both males and females, affecting the upper arms and legs. Many forms of LGMD have been identified, showing different patterns of inheritance; (e.g. autosomal dominant vs autosomal recessive classified as LGMD1 and LGMD2 respectively). Dominant LGMDs are less frequent than the recessive forms and usually show adult onset whilst recessive LGMDs usually have childhood or teenage onset. Though a person normally leads a normal life with assistance, death can occur from cardiac complications.

Mutations at more than 50 loci have been shown to cause LGMD. The overall frequency of LGMD is one in 15 000 (Emery 1991). LGMD2I is the commonest muscular dystrophy in certain northern European countries, whereas a higher incidence of sarcoglycanopathy is detected in northern Africa. LGMD2B and LGMD2A predominate in an Australian cohort (Lo, Cooper et al. 2008).

An overview of the clinical features of muscular dystrophies presenting with limb-girdle muscle weakness are summarised in Table 2, including information of the predominance of certain subtypes in certain populations.

Disease Name (Synonym)	Gene Symbol	Locus	Symptoms	Onset (Average)	Wheelchair Bound	Populations with Mutations
LGMD1A (Myotilinopathy)	MYOT	5q31.2	Proximal weakness, progressive distal weakness. Tight Achilles tendons; nasal, dysarthric speech (50%), respiratory insufficiency and dysphagia	18-40 years		German, Argentinian, Turkish, Japanese
LGMD1B	LMNA	1q22	Proximal lower limb weakness Mild contractures of elbows,	Birth to adulthood		

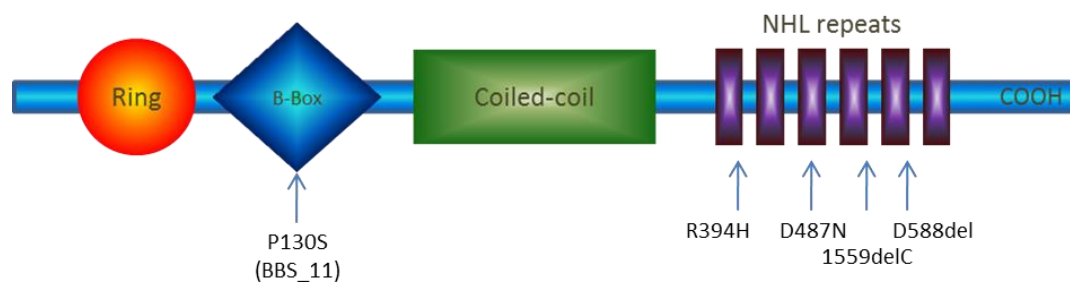
			Arrhythmia and other cardiac complications (25-45 years), Sudden death			
LGMD1C (Caveolinopathy)	CAV3	3p25.3	Mild-moderate proximal weakness Cramping, Rippling muscle disease, Calf hypertrophy	~5 years		Italian
LGMD1D	DES	2q35	Proximal muscle weakness Cardiac conduction defects, dilated cardiomyopathy	<25 yrs	All individuals remain ambulatory	
LGMD1E	DNAJB6	7q36.3	Proximal or distal weakness in the lower limbs.	18-40 years	Wheelchair bound at age 45-62 years	
LGMD1F	Unknown	7q32.1-q32.2	Proximal lower and upper limb weakness, later distal weakness.	1-58 years (juvenile-onset and adult-onset form)		Spanish
LGMD1G	Unknown	4q21	Proximal lower limb weakness; proximal upper limb weakness in later stages Progressive limitation of finger and toe flexion.	30-47 years		Brazilian-European
LGMD1H	Unknown	3p25.1-p23	Proximal lower limb weakness Muscle hypotrophy of limb girdle muscles with calf hypertrophy.	16-50 years (39)		Italian
Calpainopathy (LGMD2A)	CAPN3	15q15.1	Proximal (normal hip extensors and adductors). Difficulty to run, walk, toe walk; calf muscle atrophy.	2-40 yrs (8-15 yrs)	11-28 yrs after onset	Amish, La Reunion Island, Basque (Spain), Turkish
Dysferlinopathy (LGMD2B)	DYSF	2p13.2	Distal and/or pelvic-femoral Difficulty to tip-toe; difficulty run, walk; transient calf muscle hypertrophy (rare).	17-23 yrs		Libyan Jewish Australian
Gamma-sarcoglycanopathy (formerly SCARM1) (LGMD2C)	SGCG	13q12.12	Proximal weakness. difficulty to run and walk; calf muscle hypertrophy.	3-15 yrs (8.5 yrs)	~15 yrs	North Africans; Gypsies
Alpha-sarcoglycanopathy (LGMD2D)	SGCA	17q21.33		Childhood - adulthood		None
Beta-sarcoglycanopathy (LGMD2E)	SGCB	4q12	Cramps, exercise intolerance	Adolescent - young adulthood		Amish
Delta-sarcoglycanopathy (LGMD2F)	SGCD	5q33.3		Childhood - adulthood		Brazilian
LGMD2G	TCAP	17q12	Lower limb and proximal upper limb weakness Difficulty to run, walk; foot drop	9-15 yrs	~18 yrs after onset	Brazilian, Italian
LGMD2H	TRIM32	9q33.1	Proximal lower limb, neck and facial weakness. Waddling gait, difficulty with stairs, calf muscle wasting.	1-9 yrs	Late in life	Manitoba Hutterites
LGMD2I	FKRP	19q13.32	Proximal weakness; upper >	1.5-27 yrs	23-26 yrs	Northern

(MDDGC5)			lower limb Difficulty to run, walk; calf muscle hypertrophy, rare/late contractures/scoliosis	(11.5 yrs)	after onset	European
LGMD2J	TTN	2q31.2	Proximal weakness	5-25 yrs	Average 20 yrs after onset	Finland, Moroccan, Sudanese
LGMD2K (MDDGC1)	POMT1	9q34.13	Mild weakness; proximal > distal Fatigability, difficulty climbing stairs and running; cognitive delay with limited language development, hypertrophy of calves and thighs	1-3 yrs	varies	Turkish
LGMD2L	ANO5	11p14.3	Proximal weakness, pelvic-femoral or distal in the lower limbs. Difficulties standing on toes, Contractures (wrist, finger, TA)	Late teens-50s		Northern European
LGMD2M (MDDGC4)	FKN	9q31.2	Proximal weakness; lower > upper limb Difficulties climbing stairs, severe weakness after illness; steroid responsive	4 mo - 4 yrs	Not reported	Japanese
LGMD2N (MDDGC2)	POMT2	14q24.3	Slowness in running and getting up, scapular winging and mild lordosis; intellectual disability	18 mo; asymptomatic at 5 yrs		Unknown
LGMD2O (MDDGC3)	POMGNT1	1p34.1	Proximal weakness > distal. Difficulty standing from the sitting position and climbing stairs; severe myopia, ankle contractures	12 yrs		Unknown
LGMD2Q	PLEC	8q24.3	Proximal weakness. Delayed achievement of motor milestones, difficulties climbing stairs.	2-3 yrs		Turkish

**Table 2: Clinical Features of LGMD.**

### *LGMD2H*

Mutations in TRIM32 are responsible for LGMD2H. The first TRIM32 mutation described, p.Asp487Asn, is a founder mutation in the Hutterite population of North America with one sibling pair identified in a non-Hutterite family in Germany (country of Hutterite origin). Sarcotubular myopathy (STM), also found in the Hutterite population, is caused by the same mutation in TRIM32 (Schoser, Frosk et al. 2005; Borg, Stucka et al. 2009).



**Figure 11: TRIM32 mutations fall within the BBox and NHL domain.** Mutations in the BBox domain demonstrate a role in Bardet-Biedl syndrome whereas those located in the NHL repeats are linked to Limb-Girdle Muscular Dystrophy Type 2H (indicated by the blue arrows).

Subsequently, additional mutations in TRIM32 were identified in patients with LGMD (Figure 11); c.1180G>A (p.R394H) (Saccone, Palmieri et al. 2008)], one codon deletion [c.1761–1763delGAT (p.D588 del) (Saccone, Palmieri et al. 2008)], three frameshift mutations [c.1559delC (p.T520TfsX13) (Saccone, Palmieri et al. 2008), c.1753–1766dup (p.I590LfsX38) (Cossee, Lagier-Tourenne et al. 2009), c.1560delC (p.C521VfsX13) (Borg, Stucka et al. 2009)] and one intragenic deletion that removes the entire open reading frame [del 30 586 bp + insert 2 bp (Borg, Stucka et al. 2009)]. Similar to the previously identified D487N mutation, all these new mutations occurred in the C-terminal NHL domains of TRIM32.

Disease severity ranges from asymptomatic to severe proximal weakness. Facial weakness and a "flat smile" are common (Weiler, Greenberg et al. 1998; Saccone, Palmieri et al. 2008). Affected individuals can maintain their ability to walk well into adulthood with some reports of patients remaining capable of walking (with difficulty) into their sixties (Weiler, Greenberg et al. 1998; Saccone, Palmieri et al. 2008). Irreversible loss of motility has been reported after prolonged immobilization (Saccone, Palmieri et al. 2008).

LGMD2H biopsies show myopathic features of central nucleation, fiber splitting, Z-line streaming, and a dilated sarcotubular system with vacuoles (Jerusalem, Engel et al. 1973; Shokeir and Kobrinsky 1976; Shokeir and Rozdilsky 1985; Muller-Felber, Schlotter et al. 1999; Frosk, Weiler et al. 2002; Schoser, Frosk et al. 2005; Saccone, Palmieri et al. 2008).

*Drosophila* studies demonstrate that the TRIM32 ortholog Thin (*tn*) is a key structural protein maintaining myofibrillar stability. Myofibrils in *tn* mutants unbundle during development, leading to muscle wastage over time. The gaps in the myofibrils of *tn* mutants appear analogous to the gaps in the myofibrils of diseased muscle tissue from individuals with muscle. The muscle defects observed in *Drosophila* are more pronounced than those seen in TRIM32 deficient mammals. It is likely that Thin plays a greater functional role in *Drosophila* while it is likely that multiple mechanisms contribute to the muscle breakdown that occurs in humans suffering from LGMD2H myopathies (LaBeau-DiMenna, Clark, et al. 2012).

A TRIM32 knock-out mouse model recapitulates human muscular dystrophy phenotypes seen in LGMD2H and sarcotubular myopathy, demonstrating similar muscle morphology as patient muscle biopsies. Moreover, it allowed for the uncovering of a neurogenic component caused by disruption of the TRIM32 gene, suggesting a novel role for TRIM32 in the nervous system (Kudryashova, Struyk et al. 2011; Kudryashova, Wu et al. 2009). Additionally, it has been shown that TRIM32 deficiency plays a role in premature senescence of myogenic cells, leading to a lack of adequate muscle regrowth after atrophy. This premature senescence contributes to cell differentiation and proliferation defects in myogenic cultures, leading to impaired myotube formation (Kudryashova, Struyk et al. 2011; Kudryashova, Kramerova et al. 2012). During fasting-induced atrophy, Trim32 catalyses the loss of thin filament and Z-band components through effects on the key cytoskeletal protein – desmin (Cohen, Zhai et al. 2012). In muscle, desmin forms intermediate filaments that are localised in the sarcolemma, mitochondria, and nuclear membrane and between adjacent myofibrils linking them laterally to the Z-lines (Lazarides and Hubbard 1976; Lazarides 1978). Mice lacking desmin exhibit misaligned sarcomeres and disorganised myofibrils in skeletal and cardiac muscle (Milner, Weitzer et al. 1996).

### Neurodevelopmental Phenotypes

Recent studies conducted by Lionel et al. have demonstrated that rare copy number variants that disrupt TRIM32 have been reported. Although their work focuses mainly on the identification of ASTN1 and ASTN2 variants, they have shown that exonic deletions affecting multiple isoforms of TRIM32 are enriched in

neurodevelopmental disorders with patient phenotypes ranging from language delay and anxiety to gross motor delay and fine motor delay (Lionel, et al. 2014).

TRIM32 expression levels have also been linked to autism spectrum disorder, attention deficit disorder, obsessive compulsive disorder, and stress-related anxiety- and depression-like behaviours when exposed to mild stress (Ruan, Wang, et al. 2014; Lionel, et al. 2011, 2014).

Combined with findings that TRIM32 expression levels in the mouse neocortex have been shown to determine the post-differentiation fates of neuronal stem cells and that TRIM32 is a key regulator of neural differentiation, it would be of interest to replicate some of our findings in neural cell lines to assess whether the TRIM32-RAP2 interaction also plays a role and to try and understand the implications on the above conditions (Schwamborn, Berezikov et al. 2009; Sato, Okumura et al. 2011).

## **NOEY2/DIRAS/ARHI**

### **The DIRAS family**

The Distinct subgroup of the RAS family (DIRAS) was identified as a member of the RAS family by Kontani et al (Kontani, Tada et al. 2002). They are 26kDa GTPases comprised of 229 amino acids. Similarly to other members of the RAS family; they are localised to the plasma membrane/vacuole membrane, in a CAAX dependent manner.

### **NOEY2/DIRAS3/ARHI**

The tumour suppressor NOEY2, also designated DIRAS3 and Aplysia RAS Homology member I (ARHI), was identified as a deletion occurring on chromosome 1p31 in 28–50% of breast cancers and 40% of ovarian cancers (Hoggard, Brintnell et al. 1995; Loupart, Armour et al. 1995; Nagai, Negrini et al. 1995; Yu, Xu et al. 1999; Peng, Xu et al. 2000). Aberrant NOEY2 signalling is also found in other cancers, showing decreased expression in uterine serous papillary carcinoma and squamous NSCLC. Decreased expression of NOEY2 also occurs in follicular thyroid carcinoma (FTC), but not in classic papillary thyroid carcinoma (PTC) or follicular adenoma (FA). FTCs show strong allelic imbalance with reduction in copy number/loss of heterozygosity



in 69% of cases, compared with less than 10% for FAs (Weber, Aldred et al. 2005). However, studies are predominantly carried out in breast and ovarian cancers.

*NOEY2* spans approximately 8 kb and contains one promoter, two exons and one intron. Exon 1 contains 83 non-translated nucleotides and is connected to exon 2 with a 3.2-kb intron. The entire protein-coding region of 687 bp is located within exon 2 of 1401 bp and encodes a 26-kDa protein. Although *NOEY2* and *DIRAS* family members share 50–60% amino acid homology with *RAS* and *RAP* family members (Table 3), and have the similar highly conserved GTP-binding domains with high GTPase activity, the function differs from that of the *RAS* and *RAP*, e.g. *DIRAS* proteins do not bind *RAF* or *PI3K* (Yu, Xu et al. 1999). *NOEY2* also differs from *HRAS* in residues critical for GTPase activity and for putative effector function. The substitutions within the GTP binding domain of *NOEY2* are consistent with the mutations of *RAS* responsible for its constitutive activation. Correspondingly, *NOEY2* has been found predominantly in its active GTP-bound state in cells (Luo, Fang et al. 2003).

<b>NOEY2:</b>	<b>Homology</b>
RAP1A	56%
RAP1B	58%
RAP2A	62%
RAP2B	59%
KRAS	59%
HRAS	54%
NRAS	51%

**Table 3: Homology of *DIRAS3* compared with various *RAS* family members.**

*NOEY2* has a distinctive 34aa N-terminal extension that is critical for the suppression of tumour growth. Cleavage of this N-terminal region abrogates its tumour suppressor activity (Luo, Fang et al. 2003). Additionally, *NOEY2* is constitutively expressed in normal breast and ovarian epithelial cells, but is lost or dramatically down-regulated in 40% of ductal carcinomas in situ, 70% of invasive breast cancers and 80% of ovarian cancers (Yu, Xu et al. 1999; Hisatomi, Nagao et al.

2002; Wang, Hoque et al. 2003). Studies by Wang *et al* have shown an inverse correlation between NOEY2 expression levels and tumour invasiveness (Wang, Hoque et al. 2003).

Re-expression of NOEY2 in breast and ovarian cancers, but not in lung cancer cell lines, suppressed clonogenic growth of tumour cells and induced apoptosis (Bao, Le et al. 2002; Luo, Fang et al. 2003; Rosen, Wang et al. 2004; Nishimoto, Yu et al. 2005), while in SKOV3 ovarian cancer cells autophagy was also induced. Overexpression of NOEY2 causes down regulation of cyclin D1 promoter activity and induces p21<sup>WAF1/CIP1</sup> causing cell cycle arrest and growth inhibition in cell cultures (Xu, Xia et al. 2000).

NOEY2 mutations have rarely been reported (Yu, Xu et al. 1999). However, NOEY2 promoter hypermethylation and transcriptional silencing have been identified in the breast hyperplastic cell line (MCF10A cell line), pre-malignant cell line (MCF10AT), invasive breast cancer line (MCF10CA1a, MCF10CA1d, MCF10CA1h) and primary breast cancer (Yang, Klinkebiel et al. 2005).

### **NOEY2 is an imprinted tumour suppressor**

Genomic imprinting is an epigenetic process that involves DNA methylation and histone modifications in order to achieve monoallelic gene expression without altering the genetic sequence. Imprinted alleles are silenced such that the genes are either expressed only from the non-imprinted allele inherited from the mother (e.g. H19 or CDKN1C), or in other instances from the non-imprinted allele inherited from the father (e.g. IGF-2). These epigenetic marks are established in the germline and are maintained throughout all somatic cells of an organism (Reik, Dean et al. 2001).

Appropriate expression of imprinted genes is important for normal development, with numerous genetic diseases associated with imprinting defects including Beckwith–Wiedemann syndrome, Silver–Russell syndrome, Angelman syndrome and Prader–Willi syndrome (Eggermann, Eggermann et al. 2008; Williams, Driscoll et al. 2010). Forms of aberrant genomic imprinting have been implicated in oncogenesis through loss of tumour suppressor gene regulation, e.g. the WT1 gene, in particular, is part of an imprinted cluster with INS, IGF2, H19, and p57KIP2 on chromosome 11p (Zhang, Shields et al. 1993).

Genomic imprinting and monoallelic gene expression play an important role in tumourigenesis (Barlow 1995). Sapienz was the first to incorporate genetic imprinting into Knudson's two "hit" tumourigenesis model. If an imprinted gene is involved, the first hit can be explained by the non-expression of one of the alleles because of the imprinting process, only preserving the function of monoallelic expression. The second hit may be mutational or may result from a loss of all or part of the chromosome carrying the remaining functional suppressor allele, resulting in total loss of the function of tumour suppressor (Sapienza 1991).

*NOEY2* has been identified as a maternally imprinted gene and was the first imprinted tumour suppressor gene identified in adult tumours (Luo, Fang et al. 2003). In normal cells, the maternal copy is silenced by methylation early in embryonic development and *NOEY2* is expressed in all normal cells only from the paternal allele (Yu, Xu et al. 1999). Thus, the change of the allelic gene inherited from the father (such as loss of heterozygosity, mutation and DNA methylation) would directly affect the expression of protein and its function as tumour suppressor, resulting in malignant transformation.

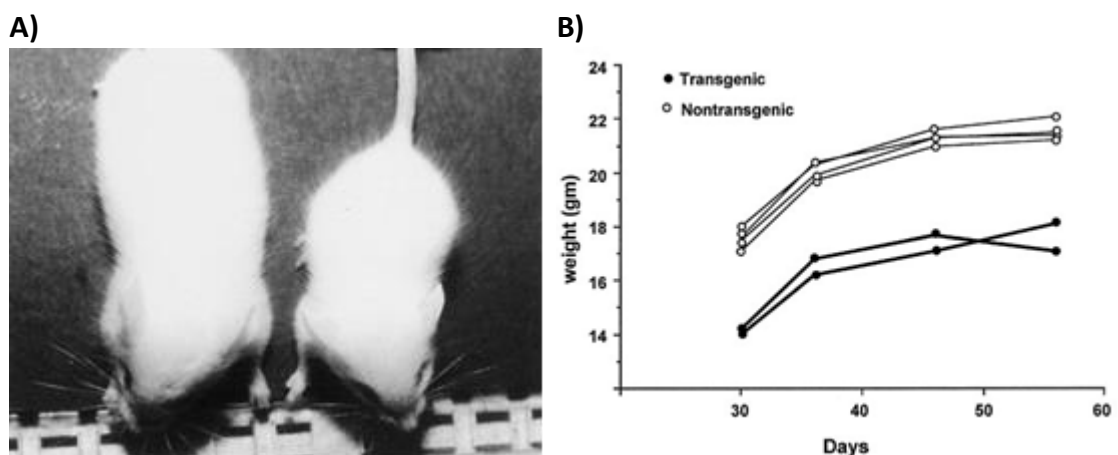
Three potential CpG islands, of approximately 300bp (base pairs) each, were found within the promoter and exons of the *NOEY2* gene. CpG island I is located about 1 kb upstream of the transcription initiation site; CpG island II is near the transcription initiation region; and CpG island III is located in the protein encoding region of exon 2. CpG island II is particularly important because it spans the 5'-upstream region of *NOEY2*, including the transcription initiation site and a portion of exon 1 (Yu, Fujii et al. 2003).

*NOEY2* CpG islands are partially methylated (one allele) in normal breast epithelial cells and although many cancer cell lines completely lack *NOEY2* expression, the extent to which the CpG islands are methylated differs (Yuan, Luo et al. 2003). Treatment with CpG demethylating agents and/or histone deacetylase inhibitors can reactivate both the silenced and imprinted alleles of *NOEY2* (Fujii, Luo et al. 2003). Reactivation of *NOEY2* expression by these reagents is related to the methylation status of the CpG islands in the *NOEY2* promoter. Histone H3 lysine-9/18 acetylation levels associated with *NOEY2* in normal cells are significantly

higher than those in breast cancer cell lines that lack NOEY2 expression. Treatment with a CpG demethylating agent and/or histone deacetylase inhibitor increases NOEY2 expression in breast cancer cells, with a corresponding increase in histone H3 lysine-9/18 acetylation and decrease in histone H3 lysine-9 methylation (Fujii, Luo et al. 2003).

### NOEY2 mouse model

Mouse homologues exist for DIRAS1 and 2, however no mouse homologue for DIRAS3/NOEY2 has been found. NOEY2 transgenic mice were therefore generated with NOEY2 overexpression driven by the cytomegalovirus (CMV) promoter. Transgenic mouse models overexpressing NOEY2 have shown impaired organ development and 10-40% lower body weight than the wild-type mouse, as seen in Figure 12. Morphological alterations are seen in neurons of the hippocampus and in Purkinje cells of the cerebellum. The severity of the phenotype has been shown to correlate with the level of transgene expression (Xu, Xia et al. 2000).

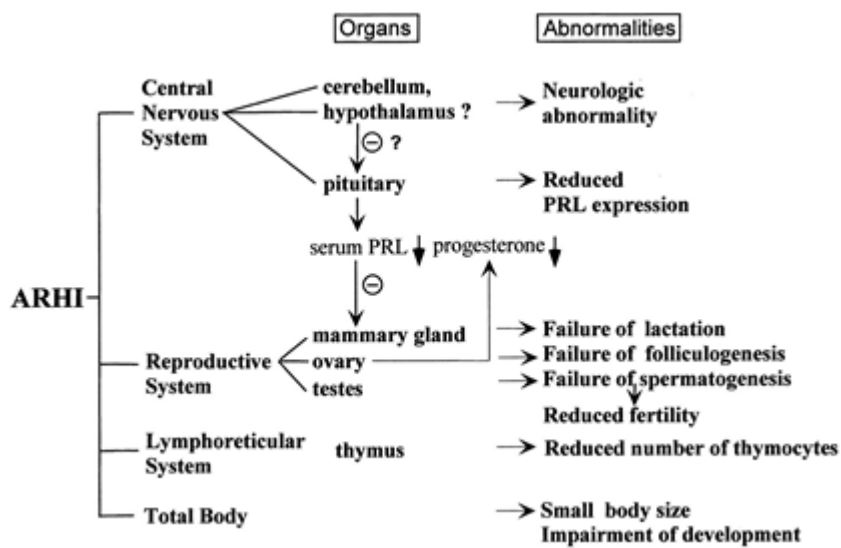


**Figure 12: Over-expression of NOEY2 reduces weight.** A) Picture of WT (left) and NOEY2 transgenic (right) mice. B) Body weight of WT and NOEY2 transgenic mice. Figure from (Xu, Xia et al. 2000).

The NOEY2 transgenic mouse model shows impaired mammary gland development and lactation with 8/22 mice unable to lactate and 4/22 showing a reduced ability to lactate. The failure of ovarian folliculogenesis in female mice results in decreased fertility/sterility while inability of Sertoli cells to mature in male mice leads to sterility (Xu, Xia et al. 2000).

Transgenic mice also show lower levels of progesterone and oestrogen receptor in mammary glands and ovaries with decreased prolactin and progesterone levels,

leading to a decrease in mammary tissue proliferation. NOEY2 inhibits prolactin (PRL) secretion and acts as a negative regulator in murine growth and development. These data suggest that NOEY2 is a negative regulator of growth and development of the breast and reproductive systems in mice (Xu, Xia et al. 2000). Interestingly, PRL knockout mice share many similarities with NOEY2 transgenic mice; they also show deficiency in lactation and are infertile. However, there is no overall change seen in body size (Figure 13)(Xu, Xia et al. 2000).



**Figure 13:**  
**Downstream effects of NOEY2 (ARHI) overexpression.**

Many inhibitory effects were seen in the transgenic mice, affecting multiple systems. Major abnormalities include failure to lactate, reduced fertility and small body size (Xu, Xia et al. 2000).

## DIRAS1

Other members of the DIRAS family have also been shown to have tumour suppressor function. DIRAS1/Rig has also shown loss of expression in primary neural tumours, suggesting a role as a tumour suppressor and perhaps indicating a subgroup specific function (Ellis, Vos et al. 2002).

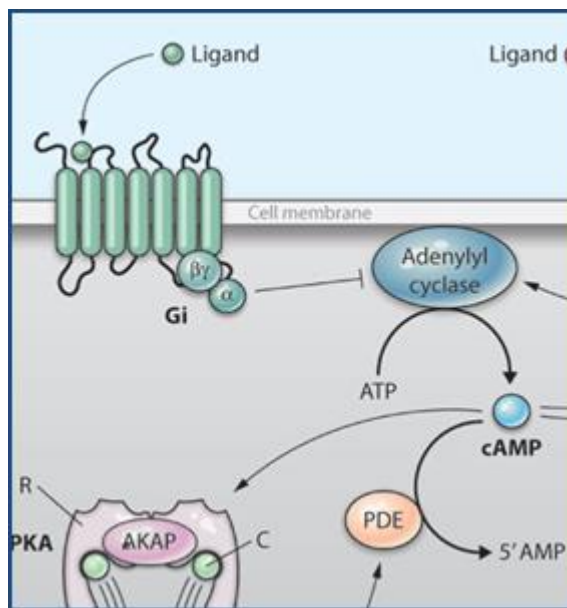
## Protein Kinases

Protein kinases are part of a large family of proteins that regulate a plethora of biological processes and their aberrant signalling is associated with many diseases. Protein kinase A/cyclic adenosine monophosphate (cAMP/3'-5'-cyclic adenosine monophosphate)-dependent protein kinase (PKA) serves in many ways as a

prototype for the entire superfamily of eukaryotic protein kinases, having played a central role in the unravelling of the mechanisms that regulates protein phosphorylation (for review see (Taylor, Ilouz et al. 2012; Scott, Dessauer et al. 2013)).

### cAMP signalling

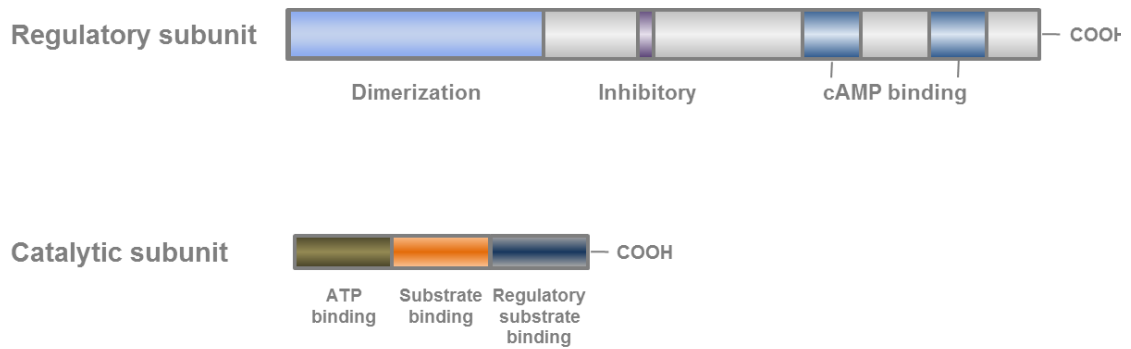
G-protein coupled receptors (GPCRs) are seven-transmembrane proteins which are activated by exchanging bound GDP for GTP upon ligand binding (Svoboda, Teisinger et al. 2004). Activation causes dissociation of the trimer into  $G\alpha$  and  $G\beta\gamma$  subunits. The  $G\alpha$  subunit inhibits adenylyl cyclase, a membrane bound-enzyme, which then converts adenosine triphosphate (ATP) to the second messenger cAMP (Figure 14) (Sunahara, Dessauer et al. 1996). cAMP is a second messenger important in many biological processes and is used for intracellular signal transduction in many different organisms, conveying the cAMP-dependent pathway. Many different physiological responses are mediated by cAMP including increase in heart rate, cortisol secretion and breakdown of glycogen and fat.



**Figure 14: PKA signalling.** Upstream signalling from GPCR leads to adenylyl cyclase converting ATP to cAMP. This then activates PKA. Figure from (Murray 2008).

### PKA

In eukaryotes, cAMP activates PKA, a ubiquitous serine/threonine protein kinase that is regulated by intracellular cAMP levels by altering the interaction between the catalytic and regulatory subunits of PKA (Bossis and Stratakis 2004).



**Figure 15: Structures of the regulatory and catalytic subunits.** The structure of the PKA regulatory subunit consists of an N-terminal dimerisation domain, an inhibitory domain (pseudosubstrate for RI and substrate site for RII) and two cAMP binding domains. The catalytic subunit (PKA-cat) has an ATP binding domain, substrate binding domain and a regulatory substrate binding domain at the C-terminus. Figure adapted from (Taylor, Kim et al. 2005).

The regulatory subunits each have two isoforms ( $\alpha$  and  $\beta$ ), which have been shown through knockout mouse studies to be functionally non-redundant (Kim, Cheng et al. 2007; Wu, Brown et al. 2007; Brown, Wu et al. 2009). Both R1 $\alpha$  and R2 $\alpha$  are ubiquitously distributed whereas R1 $\beta$  and R2 $\beta$  are detected primarily in the brain (Gamm, Baude et al. 1996). Within the cell, R1 is localised in the cytoplasm whereas R2 subunits are localised by anchoring to subcellular structures and compartments by A kinase anchoring proteins (AKAPs).

Under low levels of cAMP PKA remains an intact and inactive holoenzyme, PKA catalytic subunits (PKA-cat) are normally sequestered as an inactive tetrameric complex, consisting of two catalytic and two regulatory units, with the regulatory units blocking the catalytic centres of the catalytic units (Kim, Cheng et al. 2007; Wu, Brown et al. 2007; Brown, Wu et al. 2009). The regulatory subunits are the receptors for cAMP, and the inactive holoenzyme is activated when the concentration of cAMP rises (e.g. activation of adenylate cyclases by G protein-coupled receptors coupled to G<sub>s</sub>, inhibition of phosphodiesterases that degrade cAMP) and cAMP binds to the two binding sites on the regulatory subunits of the protein kinase. This causes dissociation between the regulatory and catalytic subunits into a dimer of regulatory subunits and two free monomeric catalytic subunits, thus liberating the catalytic sites, activating the catalytic units and enabling them to phosphorylate substrate proteins. For maximal function, each

catalytic subunit must also be phosphorylated, which occurs on T197, and helps orientate catalytic residues in the active site. The free catalytic subunits can then enter the nucleus and catalyse the transfer of ATP terminal phosphates to protein substrates at serine, or threonine residues. Since PKAs are present in a variety of cells and act on different substrates, PKA and cAMP regulation are involved in many different pathways and targets many different proteins such as CREB, CREM, ATF1.

### **PKA Inhibition**

Down-regulation of protein kinase A occurs by a feedback mechanism: One of the substrates that are activated by the kinase is a Phosphodiesterase (PDE), which hydrolyses cAMP to AMP, thus reducing the amount of cAMP that can activate PKA. Also, the catalytic subunit itself can be down-regulated by phosphorylation. Protein Kinase Inhibitors (PKI) act as a chaperone for the nuclear export of the PKA catalytic subunits and downregulate PKA activity.

### **A Kinase Anchoring Proteins (AKAPs)**

Targeting of PKA to specific sites within the cell is largely achieved by AKAPs (Wong and Scott 2004; Pidoux and Tasken 2010). AKAPs are scaffolding proteins that sequester signalling enzymes to specific subcellular environment. They prevent indiscriminate phosphorylation and form multi-protein complexes that enable the integration of cAMP signalling with other signalling events.

The regulatory subunit dimer of PKA is important for localising the kinase inside the cell. The dimerisation and docking (D/D) domain of the dimer binds to the A-kinase binding (AKB) domain of AKAPs. The AKAPs localise PKA to various locations (e.g. plasma membrane, mitochondria) within the cell. For example, an AKAP located near the nucleus of a heart muscle cell would bind both PKA and PDE which allows the cell to limit the productivity of PKA since the catalytic subunit is activated once cAMP binds to the regulatory subunits. AKAPs also introduce an additional level of regulation as PKA activity is inhibited when bound to AKAP.

### **PKA – Role in Cancer**

PRKAR1A, the gene coding for the  $1\alpha$  regulatory (R $1\alpha$ ) subunit of PKA, has already been shown to have a role in cancer, with mutations detected in multiple endocrine neoplasias as well as in Primary pigmented micronodular adrenal disease (PPNAD)



and the Carney complex. Decreased expression of PRKAR1A leads to an increase in the level of 'free' PKA-cat, thus leading to higher activity.

### **PRKAR1A; Carney Complex**

Carney complex (CNC) is a multiple neoplasia tumour predisposition syndrome that is inherited in an autosomal dominant manner. It is characterised by schwannomas, endocrine neoplasms, breast ductal carcinomas and several types of skin, liver, cardiac, and neural myxomatous tumours (Carney, Gordon et al. 1985; Stratakis, Kirschner et al. 2001).

While it is an autosomal dominant disease, CNC can occur as sporadic germline mutations. Two distinct loci have been identified for CNC that show no phenotypic differences when mutated, CNC1 & CNC2. While the gene coded for by CNC2 (2p16) is unknown, PRKAR1A has been shown to map to CNC1 (17q23-24) and is often mutated/inactivated in CNC patients, confirming its role (Kirschner, Carney et al. 2000; Kirschner, Sandrini et al. 2000). In this study by Kirschner *et al* (Kirschner, Carney et al. 2000), 22 out of 54 patients (40.7%) presented with PRKAR1A mutations.

There are many mutations identified in PRKAR1 $\alpha$  in this disease, the majority of which lead to a premature stop codon due to non-sense mediated mRNA decay (Kirschner, Sandrini et al. 2000; Robinson-White, Hundley et al. 2003). Altered PRKAR1A activity, or haploinsufficiency, are each sufficient to increase PKA activity, which predisposes to tumourigenesis (Kirschner, Carney et al. 2000; Kirschner, Kusewitt et al. 2005).

### **Involvement of PDE mutations**

Some cases with similar adrenal phenotype to CNC show PDE mutations, although these are not associated with non-adrenal features of CNC. A PDE mutation (decrease/ablation) would lead to increase cAMP levels, thereby increasing PKA pathway activity. This reinforces the involvement of the PKA pathway in this disease (Almeida and Stratakis 2010).

## Other PRKAR1A linked diseases

### McCune-Albright syndrome

McCune-Albright syndrome (MAS) is a sporadic condition resulting from *GNAS* (*guanine nucleotide binding protein alpha stimulating*) activating mutations and involving multiple endocrine and non-endocrine tumours. It shares skin abnormalities and some non-endocrine tumours with the lentiginoses of CNC. Although this is not PKA itself, *GNAS1* is the gene coding for G $\alpha$  subunit which is upstream of the PKA pathway. *GNAS* activating mutations lead to constitutive stimulation of adenylate cyclase and PKA activation and a variety of manifestations, including the classic triad of polyostotic fibrous dysplasia, café au lait skin pigmentation, and autonomous endocrine hyperfunction (Weinstein, Shenker et al. 1991). The most frequent affected endocrine tissues are pituitary, ovarian and thyroid, but bilateral macronodular adrenocortical hyperplasia can also be found in the context of MAS (Lee, Van Dop et al. 1986; Stratakis and Kirschner 1998).

Genetic defects in cAMP-binding PDEs have been described in isolated micronodular adrenocortical hyperplasia (MAH) (Stratakis 2009), significantly affecting the ability of PDEs to degrade cAMP *in vitro* (Horvath, Boikos et al. 2006; Horvath, Giatzakis et al. 2006; Horvath, Mericq et al. 2008).

### Papillary thyroid carcinoma

Papillary thyroid carcinoma is a relatively common thyroid cancer with evidence that it is caused by a number of genetic changes (OMIM 188550). Chimeric oncogenes form due to the fusion of the tyrosine kinase domain of the RET proto-oncogene to the 5-prime terminal region of PKA-R1 $\alpha$ . This has been seen in both familial and sporadic cases (Salabe 2001).

### Acute promyelocytic leukemia (APL)

APL is a malignancy of the blood and bone marrow characterised by a translocation involving the retinoic acid receptor (RARA). The PRKAR1A/RARA fusion gene is formed by the insertion of RARA distal to PRKAR1A and the deletion of 3-prime PRKAR1A, 5-prime RARA and any intervening sequences. Specifically the fusion of the R1-alpha dimerization domain to RARA may be involved in deregulation of PKA (Catalano, Dawson et al. 2007).

## Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disorder characterised by diverse dysfunctions of immune effector cells, including proliferation and cytotoxicity. PKA activity in T cells is dramatically reduced because of decreased expression of the  $\alpha$  and  $\beta$  Reg subunits. Transcript mutations (deletions, transitions, and transversions) are found in *PRKAR1A* at a frequency 7.5 times higher in SLE patients than in control T cells. No genomic mutations were identified (Laxminarayana, Khan et al. 2002).

## GPCR and G-proteins

GPCR and G-proteins have also been shown to have a role in cancer (e.g. MAS, the first GPCR identified as having oncogenic potential (Young, Waitches et al. 1986)), with activating mutations leading to ovarian, adrenal, pituitary and thyroid adenomas and carcinomas, once again highlighting the importance of this pathway.

## PRKAR1A Mouse model

*PRKAR1A* haploinsufficient mice, carrying a floxed copy of exon 2 of the murine *PRKAR1A* gene, led to the development of tumours arising in cAMP-responsive tissues, such as the bone, Schwann and thyroid follicular cells. *PRKAR1A*<sup>+/-</sup> mice developed non-pigmented schwannomas and fibro-osseous bone lesions beginning around 6 months of age. Benign and malignant thyroid neoplasias were observed in 10% of older mice (Kirschner, Kusewitt et al. 2005). Although the spectrum of tumours overlapped with what is seen in CNC patients, this mouse model did not present some of the most frequent CNC tumours, such as skin and heart myxomas and pituitary adenomas (Kirschner, Kusewitt et al. 2005).

An alternative mouse model carrying an antisense transgene for *PRKAR1A* showed approximately 50% decrease in PKA-R1 $\alpha$  protein levels similar to haploinsufficiency and consequently increased cAMP signalling. It also developed similar phenotype to CNC patients, further supporting the role of *PRKAR1A* in this disease (Griffin, Kirschner et al. 2004; Griffin, Kirschner et al. 2004). Mice developed thyroid follicular hyperplasia and adenomas, adrenocortical hyperplasia, hypercorticosterolemia, late-onset weight gain, visceral adiposity, lymphomas,

sarcomas and mesenchymal tumours. The thyroid and adrenocortical tumours showed loss of heterozygosity at the *PRKAR1A* locus (Griffin, Kirschner et al. 2004).

Adrenal-cortex specific *PRKAR1A* knockout mice developed autonomous adrenal hyper-activity and bilateral hyperplasia which are both observed in human PPNAD which is caused by proliferation of cortisol-producing foetal adrenocortical cells induced by PKA dysregulation (Sahut-Barnola, de Jossineau et al. 2010).

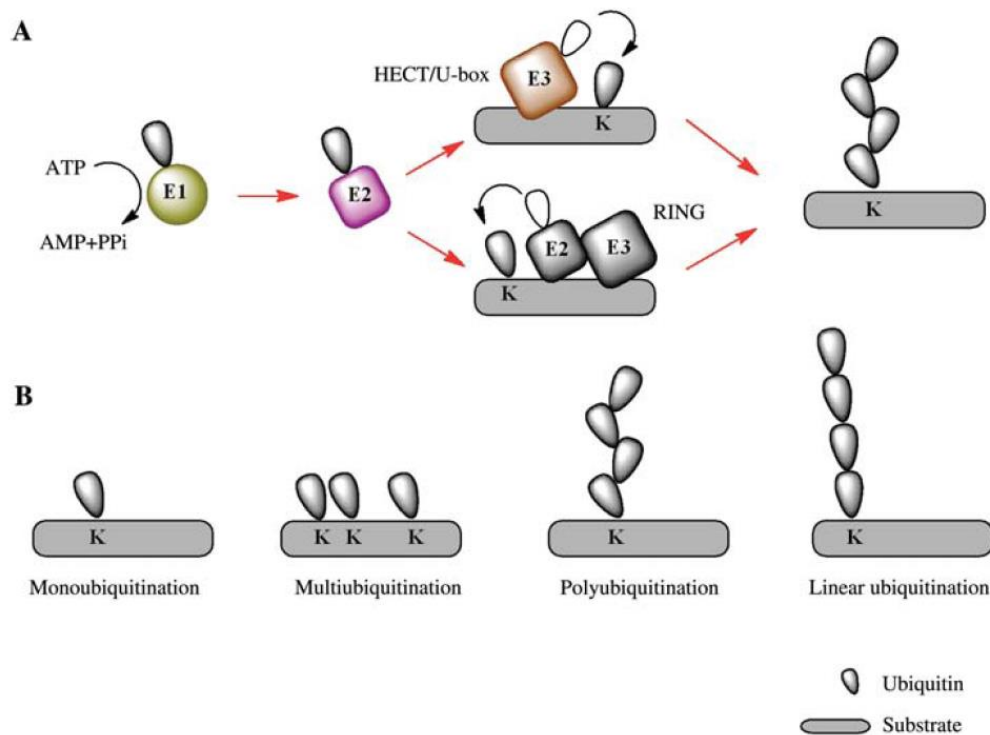
## Ubiquitination

Ubiquitination is one of the most utilised posttranslational modifications in eukaryotes and is involved in a wide range of cellular processes. Ubiquitin (Ub) alters the longevity, localisation, and/or activity of proteins via one or more covalent bonds between the C-terminal carboxyl group of ubiquitin and a nucleophilic moiety on the substrate/target protein, but is mostly known as a signal for proteasomal degradation. The ubiquitin-proteasome pathway is the principal pathway for intracellular protein degradation (Goldberg, Stein et al. 1995; Coux, Tanaka et al. 1996; King, Deshaies et al. 1996). Protein substrates are 'marked' with a poly-ubiquitin chain (Chau, Tobias et al. 1989) and then degraded to peptides and free ubiquitin by a large multimeric protease, the proteasome, which exists within all eukaryotic cells (Goldberg, Stein et al. 1995; Coux, Tanaka et al. 1996; King, Deshaies et al. 1996).

The covalent conjugation of Ub molecules onto target proteins (ubiquitination) is mediated by the action of the three enzymes E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases that together mediate the transfer of the 76-amino acid protein ubiquitin to specific lysine (K) residues on target proteins (Hershko and Ciechanover 1998; Leithe and Rivedal 2007; Willis, Townley-Tilson et al. 2010).

E1 and E2 function sequentially, E1 activating enzyme creates a thioester bond between its conserved cysteine residue and the C-terminal carboxyl group of monomeric Ub in an ATP-dependent manner (Figure 16A) (Haas and Rose 1982). The activated Ub is passed to a reactive cysteine residue of a specific E2 conjugating enzyme (E2~Ub), recruited by the C-terminal ubiquitin-fold domain of E1 (Ye and

Rape 2009). Together with an E3 ligation enzyme, E2 conjugates Ubs to either the N-terminus or internal lysine residues of targeted proteins. The E3 ligase catalyses the transfer of one Ub molecule at a time or a Ub chain to a protein target (Hochstrasser 2006). There are three types of E3 ligases; HECT (homologous to the E6-AP carboxyl terminus)-type of E3 ligases transfer activated Ub to the E3 cysteine residue and form a transient thioester intermediate between Ub and the E3, before this is ligated onto targeted proteins (Fang and Weissman 2004). RING (Really Interesting New Gene) and U-box E3 ligases do not seem to possess catalytic function and act as scaffolds for the conjugation reaction by bridging the E2~Ub and its substrate.



**Figure 16: Ubiquitination** Schematic model of sequential action of E1, E2, and E3 enzymes to create various ubiquitin tags onto their targets. (A) In an ATP-dependent manner E1 activates a ubiquitin molecule which is then passed onto the reactive cysteine residue of an E2 conjugating enzyme. E2 either passes the ubiquitin to the reactive cysteine on a HECT or U-box-type E3 enzyme to be ligated onto the target, or it cooperates with a RING-box E3 enzyme for substrate selectivity and directly adds the ubiquitin to the target itself. (B) A schematic representation of proteins that contain either mono-, multi-, or polyubiquitin tags, all resulting in different fates. Figure from (Nagy and Dikic 2010).

Ubiquitin has seven lysine residues that may serve as points of ubiquitination: K48, K63, K6, K11, K27, K29 and K33 resulting in different tertiary structures (Peng,

Schwartz et al. 2003; Pickart and Fushman 2004; Xu and Peng 2008), in addition chains can assemble on the N-terminus of a ubiquitin molecule ("linear chains") (Kirisako, Kamei et al. 2006). Following the addition of a single ubiquitin moiety to a protein substrate (mono-ubiquitination), further ubiquitin molecules can be added to the first yielding a poly-ubiquitin chain. In addition, some substrates are modified by addition of ubiquitin molecules to multiple lysine residues in a process termed multi-ubiquitination. Mono-, multi-, or poly-ubiquitination of substrate proteins are molecular signatures recognised and sorted by a diverse set of proteins containing ubiquitin binding domains (Figure 16B; (Dikic, Wakatsuki et al. 2009)). Addition of a single Ub molecule to one (mono-ubiquitination) or several (multi-ubiquitination) acceptor lysine residues of proteins causes subcellular localisation, activity, tertiary structure formation or interaction with other proteins respectively (Figure 16B). Ub binding domain-containing effector molecules discriminate poly-ubiquitin chains primarily by their tertiary structures (Komander, Reyes-Turcu et al. 2009; Xu, Duong et al. 2009).

One example is the K63 linkage, which is known to be involved in DNA damage recognition of DNA double-strand breaks. The K63 linkage is placed on the H2AX histone by the E2/E3 ligase pair, Ubc13-Mms2/RNF168. This K63 chain recruits RAP80, which contains a UIM, and RAP80 then helps localise BRCA1. This pathway will eventually recruit the necessary proteins for Homologous Recombination Repair.

Ubiquitin polymerisation occurs as its K residues serve as acceptor sites for additional ubiquitin molecules. Multiple ubiquitins can be covalently linked via K48 to form poly-ubiquitin chains, which target the ubiquitinated protein for degradation by the multi-subunit 26S proteasome (Voges, Zwickl et al. 1999; Leithe and Rivedal 2007; Willis, Townley-Tilson et al. 2010). Role of K63 chains in DNA repair and kinase activation in mammalian cells (Peng, Schwartz et al. 2003). K63 chains are also implicated in vacuolar targeting (Lauwers, Erpapazoglou et al. 2010). Poly-ubiquitination via K48 ubiquitin chains typically leads to proteasome-dependent degradation of cytosolic proteins. Endoplasmic reticulum (ER)-retained proteins are degraded in a proteasome-dependent fashion via ER associated degradation. Plasma membrane-resident receptors and transporters tend to be

vacuolar protease substrates following internalisation and sorting into multi-vesicular bodies. Multi-vesicular bodies assembly and maturation depend on the endosomal sorting complex required for transport assemblies (Katzmann, Babst et al. 2001; Babst, Katzmann et al. 2002), which are complexes that recognise proteins destined for the vacuole by binding ubiquitin (Shields, Oestreich et al. 2009). While mono-ubiquitination is usually sufficient for internalisation, multi-ubiquitination and K63 poly-ubiquitination enhance endocytic rates. Several plasma membrane-resident transmembrane proteins have been shown to require K63 poly-ubiquitination for endosomal sorting complex required for transport -dependent sorting into multi-vesicular bodies (Lauwers, Erpapazoglou et al. 2010).

In addition to families of E2 and E3 enzymes, eukaryotes express proteins with ubiquitin-deconjugating and ubiquitin-binding abilities. The former are deubiquitylating proteases that uniquely recognise and cleave the bond linking the two moieties, removing ubiquitin (D'Andrea and Pellman 1998), while the latter interact with different types of ubiquitinated proteins (Andersen, Hofmann et al. 2005). Ubiquitin-binding proteins can mediate interaction between ubiquitin-modified targets and the proteasome (Fu, Lin et al. 2010), shuttle ubiquitinated proteins from one compartment to another, or serve to modulate the activities of others in the same complex (Hicke and Dunn 2003). Alternative fates of ubiquitinated targets include proteolysis in the lytic compartment of the vacuole or the central cavity of the proteasome, nonproteolytic inhibition, and even protein activation.

The presence of membrane-localised E2 and E3 enzymes suggests that ubiquitination occurs near membrane surfaces.

A wide variety of E3 ligases have been identified and many of those are associated with diseases (Jiang and Beaudet 2004). Furthermore, muscle-specific E3 ligases have been shown to participate in muscle atrophy (Attaix, Ventadour et al. 2005).

### **Ubiquitin-like modifiers**

There are several ubiquitin-like proteins that modify cellular targets in a similar, but distinct, pathway to ubiquitin. Known Ub-like proteins include RUB (related to

ubiquitin), SUMO (small ubiquitin-like modifier), ATG8 and ATG12 (autophagy 8 and 12 respectively), MUB (membrane-anchored ubiquitin-fold protein), UFM1 (ubiquitin-fold modifier1), URM1 (ubiquitin-related modifier 1), HUB1 (homology to ubiquitin1), amongst others (Miura, Jin et al. 2007; Hochstrasser 2009; Vierstra 2009; Li and Vierstra 2012).

Ub and most Ub-like modifiers enter the same E1→E2→E3 reaction cascade that involves a signature high-energy thioester intermediate. Like Ub, conjugation of most Ub-like modifiers is transient and can be reversed by unique proteases that release the isopeptide-linked UBL moiety (Kerscher, Felberbaum et al. 2006). These “deconjugases” are highly specific for the corresponding tag and fail to recognise or poorly recognise even closely related members (e.g. Ub and RUB).

### **SUMOylation**

SUMOylation is the covalent linkage to the small ubiquitin-like modifier (SUMO) family of proteins. SUMOylation acts in most cellular compartments to adapt proteins in multiple processes including transcription, translation, cellular transport, protein interactions, cell growth and programmed cell death (Geiss-Friedlander and Melchior 2007).

Three SUMO isoforms have been identified, SUMO1, 2, and 3. SUMO is distantly related to ubiquitin (20% identity) and differs from other Ub-like modifiers by a long N-terminal extension that appears unstructured in solution. Desumoylating proteases not only disassemble SUMO conjugates but are needed to generate mature SUMO by trimming extra amino acids beyond the C-terminal di-Gly. Whereas most targets bear a single SUMO moiety, some can be modified with multiple SUMOs or poly-SUMO chains via iterative cycles of conjugation analogous to ubiquitylation (Miller, Barrett-Wilt et al. 2010; Wilkinson and Henley 2010). Based on alignments of known SUMO attachment sites, sumoylation appears to employ several loose consensus motifs; one prevalent in plants, yeast, and mammals encompasses a  $\Psi$ KXE motif (where  $\Psi$  is a large hydrophobic residue and K is the Lys where SUMO is linked) and two others identified thus far in mammals contain extended motifs rich in phosphorylated or negatively charged residues (Miller, Barrett-Wilt et al. 2010; Wilkinson and Henley 2010).



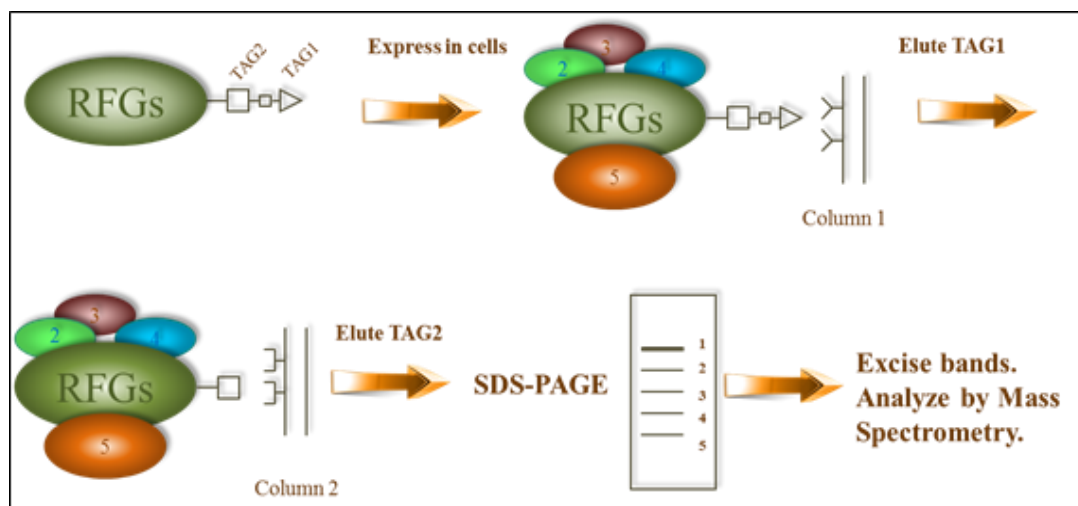
The functions of sumoylation are diverse and mostly nonproteolytic and include controls on localisation, interaction, and activity of the modified protein (Miura, Jin et al. 2007; Wilkinson and Henley 2010). One unique situation has also been reported in mammalian cells, where SUMO addition protects the I $\kappa$ B $\alpha$  protein from Ub addition by blocking accessible Lys residues (Desterro, Rodriguez et al. 1998). Some of these effects are mediated by a collection of binding partners bearing SUMO-interacting motifs.

## Research principle

The aim of this project has been to identify and validate novel effectors of RAP2, in the first instance, and NOEY2, in the second instance, in order to elucidate its function, both under normal circumstances and in disease.

## Tandem Affinity Purification

Tandem affinity purification (TAP) is an unbiased procedure for protein purification and proteome exploration in order to detect novel protein-protein interactions. After two sequential affinity column purifications using mild washing and eluting conditions, proteins co-purifying with the tagged-bait are identified by mass spectrometry. An advantage of this method is the detection of novel protein partners *in vivo* without prior knowledge of complex composition.



**Figure 17: Tandem Affinity Purification was used to pull down potential targets of RAS family members.** Using overexpressed TAP-tagged proteins, two rounds of purification, using His and Strep beads, were used in order to identify potential interactions.

Established by a research team working in the European Molecular Biology Laboratory (EMBL) in the late 1990s (Rigaut, Shevchenko et al. 1999; Puig, Caspary et al. 2001) it is used as a tool for proteome exploration and has facilitated the characterisation of several protein complexes (Caspary, Shevchenko et al. 1999; Rigaut, Shevchenko et al. 1999; Bouveret, Rigaut et al. 2000; Puig, Caspary et al. 2001). The first large-scale application of this technique was in 2002, where it was used to develop a visual map of the interaction of more than 230 multi-protein complexes in a yeast cell by systematically tagging the TAP tag to each protein<sup>iv</sup>.

Using this technique, identified targets were then validated and investigated as described below.

# CHAPTER 2: Materials and Methods

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## MATERIALS

### Reagents

Low melting point agarose (15517022), Kanamycin (11815-024), Bromophenol blue, newborn Bovine serum (16010-159), PCR primers, foetal bovine serum (FBS), Trypsin (25300054), D-MEM (41966029) and Opti-MEM (11058021) were from Invitrogen (Paisley, UK). Complete, EDTA-free Protease inhibitors were obtained from Roche (Welwyn Garden City, UK). Skimmed Milk powder was from Tesco. LB agar, Triton-X100, Polybrene hexadimethrine bromide, Coomassie Brilliant blue, Phalloidin (P1951), Hoechst stain solution (H6024-10ML), PEI 1mg/ml, Paraformaldehyde (PFA), Glycine and Flag beads were obtained from Sigma Aldrich (Haverhill, UK). Amersham Hybond Polyvinylidene difluoride (PVDF) membranes, autoradiography Fuji Film, Glutathione sepharose 4FF beads (17-5132-02), Protein A Sepharose 4 fast flow beads (17-5280-01), Protein G Sepharose 4 Fast flow beads (17-0618-01) and Streptavidin Sepharose high performance beads (17-5113-01) was purchased from GE Healthcare (Buckinghamshire, UK). The LumiGlo/LumiGlo reserve chemiluminescence detection reagents were purchased from Insight Biotechnology Limited (Middlesex, UK). PfuUltra™ high-fidelity DNA polymerase and 10× PfuUltra™ HF reaction buffer from Stratagene (CA, USA). Image clone, p8.91, pMDG and shRNA were ordered from Open Biosystems (MA, USA). Human fetal brain Poly A+ RNA and Human placental pLIB cDNA library (PT3230.1) were from Clontech (St-Germain-en-Laye, France). Methanol and coverslips (diameter: 13mm for 24 wells, Cat.-No. 631-0149) were from VWR (Leicestershire, UK). Mounting solution (S3023) from Dako Cytomation Ltd (Cambridgeshire, UK). MG-132 from Calbiochem (Nottingham, UK). Magnesium and Calcium free PBS tablets (BR014G) were from Fisher Scientific (Loughborough, UK). FACS tubes (2053-001, polypropylene, 5.4ml) were from Elkay.

Primary antibodies against AF6 (H-106), BRAF (F7), HA (F-7), HA (Y-11), PP1c (E9), RGL2 (F-30.1), RGL2 (H-120), Trim32 (H-204), Ubiquitin-HRP (P4D1), were from Santa Cruz Biotechnology (Heidelberg, Germany). AF6 (35/AF6), RAP1 (clone 3), RAP2 (12/RAP2) antibodies were from BD Transduction Laboratories (Oxford, UK). Antibody against FLAG (M2) was obtained from Sigma-Aldrich (Steinheim, Germany). MINK (A302-191A), NIK/MAP4K4 (A301-502A) and TNIK (A302-695A) antibodies were from Bethyl Laboratories (Cambridge, UK). TRIM32 (10326-1-AP) from Protein Technology (Manchester, UK). TRIM32 (ab50555) from Abcam (Cambridge, UK).

Anti-mouse IgG, peroxidase-linked species-specific whole antibody, from sheep (NA 931) and Anti-rabbit IgG, peroxidase-linked species-specific whole antibody, from donkey (NA 934) from Amersham. Goat Anti-Mouse-HRP, Light Chain Specific (115-035-174), Mouse mAb Anti-Rabbit-HRP, Light Chain Specific (211-032-171), Goat Anti-Rabbit-HRP, Fc Fragment Specific (111-035-046), Rabbit Anti-Mouse-HRP, Fc Fragment Specific (315-035-046) from Stratech Scientific.

## Buffers and solutions

Reagent	Recipe
<u>General Buffers and solutions</u>	
<b>0.5M EDTA pH 8.0</b> (Ethylenediaminetetra-acetic acid)	146.13g EDTA in total of 1L ddH <sub>2</sub> O adjusted to pH 8.0 with NaOH
<b>1 x PBS</b>	137mM NaCl, 2.7mM KCl, 10.1mM Na <sub>2</sub> HPO <sub>4</sub> *H <sub>2</sub> O, 1.76mM KH <sub>2</sub> PO <sub>4</sub> , 1mM CaCl <sub>2</sub> , 0.5mM MgCl <sub>2</sub> , pH 7.4 with HCl
<b>PBS-T buffer</b>	10 x PBS, 0.1% Tween20
<b>PBS-E lysis buffer</b>	1 x PBS, 1% Triton-X100, 1mM EDTA (1mM DTT, protease and Phosphatase inhibitor cocktail added for lysis)

**PBS-M lysis buffer** 1 x PBS, 1% Triton-X100, 5mM MgCl<sub>2</sub> (1mM DTT, protease and Phosphatase inhibitor cocktail added for lysis)

**1M Tris pH8** 121.1g Tris in total of 1l ddH<sub>2</sub>O

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### Western Blotting

**1° Antibody buffer** 3% BSA, PBS-T, 0.02% NaN<sub>3</sub>

**2° Antibody buffer/Blocking solution** 5% Milk/PBS-T

**NuPAGE MOPS Running buffer** 209.2g MOPS (free acid); 121.2g Tris, pH 8.8; 0.2% SDS; 6g EDTA

**NaN<sub>3</sub> (Sodium Azide)** 10% (w/v) NaN<sub>3</sub>, 500x stock solution

**20x Transfer buffer** 80.16g Bicine; 104.6g Bis-Tris (free acid); 6g EDTA  
(add 10% MeOH when making 1x)

**Phosphatase inhibitor cocktail** 10mM Sodium Fluoride; 2mM Sodium Orthovanadate; 2mM Sodium Pyrophosphate, decahydrate; 2mM beta-glycerophosphate

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### FACS

**Staining Buffer** 50µg/ml PI, 100µg/ml RNase (DNase free), PBS + 0.05 % Triton-X100

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**Table 4: List of buffers and reagents**

## Kits used

<b>Kit</b>	<b>Company</b>
<b>PrimeScript One Step RT-PCR kit Ver.2</b>	TaKaRa
<b>SuperScript One-Step RT-PCR for long templates</b>	Invitrogen
<b>QIAquick gel extraction kit</b>	QIAGEN
<b>QIAquick PCR purification kit</b>	QIAGEN
<b>GATEWAY Cloning technology</b>	Life Technologies
<b>QIAprep Spin Miniprep kit</b>	QIAGEN
<b>PureLink HiPure Plasmid Midiprep kit</b>	Invitrogen

**Table 5: Kits used**

## Mammalian cell culture

All media are stored at 4°C and used within 1 month.

Cell type	Tissue of origin	Media	Additives
<b>C2C12</b>	Mouse myoblast	DMEM (Dulbecco's Modified Media)	<i>Growth medium:</i> 20% FBS <i>Fusion medium:</i> 2% HS
<b>HEK293T</b>	Human Embryonic Kidney	DMEM	10% FBS
<b>HMLE</b>	Immortalised human mammary epithelial cells	DMEM F12	5% HS, 20ng/ml EGF, 0.5µg/ml hydrocortisone, 100ng/ml cholera toxin, 10µg/ml insulin, 2mM L-glutamine
<b>U2OS</b>	Osteosarcoma	DMEM	10% FBS

**Table 6: Mammalian cell culture**

## METHODS

### Generation of constructs

#### *Polymerase chain reaction*

Human placental cDNA and image clones were used as a template for PCR of various genes, using Pfu Ultra High-Fidelity DNA polymerase enzyme. Samples were then run on 1.5% gel: TAE/agarose gel using 1 x sample loading buffer, with a 1kb ladder to allow the estimation of their size. This was compared to their literature values in order to ascertain whether the correct size fragment had been obtained.

For those samples that did not work on PCR from a cDNA library, RT-PCR was done on human foetal brain Poly A+ RNA (from Clontech). The “Invitrogen SuperScript One-Step RT-PCR for long templates” and TAKARA “PrimeScript One Step RT-PCR kit Ver.2” kit were used.

### *Extraction*

PCR products were purified following the QIAquick PCR purification kit as per manufacturer’s instructions. Then samples were digested with restriction enzymes, and then run on a wide combed, 1.5% agarose/TAE gel stained with GelSTAR. These were then cut using a scalpel, and placed in individual microcentrifuge tubes. Using the QIAquick gel extraction kit, these were then gel purified as per the manufacturer’s instructions.

### *Ligation*

Digested inserts were ligated into pENTR vector (cut with corresponding restriction enzymes) using T4 DNA ligase, overnight at 4°C.

### *Transformation*

2.5µl of the ligation mix was then added to 25µl DH5α sub-cloning efficiency competent cells from Invitrogen. These were then left on ice for 30 mins in order to allow the ligation mix to bind to the outside of the cells. Cells were permeabilised by heat shock at 42°C for 45 sec, allowing the vectors to enter, and placed on ice for a further 2 min in order to reduce permeability of the cells. 300µl of room temperature LB broth was then added to each sample, and incubated for 1h at 37°C, in order to allow the cells to recover from the shock and for the antibody resistance, coded for by the vector, to be expressed by the cells. The samples were then plated on Kanamycin LB plates, and incubated overnight at 37°C.

Colonies were picked, plasmid DNA was extracted using Qiagen spin Miniprep kits, and constructs were sequenced to confirm insertion of the correct gene. Miniprep DNA was combined with the required destination vector (e.g. DNA3-FLAG, myc, DEST-27 (GST), pLEX-, etc.) and clonase II mix was added to perform recombination reaction. Reaction was transformed and plated onto Ampicillin LB plates, from which colonies were picked and DNA was isolated.



## Cell Culture

For experimental purposes all cell lines were used at the lowest possible passage number. The cell lines used are adherent cells, cultured in a humidified atmosphere at 37°C and 5% CO<sub>2</sub>. Cells were grown to approximately 90% confluence before passaging. The media was aspirated and the cells were washed with PBS solution and incubated with 0.05% Trypsin/EDTA (1ml/10cm or 2ml/15cm dish) at room temperature for 2-3 minutes to detach. Pre-warmed cell culture media was added to inactivate the trypsin and cell clumps were disrupted through gentle pipetting. The suspension was then diluted and cells re-plated as required. C2C12 cells were differentiated by growing to ~80% confluence and replacing 20% FBS growth medium with 2% HS fusion medium.

## DNA Transfection

Cells were transfected on the same day as seeding. For a single transfection assay,  $1.3 \times 10^6$  cells/well (6wp) or  $10 \times 10^6$  cells/10cm plate were transfected with 2µg or 8µg of plasmid DNA respectively in 200 µL Opti-MEM (Gibco) with 8µg or 32µg polyethylenimine (PEI, stock solution 1mg/ml). After 30 min incubation at RT, the transfection solution was then added to the cells and then the media was changed after 4-6h. Next day, 2mL of media was added to each well for boosting cells and cells were harvested the following day.

For co-transfection assays, equal amounts of indicated plasmids were used, excluding ubiquitination assays for which HA-tagged ubiquitin was transfected at a higher ratio of 2:1:1 (w:w).

## Lentivirus generation

In order to generate lentivirus,  $3 \times 10^6$  HEK-293T cells were seeded on 6cm dishes in 3ml media. After allowing the cells to settle for 3h, they were transfected with the lentiviral plasmid, using p8.91 (0.98µg/µl), pMDG (0.5µg/µl), lentiviral construct (0.5µg/µl) and PEI 1mg/ml. Medium was replaced 4-6h later and again 24h later. 48h after transfection, supernatant containing virus was aliquoted into Polybrene to a final concentration of 10µg/ml and snap frozen (and stored at -80°C). Media was replaced and supernatant collected after a further 24 and 48h.

## **Lentiviral Infection**

Cells were seeded into 6wp and allowed to grow overnight to 50% confluence. Next day, frozen virus stocks in 1ml aliquots including 5 µg/ml Polybrene were thawed at 37°C and then spun for 1 min at 16000 rpm at 4°C in order to pellet residual cells in the supernatant. Then cell medium was replaced by 1ml of the virus/polybrene solution. After 4h of virus supernatant incubation, the virus supernatant was removed and replaced with 2ml of growth medium for 24h before adding the appropriate selective agent.

## **Protein extraction**

Proteins were isolated using 350µl/6wp or 1ml/10cm PBS-M lysis buffer or PBS-E lysis buffer for interaction and ubiquitination assays, respectively. Lysates were cleared of insoluble debris by centrifugation at 16,000 x *g* for 10 mins at 4°C.

## **Immunoprecipitation/pull-down**

Proteins were purified using the corresponding beads: GST-glutathione beads (Amersham), Flag-Flag beads (Sigma). Lysates were rotated with the beads for 2h at 4°C. These were washed x 3 with lysis buffer without protease or phosphatase inhibitors and drained with a needle. Samples were resuspended in 20µl (6wp) or 30µl (10cm) LDS.

## **SDS-polyacrylamide gel electrophoresis**

Samples were heated at 70°C for 10min to denature the proteins and an aliquot of the cell lysate (2µl) or immunoprecipitate 10µl (6wp) or 15µl (10cm) were loaded as specified onto the gel alongside a Precision Plus Protein Prestained Standards (BioRad). Proteins were separated using a 4-12% Bis/Tris gradient gel (Invitrogen). Gels were run using MOPS running buffer at 150V until the dye reached the bottom of the gel. The proteins were transferred to a methanol-activated polyvinylidene fluoride membrane (PVDF) membrane using Transfer buffer supplemented with 10% methanol (v/v) at 90 V for 90 min at 4°C using an BioRad Transfer system.

## **Western Blot**

Membranes were stained with Coomassie (0.2% (w/v) Brilliant Blue Reagent (Sigma), 10% acetic acid (BDH), 50% methanol (BDH)) and blocked in PBS-T containing 5% skimmed milk prior to incubation with antibodies. Primary antibodies

were diluted in 3% BSA/PBS-T supplemented with 0.02% NaN<sub>3</sub> at 4 °C overnight. Membranes were washed three times with PBS-T and incubated for 1h at room temperature with secondary antibody, followed by washing three times with PBS-T. Binding of each antibody was detected using Lumiglow and Lumiglow reserve. The emitted fluorescence was detected using Photography film (Fujifilm) on SRX-101A x-ray developer.

### **Tandem Affinity Purification (TAP)**

TAP was performed from 10x15cm dishes of various cell lines (as specified) transiently transfected with TAP6-tagged constructs, as shown. The TAP6-tag contains streptactin-histidine-SBP and Flag tags. All steps were performed at 4°C. Cells were lysed with PBS-E lysis buffer: 1 x PBS, 1mM EDTA, 1% w/v Triton-X100, 1mM DTT, Protease inhibitor cocktail (Roche) and Phosphatase Inhibitor solution (Sigma). Clarified pooled lysates were incubated with Streptavidine Sepharose High Performance beads (GE Healthcare) and washed extensively with PBS containing 0.1% TX-100. Bound proteins were eluted with PBS / 0.1% TX-100 / 2mM Biotin and the eluate incubated with TALON Metal Affinity Resin (Clontech). After washing, bound proteins were eluted with 200mM Imidazole, concentrated by TCA precipitation, separated on 4-12% NuPAGE gels (Invitrogen) and stained with Simply Blue SafeStain (Invitrogen). Samples were then sent off to collaborators in San Francisco for Reversed-phase liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS) analysis.

### **Cell Cycle Analysis**

Two days after retroviral infection with YFP-tagged protein, cells were spun at 400xg for 4min and supernatant was removed and pellet was washed once with PBS. After removing the supernatant once again, the pellet was re-suspended in remaining PBS and fixed in cold 70% EtOH. Samples were then stored at 4°C overnight to fix. Samples were spun at 800xg for 4min and re-suspended in 750µl of staining buffer (50µg/ml PI (Sigma), 100µg/ml RNase (DNase free, Sigma), 0.05 % Triton X-100). Cells were treated with Ribonuclease to ensure that only DNA was stained. Cells were then incubated in the dark for 30min at RT, prior to FACS analysis using a CyAn ADP (Becton Coulter). Debris and cell doublets were excluded and the PI staining intensity was recorded as a measure of DNA content. For each

sample at least 10,000 events were acquired and data analysis was performed using Summit software. The cell cycle of YFP-positive cells is shown, no changes were seen in the YFP-negative population of cells.

### **Colony Formation Assay**

Cells infected with lentivirus overexpressed proteins were counted and seeded in 10cm plates at the same density. Cells were allowed to grow until the control reached ~90% confluence and then stained with crystal violet dye (Sigma; prepared as 0.5% solution in a 10% methanol/water mix).

### **Proliferation assay**

Cells infected with lentivirus overexpressed proteins were counted manually and seeded in 24 well plates at various densities, depending on the cell line and rate of proliferation. They were then placed in the IncuCyte (Essen Instruments) and well density was measured every 2h until dense. Medium was changed regularly (every 48h) between readings. Readings were analysed using Excel.

### **Wound healing assay**

Cells were seeded in quadruplicate in 24 well plates (Imagelock plates distributed by Essen Instruments), grown to a confluent cell monolayer and wounded by a woundmaker (Essen Instruments) using 10  $\mu$ L sterile tips. The cells were then washed with PBS twice and 500  $\mu$ L media containing 10% FBS added. Plates were placed in the Essen Instruments IncuCyte and the wound area was monitored over time. Pictures were automatically taken every 2h for up to 2 days. Wound healing data was analysed by tracking the confluency (cell repopulation of the wound in %) over a period of time and plotting a graph over time by IncuCyte software version 2010A.

### **Localisation**

Cells were seeded on coverslips and allowed to attach. Cell monolayers were washed once with PBS and fixed with 4% PFA for 15 min at RT (250 $\mu$ L per well). PFA was aspirated and cells were washed 3 x 3 min with PBS before permeabilising with 0.1% Triton-X100 for 10 min at room temperature. The Triton-X100 was aspirated and coverslips washed (3 x 3 min with PBS) and transferred to a wet chamber. Cells were stained with Hoechst (1:10000) and Phalloidin (1:2000) diluted in 2% BSA/PBS

for 10 min at RT to visualise the nucleus and actin cytoskeleton, respectively, where indicated. After 3 x 5 min wash with PBS, salts were removed by dipping each coverslip into H<sub>2</sub>O. Coverslips were dried and gently dropped onto the mounting solution (Dako Fluorescent mounting medium, S3023, Lot 10022881) that had been placed on slides.

# Chapter 3: Validation of the TAP interaction

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## **TAP Identified Novel Targets of RAP2**

Tandem affinity purification (TAP) is an unbiased approach that allows high throughput identification of protein interactions. In order to identify novel interacting proteins of RAP2 that may shed light on its function, Dr Rodriguez-Viciano had performed three independent TAP purifications using the TAP-tagged constitutively active V12 mutant of RAP2. Initially, TAP-RAP2 V12 was stably expressed by retroviral infection in A549 and HEK-293 cells. After purification and elution only 2 and 3 proteins respectively were identified by mass spectrometry as co-purifying specifically with RAP2 but not other control baits (Table 7). The E3 ubiquitin ligase TRIM32 was the only protein that was identified in both purifications. RAP1GDS/SmgGDS is known to interact in a GTP-independent manner with multiple RAS family GTPases (Riess, Epplen et al. 1993). RADIL is a RA-domain containing protein that was subsequently shown to interact with RAP proteins (Smolen, Schott et al. 2007; Ahmed, Daulat et al. 2010). TBC1D10B functions as a GAP for several members of the Rab family (Ishibashi, Kanno et al. 2009).

A third TAP purification was performed at a later time by transiently transfecting TAP-RAP2 V12 in HEK-293T cells. The higher levels of expression achieved by this approach coupled with the higher sensitivity of the mass spectrometry facilities used by our collaborators to analyse these samples, led to the identification of many more co-purifying proteins including several additional RAP2-specific interactors (Table 7). TRIM32 was again specifically co-purified with RAP2 for the third time. Other known RAP2 effectors such as AF6, RGL2, MINK, TNIK and NIK, were also identified, confirming the higher sensitivity of this approach. In addition to TRIM32, this TAP also identified the VPS29 and VPS35, which are known to form a complex (Nothwehr, Ha et al. 2000; Collins, Norwood et al. 2008; Swarbrick, Shaw et al. 2011) as RAP2 co-purifying proteins.

ID	Unique peptides	% Coverage
RAP2A	11	57.9
TRIM32	66	75.7
RADIL	30	32.3
TBCD10B	3	6.8

**A) TAP-RAP2A in 293**

ID	Unique peptides	% Coverage
RAP2A	13	44.3
TRIM32	36	50.4
RAP1GDS	9	18.5

**B) TAP-RAP2A in A549**

ID	Unique peptides	% Coverage
RAP2A	35	90.2
MLLT4/AFADIN	71	44.8
TNIK	67	47.9
MAP4K4	27	19.6
MINK1	23	13.6
VPS35	14	21.2
VPS29	3	17
OBSL1	4	2.8
RGL2	3	10.7
RAP1GDS1	2	4.3
TRIM32	1	1.7

**C) TAP-RAP2A in 293T**

**Novel interactions**

- **TRIM32**
- VPS29
- VPS35
- TBC1D10B

**Known interactions**

- TNIK
  - MAP4K4
  - MINK1
- } Rap2 specific
- RGL2
  - MLLT4/AF6
- } Binds Ras family proteins

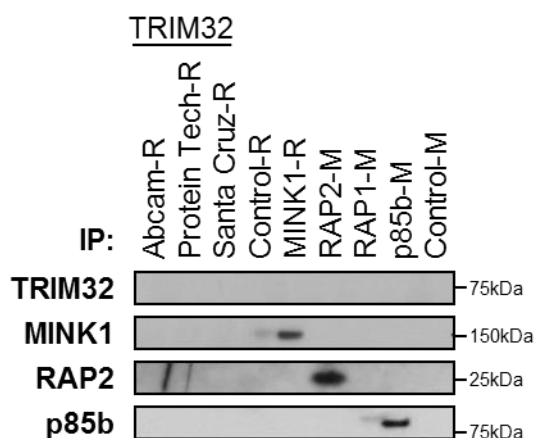
**Table 7: Novel and known RAP2 interactions identified by TAP screen.** A-C) Three independent TAP experiments conducted in HEK-293, A549 and HEK-293T cell lines identified several potential interactors of RAP2. Due to a difference in the sensitivity of the mass spectrometer used, more targets were picked up on the final set of TAP data. D) Data summarised showing identified potential novel interactors of RAP2, including TRIM32 (highlighted in red) which was pursued for further study. The successful detection of known RAP2 interactors serves as an internal positive control for this technique.

## Antibody characterisation

Various commercially available antibodies against TRIM32, MINK1, RAP1 and RAP2 proteins were ordered for use in subsequent experiments. These were characterised, and their specificity tested, and the ability of the antibodies to specifically immunoprecipitate was determined, following the protocol described in the Methods section.

MINK1, RAP2 and p85 were specifically detected by western blot with their respective antibodies in the lanes in which MINK1, RAP2 and p85 were immunoprecipitated (Figure 18).

While the TRIM32 antibodies were able to detect overexpressed and endogenous TRIM32 by Western blotting, they are not able to specifically immunoprecipitate endogenous TRIM32 (Figure 18), therefore limiting the possibility of working with the endogenous protein by immunoprecipitation.



**Figure 18: RAP2, MINK1 and p85b antibodies are able to immunoprecipitate endogenous proteins, TRIM32 is not.** HEK-293T lysates were incubated with Protein A or G beads and the indicated antibody for up to 4h. Lysates were then washed and run on an SDS-PAGE gel. The level of protein pulled-down was detected using western blot. Negative controls of closely related antibodies (Rabbit and Mouse SQSTM1 antibody) were used for both R- and M-, and did not show any bands. This reinforces the fact that the bands that were detected are likely to be specific. RAP2, MINK1 and p85b antibodies were able to immunoprecipitate the respective proteins whereas the other antibodies tested (TRIM32, RAP1) were not.

## Generation of constructs

To further explore protein-protein interactions, overexpression of a battery of tagged constructs (Table 8) was generated as described in the Methods section.



Each of these genes were ligated to ENTR Gateway vector (Gateway®, Invitrogen) and mutagenesis performed where appropriate and verified by sequencing. Gateway technology was then used to transfer the inserts into different Gateway-compatible Tagged-destination vectors for expression in mammalian cells. The resulting tagged proteins (Flag-, GST-, Myc-, TAP6 tags) were used in subsequent experiments.

Mutations at residues G12 and Q63 render RAP proteins insensitive to regulation by GAP resulting in a constitutively active GTP bound state. Therefore, G12V and Q63L mutations were generated to simulate constitutively active RAP1 and RAP2 proteins.

The RAP V12A38 and L63A38 mutants were generated because mutations at A38 in the effector domain prevent binding to downstream effectors, even when the protein is GTP-bound. These constructs were therefore used to demonstrate whether or not an interacting protein behaves as an effector of RAP.

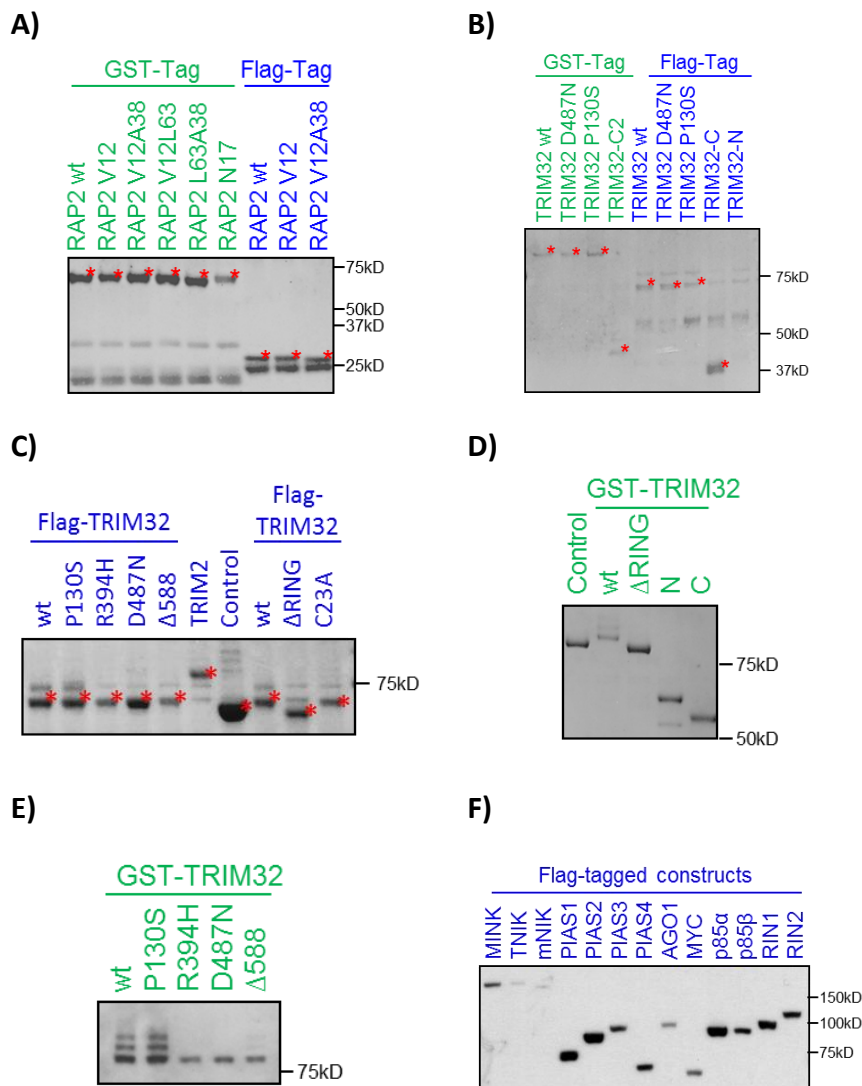
The T35S substitution in the effector domain of HRAS has previously been shown to differentially disrupt its ability to interact with some effectors (e.g. RalGDS, PI3K) but not others (RAF1). The equivalent mutant was generated in RAP2 to test whether it could differentially disrupt the interaction with TRIM32 or other RAP2 effectors.

As mentioned previously, there are five main TRIM32 mutants that have been identified; TRIM32 P130S (found in Bardet-Biedl syndrome) and TRIM32 R394H, D487N, 1559delC and  $\Delta$ 599 (identified in patients with LGMD2H). These, with the exception of 1559delC, were generated by PCR-site direction mutagenesis and were used to study whether they affected the interaction of TRIM32 with other proteins. Additionally,  $\Delta$ RING deletes the RING domain while C23A disrupts the ability of the RING domain to function as an E3 ligase. These mutants were later used in order to determine whether the ubiquitin ligase function of TRIM32 is required for the RAP2-TRIM32 interaction to take place. Finally, truncation products named TRIM32-C, -N and -C2 were generated in order to map the regions of TRIM32 mediating interactions.

Gene	Mutations status	Properties
RAP1	Wt V12 V12 A38	Wt Constitutively active mutant Effector domain mutant
RAP2	Wt V12 V12 A38 V12 T35S N17 L63 L63 A38	Wt Constitutively active mutant Effector domain mutant Effector domain mutant Dominant negative Active mutant Effector domain mutant
TRIM32	Wt P130S R394H D487N Δ588 ΔRING C23A N C C2	wild type BBS mutant LGMD2H mutant LGMD2H mutant LGMD2H mutant Deletion of RING domain (aa 20-63) Inactivating mutation in RING domain N-terminal half (aa 1-308) C-terminal half (aa 308-end) C-terminal fragment (aa 440 to end)
MINK	Wt	wild type
TNIK	Wt	Wt
mNIK	wt	wt mouse isoform (kind gift from D.Barber)
RIN1	Wt	Wt
RIN2a	Wt	Wt
RIN3	Wt	Wt
TBC10D	Wt	Wt
VPS25	Wt	Wt
VPS39	Wt	Wt
MRAS	Wt L63 L63A38	Wt Constitutively active mutant Effector domain mutant
NRAS	Wt V12 V12A38	Wt Constitutively active mutant Effector domain mutant
KRAS	Wt V12 V12A38 DUTR V12 DUTR V12A38 DUTR	Wt Constitutively active mutant Effector domain mutant Deleted untranslated region Constitutively active mutant, deleted untranslated region Effector domain mutant, deleted untranslated region
Ub	8x Ub	kind gift from P. Meier and H. Plun-Favreau

**Table 8: List of Constructs generated.** Shown above, the different constructs generated for subsequent use.

All generated constructs were sequenced in order to confirm correct protein sequence and correct insertion of mutations. In order to assess protein expression, each of these constructs was transfected into HEK-293T cells, and protein expression was assessed by immunoprecipitating samples with the associated tag (e.g. Flag-, GST-) as indicated and Western blotting on SDS-PAGE gels. Blots were stained by coomassie (A-C, Figure 19) or probed using specific tag antibodies (e.g. Flag-, GST-, Myc-, TAP6; D-F, Figure 19). All the transfected constructs encoded for proteins at the predicted size, taking into account the additional size of the tag.



**Figure 19: Example of Coomassie and Western blot data demonstrating expression levels of proteins.** HEK-293T cells transfected with the indicated constructs were lysed after 48h, immunoprecipitated with the correlating tag and separated on SDS PAGE gels. In the Western blots shown above, construct expression is indicated with a red star. Protein expression is seen at the predicted size for each of the protein + tag above. A-C). Protein expression is detectable by Coomassie-Coomassie staining in all but TRIM32-N (figure B, lane 9). This is, however, detectable by wb using the Flag antibody (D-F). D-F) GST- or Flag-antibody was used in order to detect protein expression. All proteins were detected at the predicted size.

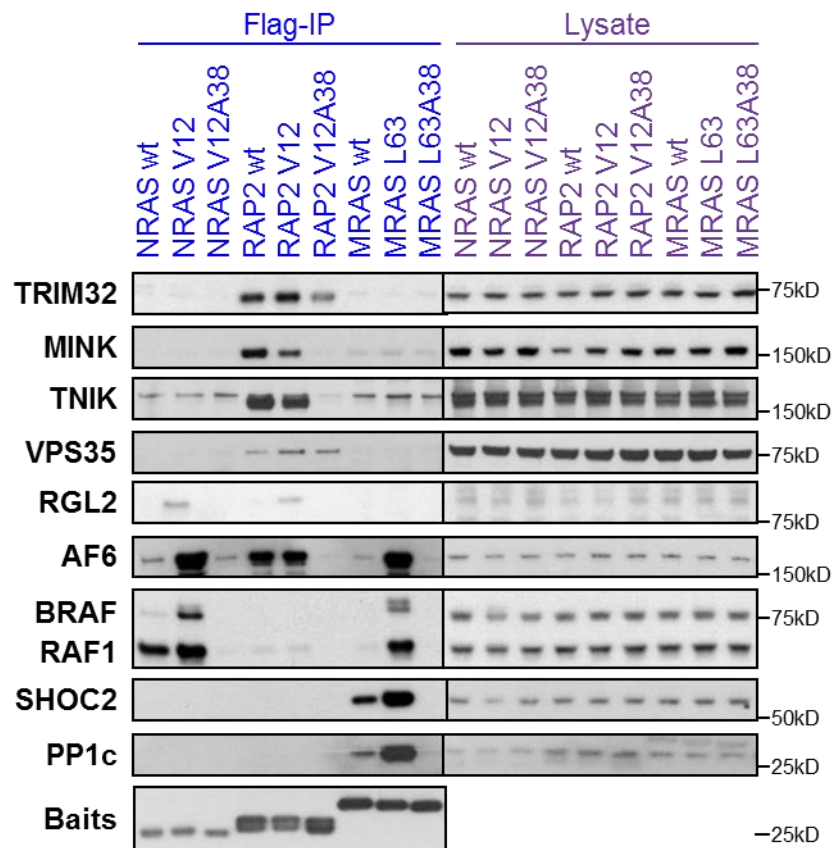
Expression levels of immunoprecipitated GST- and Flag-RAP2 constructs can be clearly detected by Coomassie staining of the blot (Figure 19A), with slightly lower expression of the GST-RAP2 N17 (dominant negative) construct (lane 6).

TRIM32 GST- and Flag-tagged constructs demonstrate lower expression levels that are barely detectable by Coomassie staining (Figure 19B), and in fact no detectable band is seen for Flag-TRIM32-N (lane 9). These constructs were re-run using more of the sample and in some cases the band was now detectable by Coomassie (Figure 19C) and in others, the proteins were detected using the corresponding antibody, GST- or Flag as indicated (Figure 19D-E).

Interestingly, the GST-TRIM32 constructs show laddering on the wt and the P130S mutant seen in BBS (lane 1-2) but not on the remaining LGMD2H mutants (lanes 3-5, Figure 19E). Similarly, when using the Flag-TRIM32 construct, a double band can be seen with wt and P130S but not TRIM32 R394H, D487N or  $\Delta$ 588 LGMD2H mutants (Figure 19D). When detected with an anti-ubiquitin antibody, these extra bands/laddering were shown to be ubiquitinated TRIM32 proteins (see later).

### **Validation of RAP2 interactions identified by TAP**

In order to validate and characterise the interactions detected by TAP (Table 7), Flag-tagged versions of either wild type (lane 4), constitutively active (V12, lane 5) and effector domain mutants (V12-A38, lane 6) of RAP2 were transiently transfected into HEK-293T cells. As controls for the specificity of the interactions, equivalent sets of wt and mutant versions of the closely related NRAS (lane 1-3) and MRAS (lane 7-9) members of the RAS family were also expressed in parallel. After immunoprecipitation of the tagged GTPase with FLAG-beads, associated endogenous proteins were detected by western blotting with the appropriate antibodies (Figure 20).



**Figure 20: Endogenous TRIM32 immunoprecipitates with Flag-RAP2 wt and RAP2 V12.** HEK-293T cells were transfected with Flag-RFG. Lysates were taken on day 3 and immunoprecipitated using Flag-beads. Proteins were separated using SDS-PAGE and Co-purifying proteins were detected by WB using endogenous antibodies. TRIM32 was shown to interact specifically with RAP2, and not NRAS or MRAS, with slightly stronger binding to the active RAP2 V12 mutant and a slight disruption in the interaction with the defective RAP2 A38 mutant. MINK and TNIK were also shown to bind specifically to RAP2, which is disrupted in the presence of the RAP2 A38 mutation. VPS35 was also shown to interact specifically with RAP2, although the interaction is not disrupted by the effector domain mutant RAP2 A38.

Endogenous TRIM32 was found to co-immunoprecipitate specifically with RAP2 but not NRAS nor MRAS (Figure 20). The interaction was slightly stronger with the V12 mutant (lane 5) and partially disrupted by the A38 mutation (lane 6), consistent with the activation- and effector domain-dependent interaction of an effector protein (see also later).

The MINK and TNIK family of GCK kinases was also shown to co-immunoprecipitate specifically with RAP2 but not any other RAS family GTPase tested. The interaction in both cases was strongly disrupted by the A38 substitution in the RAP2 effector domain (lane 6), consistent with their published role as RAP2 effectors (Taira, Umikawa et al. 2004; Nonaka, Takei et al. 2008). However, surprisingly, it was reproducibly observed that the wild type (lane 4) protein interacts more strongly than the V12 mutant (lane 5). This behaviour was also seen with NIK in other experiments (see Figure 21). Our data is consistent with binding in an effector domain-dependent manner, however, with a preference for the wt protein over the V12 mutant. We do not know the reason for this discrepancy.

VPS35, which has not been previously reported to interact with RAP2 but was identified by TAP, can also be seen to co-immunoprecipitate specifically with RAP2 (lane 4-6) but not other RAS family members (Figure 20). In this case however, the interaction was not disrupted by the A38 substitution (lane 6). This suggests that VPS35 is a novel RAP2 interacting protein that may not function as a RAP2 effector, but could be involved in an upstream regulatory role, for example in mediating RAP2 transport (see discussion).

RGL2 is a member of the RALGEF family that is known to interact with RAP2 and NRAS in an effector domain dependent manner, more strongly with the V12 (lanes 2 and 5) than the wt (lane 1 and 4), with no binding to the A38 mutant (lane 3 and 6) as seen above (Figure 20).

AF6, that co-purified with TAP-RAP2 and is a known RAS family effector, can be shown to strongly co-immunoprecipitate with RAP2 as well as NRAS and MRAS in a manner that is strongly disrupted by the A38 effector domain mutations (Figure 20). This shows that AF6 is a promiscuous RAS family effector. In the case of NRAS and MRAS, the interaction takes place preferentially with the activating V12 or L63 mutants (lane 2, lane 8 respectively). In contrast however, no difference can be seen between the wild type and V12 mutant (lane 4, lane 5 respectively) of RAP2 in its ability to interact with AF6. This could reflect the possibility, that unlike other RAS family GTPases, the wild type RAP2 protein may already be predominantly GTP-bound in the cell in some contexts.

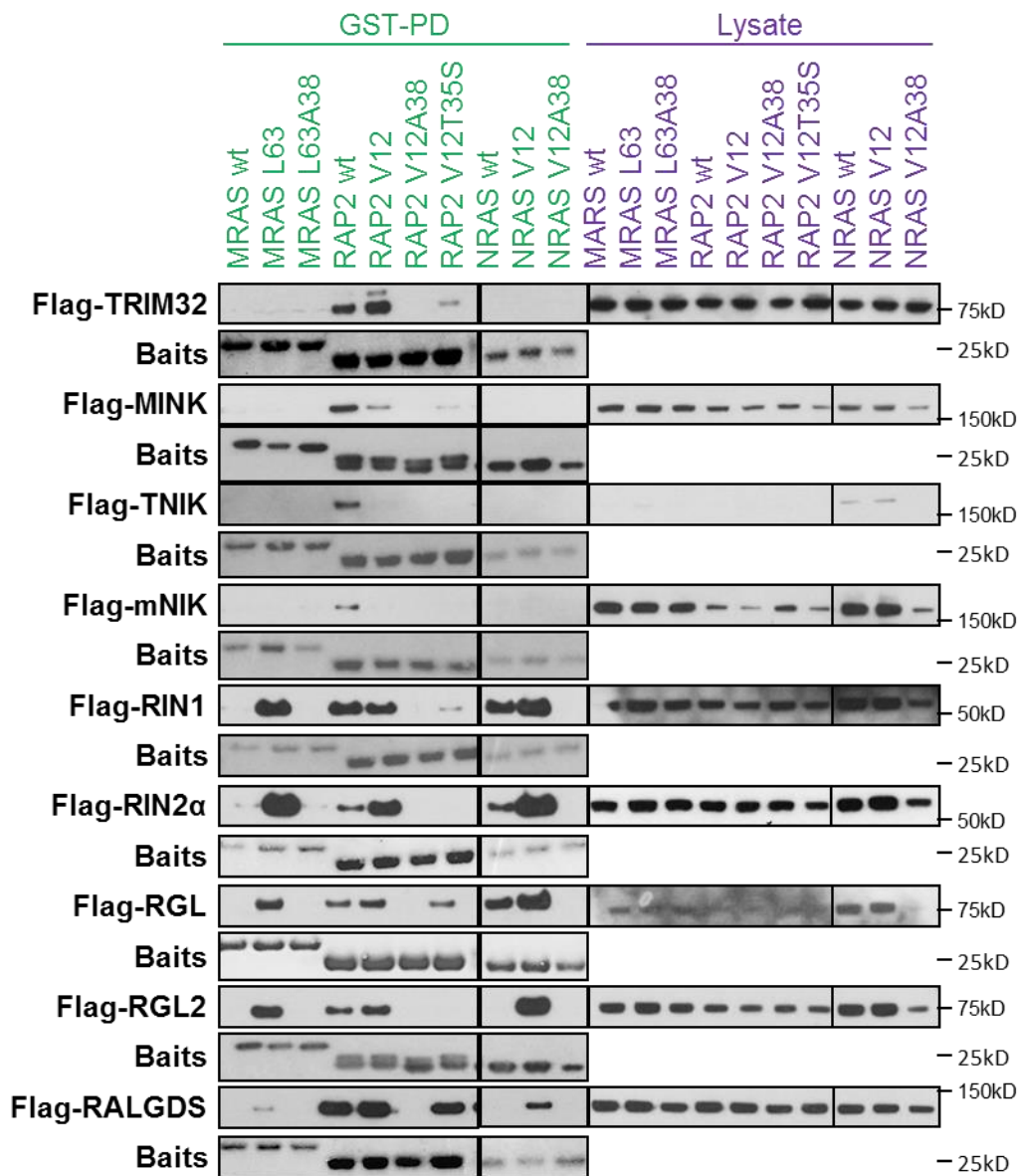
The BRAF and CRAF/RAF1 kinases interact in an effector domain dependent manner with NRAS and MRAS, binding more strongly to the activating mutants (lane 2 and 8), than the wt (lane 1 and 7) with no binding seen with the A38 (lane 3 and 9). However, no binding is observed to RAP2.

SHOC2 and PP1C behave as specific MRAS effectors, with demonstrably stronger MRAS L63 (lane 8) binding than wt (lane 7), and disrupted A38 binding (lane 9), and no interaction with any of the other RFGs.

### **Assessing interaction with further RAP2 mutants**

To further characterise the specificity of these interactions, the interaction of RAP2 with TRIM32 as well as other effectors was also tested in co-transfection assays. GST-tagged RAP2, MRAS and NRAS wild type, activating and effector domain mutants were co-transfected in HEK-293T cells together with Flag-tagged versions of TRIM32 or the indicated proteins (Figure 21).

The A38 mutation in the effector domain of H/K/NRAS oncoproteins leads to full loss-of-function and is known to disrupt the interaction of RAS proteins with all known effectors. However, other substitutions within the RAS effector domain are known to create partial loss-of-function mutants by differentially disrupting the ability to interact with some effectors but not others. HRAS T35S for example can no longer interact with RALGDS or PI3K, while still being able to interact with RAF kinases and activate the RAF-ERK pathway (although with decreased efficiency)(White, Nicolette et al. 1995; Rodriguez-Viciano, Warne et al. 1997). These partial loss-of-function mutants have provided useful experimental tools to assess the contribution of the different RAS effector pathways to the known biological effects regulated by RAS. To test whether the equivalent T35S substitution in RAP2 could differentially disrupt the interaction with TRIM32 and/or other effectors, this mutation was also generated and tested.



**Figure 21: The RAP2-V12T35S mutant partially disrupts the interactions with TRIM32 and RIN1, abrogates interactions with MINK, TNIK, NIK, RIN2 $\alpha$ , and RGL2 and appears to have no effect on interactions with RGL and RALGDS.** HEK-293T cells were transiently co-transfected with GST-tagged RFG constructs and Flag-tagged interactors, and immunoblotted using the Flag antibody. Flag-tagged construct expression levels were analysed by western blotting the lysates with the Flag antibody. TRIM32 interacts specifically with RAP2 in an effector domain dependent manner. This interaction is also partially disrupted in the presence of the RAP2 T35S mutation. The specific MINK, TNIK and NIK interaction with RAP2 is abrogated in the presence of the RAP2 A38 effector domain mutation, and almost entirely disrupted in the presence of the RAP2 T35S mutation. RIN1 and RIN2 interact with NRAS and MRAS in an activation- and effector-domain dependent manner. RIN1 appears to bind more strongly to RAP2 wt than RAP2 V12, with complete and partial disruption on A38 and T35S mutations respectively. RIN2 again interacts in an activation- and effector-domain dependent manner with RAP2, and this interaction is abolished with the RAP2 T35S mutation.



In co-transfection assays TRIM32 was again shown to interact specifically with RAP2 but not NRAS, MRAS (Figure 21) or any other RAS family GTPase tested, including RAP1, KRAS, DIRAS1, DIRAS2 or DIRAS3 (data not shown). This shows, that even under conditions of overexpression, TRIM32 is a very specific RAP2 effector. RAP2 V12 interacted more strongly than the wild type whereas the A38 mutation severely disrupts the interaction. The T35S mutation also strongly disrupts the interaction with TRIM32 although not as strongly as D38A.

MINK, TNIK and NIK also interact specifically with RAP2 but not any other RAS family GTPases. However, contrary to what has been reported in the literature (Taira, Umikawa et al. 2004; Nonaka, Takei et al. 2008); we consistently see preferential binding to RAP2 wt than RAP2 V12 (Figure 21).

As seen for TRIM32, D38A mutation severely disrupts the interaction with MINK whereas T35S has a partial effect. In this type of co-transfection assay, TNIK and NIK could be detected binding to wild type (but not V12 as the signal was likely too weak) RAP2. Because the A38 and T35S mutants were only generated within the background of the V12 mutation, the effect of only the A38 mutant on the interaction with TNIK and NIK could not be determined.

RIN1 and RIN2 show strong activation- and effector domain-dependent interactions with NRAS (lane 1-3) and MRAS (lane 8-10) and both also interact with RAP2 and mutations in the effector domain also strongly disrupt this interaction, with RAP2 A38 completely abrogating the interaction (lane 6) and T35S still displaying some residual binding to RIN1 (lane 7). Intriguingly, RAP2 V12 (lane 5) interacts more strongly than RAP2 wt (lane 4) with RIN2 whereas the opposite pattern was seen with RIN1 (Figure 21).

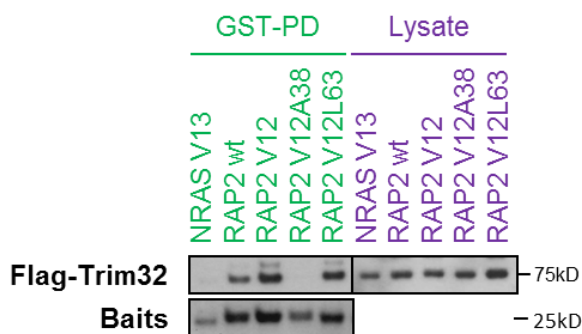
The RALGEF family (RGL, RGFL2 and RaLGDS) also interact with RAP2 and other RAS family members although with different efficiencies. For example, RaLGDS showed the strongest interaction with RAP2 followed by MRAS with clearly weaker binding to NRAS. The A38 mutation blocked interaction with all three RALGEF family members. Interestingly however, whereas the S35 mutation in RAP2 strongly disrupts the interaction with RGL2, it only has a marginal effect on the interaction

with RGL and RalGDS (Figure 21). The results of the RAP2 interactions are summarised in Table 9.

	MRAS			RAP2				NRAS		
	wt	L63	L63A38	wt	V12	V12A38	V12 T35S	wt	V12	V12A38
TRIM32				++	+++		+			
MINK				++	+					
TNIK				++						
NIK				+						
RIN1		++++		+++	+++		+	+++	++++	
RIN2		++++		+				++	++++	
RGL		+++		+	++		+	+++	++++	
RGL2		+++		+	++				++++	
RALGDS				++++	++++		++++		++	

**Table 9: Summary of RAP2 interactions.** Our data so far demonstrates that Trim32, MINK, TNIK and NIK specifically interact with RAP2. RIN1 and RIN2 interact with MRAS, RAP2 and NRAS.

In the case of some RAS and RHO/RAC family GTPases, the ‘L61’ activating mutant was shown to interact more strongly with effectors than the V12 activating mutant. In order to test the possibility that a similar scenario may be taking place with RAP2, the equivalent L63 mutation was generated in RAP2. As the L63 construct we generated did not express at the correct size, the RAP2 V12L63 mutant was used in subsequent studies (Figure 21).



**Figure 22: The RAP2 V12L63 mutant does not effect the TRIM32 interaction.**

GST-tagged RAP2 mutants were over-expressed with Flag-tagged TRIM32. Cells were lysed after 48hours and GST-tagged proteins were pulled down. Subsequently, samples were run on SDS-PAGE gel and interacting Flag-tagged proteins were assessed using the Flag antibody. GST-RAP2 V12 and GST-RAP2 V12L63 expression levels are comparable and no effect is seen on their affinity for TRIM32.

In co-transfection assays, no difference in the ability of the V12 or V12L63 RAP2 to associate with Flag-TRIM32 was detected (Figure 22). Therefore, RAP2 V12 was used in all subsequent studies.

## Summary

Having identified several potential novel RAP2 interacting proteins via the unbiased approach of TAP, we then proceeded to validate them first using overexpressed RAP2 and endogenous proteins and then co-transfecting both RAP2 and the targets.

TRIM32 binds very specifically to RAP2 but not other RAS family GTPases in an activation- and effector domain-dependent interaction. It binds preferentially to the V12 activating mutant over the wt RAP2 and the interaction is disrupted by the A38 mutation in the effector domain. Therefore, TRIM32 behaves as a novel RAP2 effector.

VPS35 was found to interact specifically with RAP2, stronger with RAP2 V12 than wt. However, this interaction was not disrupted using a construct carrying the A38 mutation suggesting that VPS35 may not function as an effector of RAP2, but it could be involved upstream of the pathway.

Further interactions were also confirmed between MINK, TNIK and NIK (previously published), in an effector domain dependent manner, consistent with their published role as RAP2 effectors (Taira, Umikawa et al. 2004; Nonaka, Takei et al. 2008). However, contrary to what has been reported in the literature, we consistently see preferential binding to RAP2 wt than RAP2 V12. This could be due to preferential binding to the GDP bound conformation of RAP2 when compared to the GTP-bound. Alternatively, the V12 mutation may affect the interaction in a guanine nucleotide independent manner.

The T35S substitution in the effector domain of HRAS has previously been shown to differentially disrupt its ability to interact with some effectors (e.g. PI3K) but not others (RAF1). The equivalent mutant was generated in RAP2 to test whether it could differentially disrupt the interaction with TRIM32 or other RAP2 effectors. The T35S substitution in RAP2 only partially disrupts the interaction with TRIM32. RAP2 V12 T35S binds weakly with RIN1, and not with Rin2. Additionally, RGL and RaIGDS

interact with RAP2 V12 T35S, whereas RGL2 does not interact with RAP2 V12 T35S. This demonstrates that similar to the HRAS T35S substitution, the RAP2 V12 T35S residue selectively disrupts interactions both between different protein families as well as within them.

Due to the robust nature of the experiments, the interesting links in the literature, and the availability of the reagents, subsequent experiments were conducted focusing mainly on understanding the RAP2-TRIM32 interaction.

# Chapter 4: Assessing the Effect of TRIM32 mutants

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TRIM32 was identified as a novel RAP2 binding protein by TAP, and validated to interact in a GTP bound, effector domain-dependent manner. In order to better understand how RAP2 may regulate TRIM32 function, the dynamics of the interaction were investigated below.

## **TAP experiments identify a network of protein interactions containing TRIM32 and RAP2 effectors**

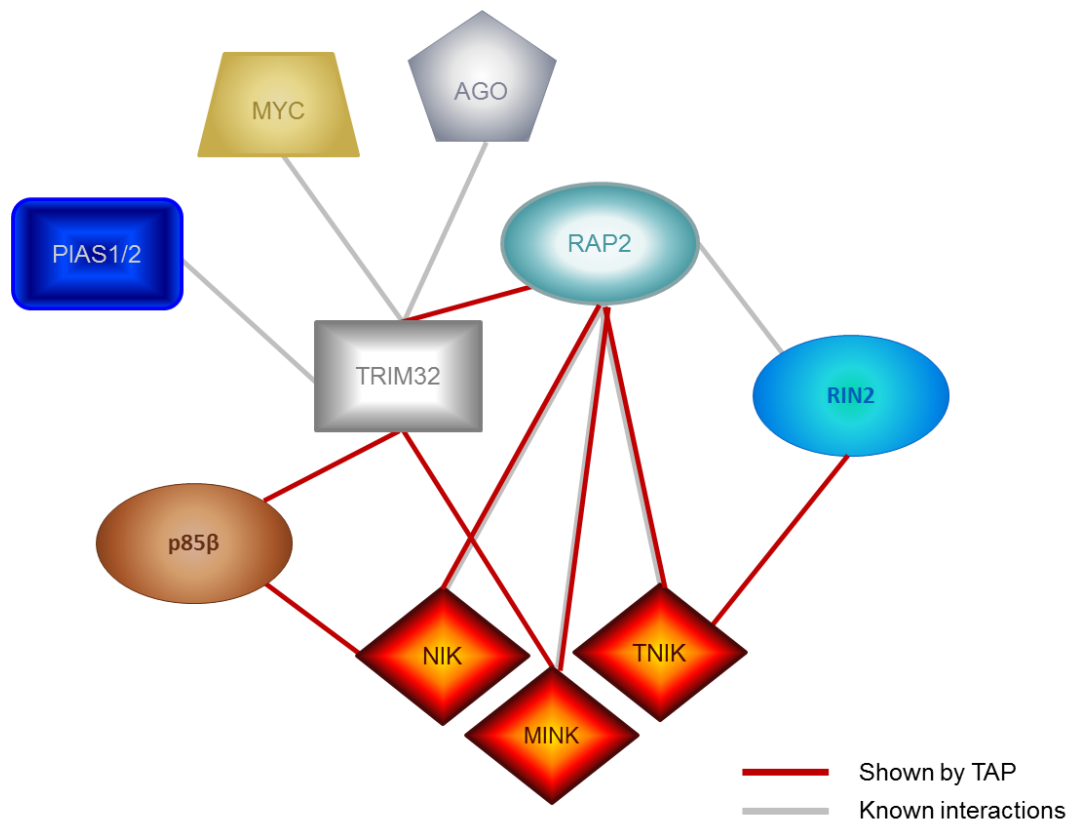
In independent TAP purifications performed by Dr Rodriguez-Viciana TRIM32 was found to specifically co-purify with other TAP baits (Table 10). Mass spectrometry identified TRIM32 as one the most prominent bands co-purifying with TAP-MINK from HEK293T cells that were also co-expressing RAP2 V12, suggesting that MINK, TRIM32 and RAP2 may form a complex *in vivo*. TRIM32 and MAP4K4/NIK were also identified among other proteins co-purifying specifically in TAP purification of the p85 $\beta$  regulatory subunit of PI3K. Both results suggest that TRIM32 and members of the MINK/NIK/TNIK family of kinases may form a complex *in vivo*. TAP of the Ras family effector RIN2 identified TNIK as a co-purifying protein.

<b>MINK (+RAP2)</b>	<b>p85β</b>	<b>RIN2</b>
<b>TRIM32</b>	NISCH	CSNK2A1
CDC37	<b>MAP4K4</b>	CSNK2B
RAP2a	TBC1D15	RRAS2
14-3-3s	14-3-3s	<b>TNIK</b>
LONP1	<b>TRIM32</b>	RIN3
CRBN	CBL	SH3GL1
CDC123	IRS2	CAMK2D
	IRS1	CAMK2G
	INSRR	CAMK2B
	PIK3CB	
	PIK3R1	
	EGFR	
	ERBB2	

**Table 10: TAP-MINK, p85β and RIN2.** TAP-MINK and TAP-p85β identified TRIM32 (red) and as a potential target. TAP-p85β and TAP-RIN2 both detect members of the Ste20 kinases (green) as potential targets.

This data is derived from three separate TAP-purifications using TAP - MINK, -p85β and -RIN2 as indicated (top row) and the proteins listed below are those identified by TAP as potential interactors with the bait.

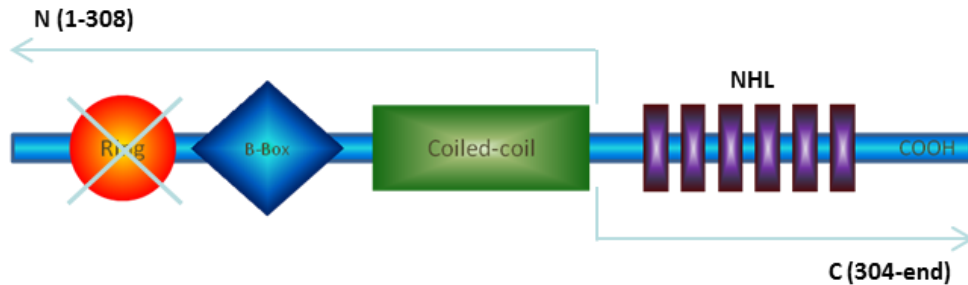
A schematic was generated displaying interactions published in literature as well as those newly identified potential interactions in order to represent the possible signalling network we wished to investigate and help identify areas of overlap/interest for further study (Figure 23).



**Figure 23: Schematic of interactions linking RAP2 to known and novel interactions.** Grey lines indicate interactions that have previously been published in the literature, whereas red lines indicate those identified by TAP.

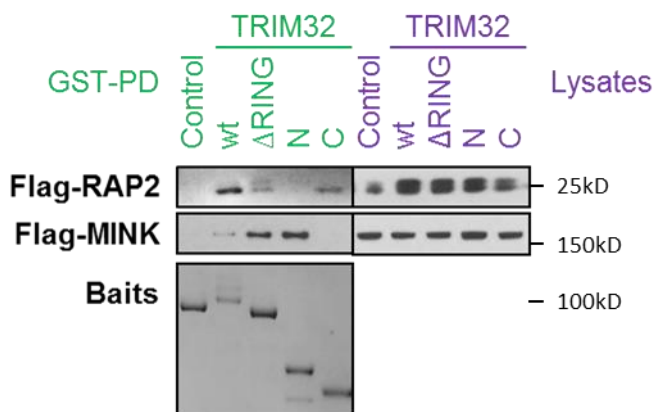
It is apparent from the schematic that RAP2 and TRIM32 may be part of a network of protein interactions with the MINK/TNIK/NIK members of the Ste20 kinase family. There are several potentially novel pathways that can be inferred from a combination of what has been published in the literature and what has been identified from the various TAP experiments. Therefore, these genes were cloned by PCR and expression constructs generated for use in subsequent experiments in order to map the interaction site or assess whether RAP2 can modulate these interactions as well as TRIM32.

## RAP2 interacts with TRIM32 C-terminus and requires the RING domain



**Figure 24: TRIM32 mutants.** Truncation mutants are indicated above (N-terminal, aa 1-308) and below (C-terminus, aa 304-end).  $\Delta$ RING is indicated by the cross on the RING domain.

In order to map the region of TRIM32 that interacts with RAP2, the N- and C-terminal halves of TRIM32 as well as a deletion mutant lacking the RING domain were expressed as GST-fusion proteins in HEK-293T cells together with Flag-RAP2 or Flag-MINK. After 48h, the cells were lysed and pulled-down using the GST-tag, and the interactions assessed using the Flag-antibody.



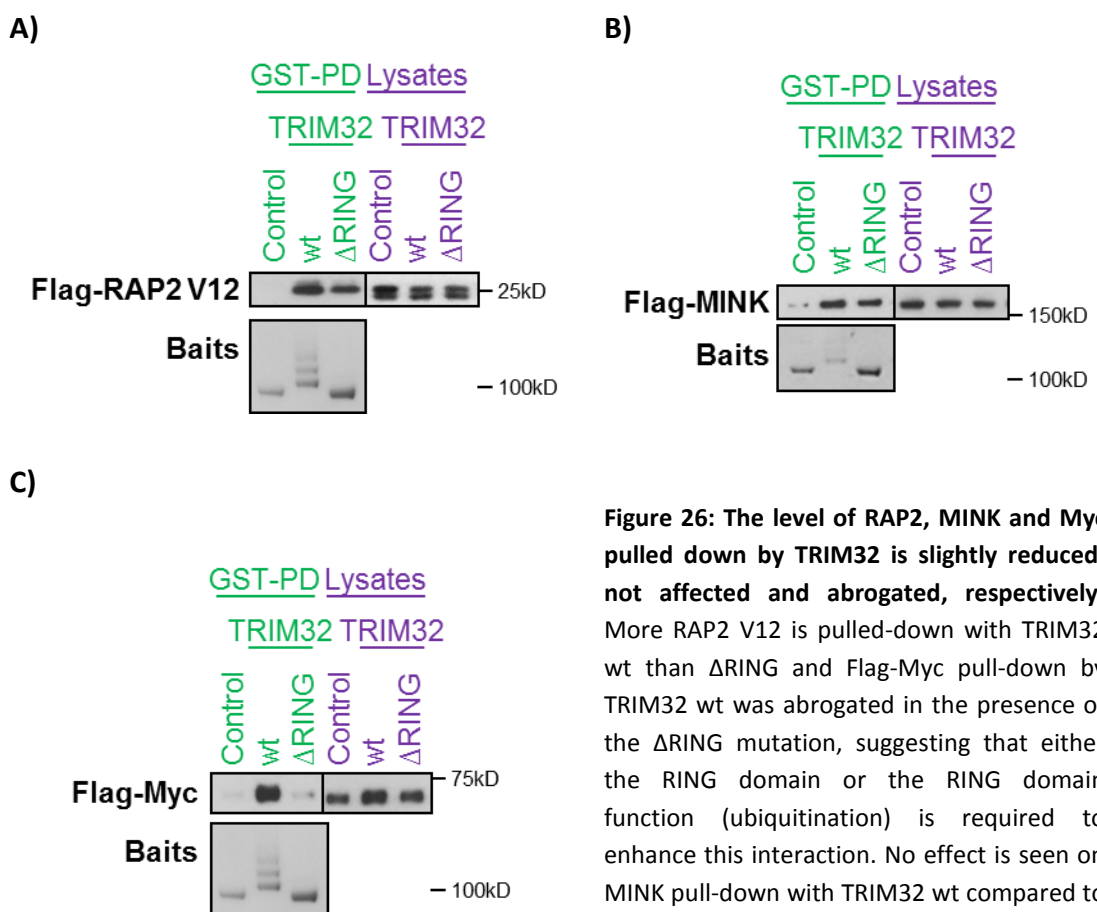
**Figure 25: RAP2 interacts with TRIM32-C-terminus.** RAP2 binds to TRIM32 C-terminus and not N-terminus with reduced interaction upon deletion of the RING domain. Conversely, MINK binds to the N-terminus and not the C-terminus and is not affected by the RING domain deletion. This is surprising as the RING domain is located in the N-terminal region, suggesting that it could be the activity (ubiquitination) of the RING domain that is enhances the RAP2-TRIM32 interaction.

GST-TRIM32 wt,  $\Delta$ RING, N-terminus or C-terminus (as indicated) were over-expressed with either Flag-RAP2 or Flag-MINK. Lysates were incubated with glutathione beads and interactions were detected using the Flag-antibody.



Flag-RAP2 binds to C- (lane 5), but not the N-terminal half (lane 4) of TRIM32, although not as efficiently as the wild type protein (note difference in bait levels). In clear contrast, MINK binds to the N- but not C-terminus of TRIM32. This confirms the MINK-TAP result and validates MINK as a TRIM32 interacting protein. It also indicates that TRIM32 uses different domains to interact with both RAP2 and MINK and could thus bring them into the same complex in vivo (Figure 25).

The RING domain is required for the E3-ligase activity of TRIM32, it works with E2 enzymes to monoubiquitinate or polyubiquitinate substrates, most likely by acting as a scaffold, bridging the E2~Ub and its substrate. This was therefore mutated in order to determine whether RAP2 and MINK are still able to interact with TRIM32 with a disrupted RING domain as this could indicate a link to TRIM32s E3 ligase activity.



**Figure 26: The level of RAP2, MINK and Myc pulled down by TRIM32 is slightly reduced, not affected and abrogated, respectively.** More RAP2 V12 is pulled-down with TRIM32 wt than ΔRING and Flag-Myc pull-down by TRIM32 wt was abrogated in the presence of the ΔRING mutation, suggesting that either the RING domain or the RING domain function (ubiquitination) is required to enhance this interaction. No effect is seen on MINK pull-down with TRIM32 wt compared to ΔRING suggesting that this is a pathway specific effect.

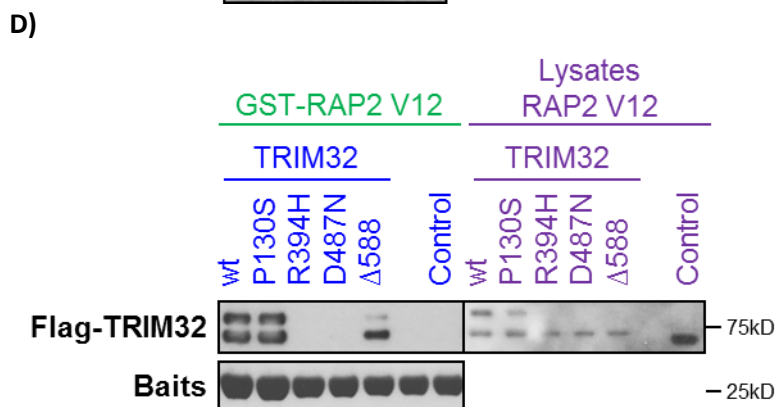
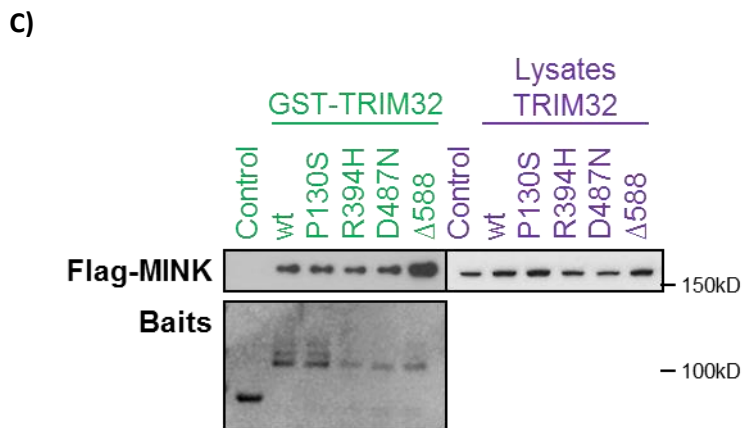
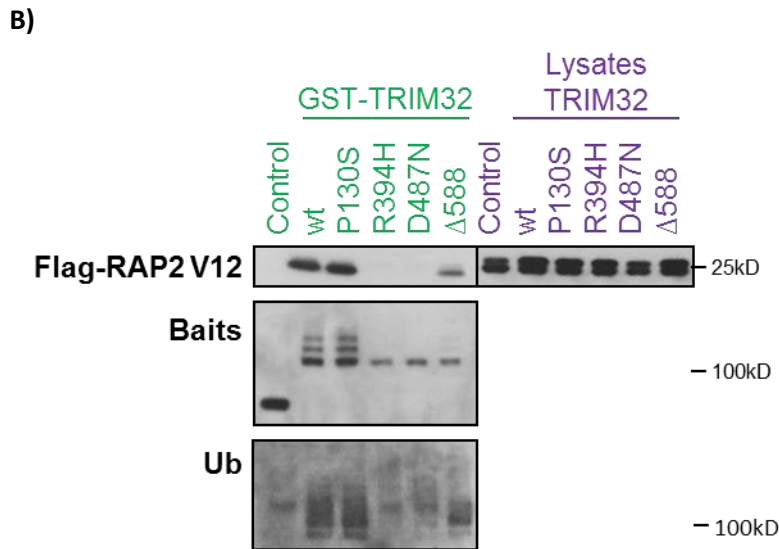
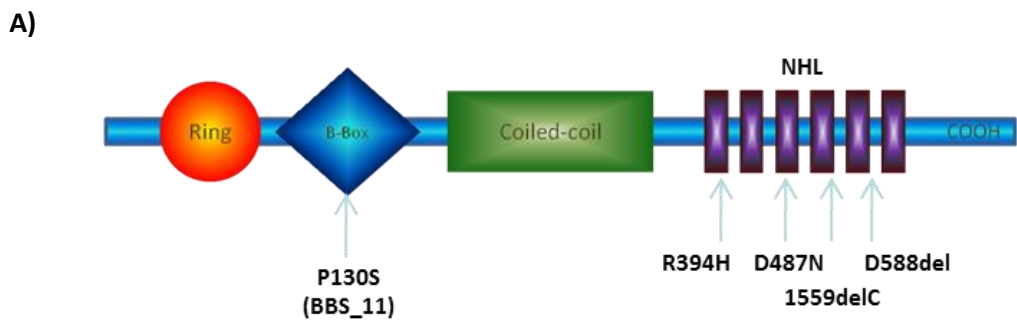
GST-TRIM32 wt or ΔRING (as indicated) were over-expressed with either Flag-RAP2, Flag-MINK or Flag-Myc. Lysates were incubated with glutathione beads and interactions were detected using the Flag-antibody. SHOC2 construct was used as an unrelated control.

MINK interaction does not require the RING domain (lane 3, Figure 26). Note that although the interaction with wt TRIM32 appears weaker, the baits in lane 2 indicate that this is likely a reflection of the lower expression levels achieved with the wild type protein. Conversely, disruption of the TRIM32-RING domain abrogates the Myc-TRIM32 interaction, seen on Figure 26C despite the stronger TRIM32 bait. Surprisingly, even though RAP2 can interact with the C-terminal half of TRIM32, deletion of the RING domain, which is located in the N-terminal half (Figure 24) also partially disrupts the interaction with RAP2.

### **TRIM32 disease mutants disrupt the RAP2 V12 interaction but not MINK**

As mentioned previously, there are five main TRIM32 mutants that have been identified in disease; TRIM32 P130S (found in Bardet-Biedl syndrome) and TRIM32 R394H, D487N and  $\Delta$ 599 (identified in patients with LGMD2H).

In order to test whether these mutations had any effect on the interactions with RAP2 or MINK, and investigate whether these interactions are involved in either disease aetiology, the mutants were overexpressed in HEK-293T cells with either RAP2 or MINK by transient transfection.



**Figure 27: TRIM32 R394H and D487N mutants disrupt RAP2 interaction.**

A) TRIM32 structure with disease mutants indicated by arrows. B-C) Flag-RAP2 V12 and GST-TRIM32 wt and mutants were overexpressed in HEK-293T cells as described previously. Lysates were then pulled down by the GST-tag, and interaction was assessed using the Flag-antibody.

Membranes were also probed with endogenous Ub antibody to assess Ub levels for the different mutants. D) Flag-TRIM32 wt and mutants and GST-RAP2 V12 were overexpressed in HEK 293T cells as described previously. A PKA-R2 $\alpha$  construct was used as an unrelated control.

When GST-TRIM32 is used to pull-down RAP2, the P130S substitution in the B-box within the N-terminus of the protein that is found in BBS has no effect on the interaction with RAP2. In contrast R394H and D487N within the C-terminal NHL repeats strongly inhibit the interaction with RAP2 whereas  $\Delta 588$  showed greatly diminished but still detectable interaction. None of the mutations had any effect on the interaction with MINK (Figure 27A).

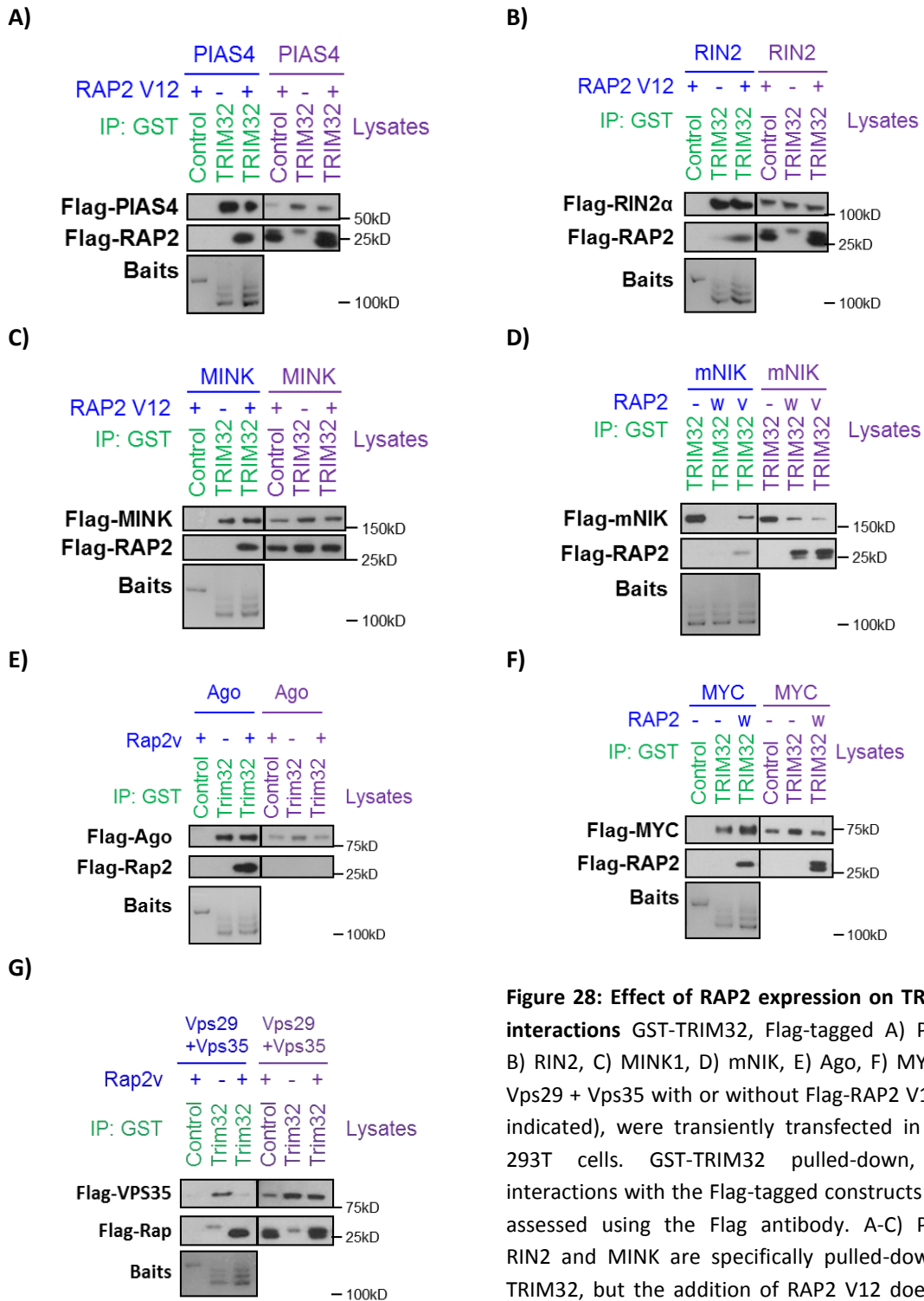
Laddering can be seen on the baits for GST-TRIM32 wt and P130S but not for TRIM32 R394H, D487N or  $\Delta 588$ . Using an antibody against endogenous ubiquitin, the laddering has been shown to correlate with ubiquitination, implicating the E3 ligase activity of TRIM32 in the RAP2 interaction (Figure 27A).

Similar results are seen when the experiment is done with immobilised GST-RAP2 pulling down Flag-TRIM32 (Figure 27A), with R394H and D487N completely disrupting the interaction,  $\Delta 588$  having a partial effect, whereas P130S behaved as the wild type. Flag-TRIM32 shows a double band which would correlate in size to mono- or di-ubiquitination of TRIM32.

### **Modulation of TRIM32 interactions by RAP2**

As RAP2 is upstream of TRIM32, demonstrated by the effector-type properties of the interaction, it is likely that RAP2 regulates the function or activity of TRIM32. Therefore, we investigated several other TRIM32 interactions which we or others had identified and assessed whether RAP2 played a role in modulating these interactions.

The ability of RAP2 to effect TRIM32 interactions was assessed by co-transfection of GST-TRIM32, Flag-tagged PIAS4/RIN2/MINK (as indicated) with or without Flag-RAP2 V12 (as indicated). GST-TRIM32 was pulled down using Glutathione beads and interacting Flag-tagged proteins were detected using an anti-Flag antibody.



**Figure 28: Effect of RAP2 expression on TRIM32 interactions** GST-TRIM32, Flag-tagged A) PIAS4, B) RIN2, C) MINK1, D) mNIK, E) Ago, F) MYC, G) Vps29 + Vps35 with or without Flag-RAP2 V12 (as indicated), were transiently transfected in HEK-293T cells. GST-TRIM32 pulled-down, and interactions with the Flag-tagged constructs were assessed using the Flag antibody. A-C) PIAS4, RIN2 and MINK are specifically pulled-down by TRIM32, but the addition of RAP2 V12 does not affect the level of protein pulled-down.

D) mNIK is pulled-down by TRIM32 and co-transfection with RAP2 V12 (even with poor expression of the RAP2 V12 construct) results in less mNIK being pulled-down by TRIM32. E) Ago is pulled-down by TRIM32 and addition of RAP2 V12 does not appear to affect this. However, despite RAP2 V12 being detected on pull down by TRIM32, such low levels are expressed in this experiment that it is not seen in the baits. F) MYC is pulled down by TRIM32 and co-expression of RAP2 results in an increase in the levels of MYC detected. G) VPS29 + VPS35 co-expression with TRIM32 enables detectable amounts of VPS35 to be pulled down by TRIM32, and this is abrogated on co-transfection with RAP2 V12. GST-CIP2A construct was used as an unrelated control.

TRIM32 is able to specifically pull down PIAS4, RIN2 and MINK and the co-expression of RAP2 V12 (with RAP1 V12 as a control) does not appear to affect the strength of the interaction, suggesting that RAP2 V12 does not modulate TRIM32s ability to interact with these proteins (Figure 28A-C).

In the case of mNIK, however, (Figure 28D) the mNIK-TRIM32 interaction is disrupted in the presence of RAP2 and this effect is seen more strongly with RAP2 wt (lane 2) than RAP2 V12 (lane 3), which correlates with previously demonstrated interaction data showing a stronger interaction between mNIK and RAP2 wt than RAP2 V12 (Figure 21). It is possible that disruption in the MINK-TRIM32 interaction may be detected in the presence of RAP2 wt as it also binds more strongly to RAP2 wt.

Overexpressed Ago is pulled down by TRIM32 with no effect seen upon addition of RAP2 V12 (Figure 28E). However, despite RAP2 V12 being detected on pull down by TRIM32, such low levels are expressed in this experiment that it is not seen in the baits, with stronger RAP2 V12 expression, it is possible that an effect may be seen, however we were not able to obtain this data.

MYC is pulled down by TRIM32 and co-expression of RAP2 results in an increase in the levels of MYC detected (Figure 28F), whereas VPS29 + VPS35 co-expression with TRIM32 enables detectable amounts of VPS35 to be pulled down by TRIM32, and this is abrogated on co-transfection with RAP2 V12 (Figure 28G). VPS29 functions as a scaffold protein that facilitates the linking of two subcomplexes for the assembly of the heteropentamer in endosomal protein sorting (Swarbrick, Shaw et al. 2011), which is a possible reason why it has not been detected on pull-down with TRIM32 whereas VPS35 has.

This series of experiments suggests that RAP2 modulates the TRIM32 interaction with downstream effectors in an effector-dependent manner.

## Summary

In this section we have demonstrated that RAP2 interacts with TRIM32 C-terminus and LGMD2H disease mutants specifically disrupt this interaction. This disruption of the RAP2-TRIM32 interaction in the presence of the LGMD2H disease mutants and

not with the mutations found in BBS, establish a link between this interaction and muscular dystrophy. Interestingly, these LGMD2H mutations are all located in the NHL domain, a motif involved in protein-protein interactions (Edwards, Wilkinson et al. 2003) and previously shown in TRIM32 to associate with and enhance the activity of Argonautes (AGO), a miRNA (Schwamborn, Berezikov et al. 2009; Kudryashova, Struyk et al. 2011). Surprisingly, even though RAP2 can interact with the C-terminal half of TRIM32, deletion of the RING domain, which is located in the N-terminal half (Figure 24) also partially disrupts the interaction with RAP2. This suggests that in the context of the full length protein, RAP2 may make multiple contacts with the TRIM32 proteins, both at the C-terminus and with the RING domain. Alternatively, the activity of the RING domain for E3 ligase activity may be important for the interaction with RAP2. In clear contrast, MINK binds to the N-terminus of TRIM32 and is not affected by mutations to the NHL or RING domain. This indicates that TRIM32 uses different domains to interact with both RAP2 and MINK and could thus bring them into the same complex *in vivo*.

It has been shown using yeast two-hybrid that TRIM32 proteins form homodimers and that LGMD2H mutations (but not the BBS P130S mutation) disrupted homodimer formation (Saccone, Palmieri et al. 2008). With the  $\Delta 588$  mutation, there was a partial effect with TRIM32 dimers still occurring, but slower growth seen in yeast two-hybrid analysis (Saccone, Palmieri et al. 2008). Therefore there is an interesting correlation between the ability of LGMD2H mutations to affect the ability of TRIM32 to interact with RAP2 (Figure 27) and to form homodimers (Saccone, Palmieri et al. 2008).

Laddering can be seen on the baits for GST-TRIM32 wt and P130S but not for TRIM32 R394H, D487N or  $\Delta 588$ . Using an antibody against endogenous ubiquitin, the laddering has been shown to correlate with ubiquitination, implicating the E3 ligase activity of TRIM32 in the RAP2 interaction (Figure 27). This is consistent with the previous result on Figure 26, where deletion of the RING domain decreased the interaction with RAP2 in the context of the  $\Delta$ RING protein and further suggests that the RING domain may make contacts with the C-terminus NHL domains, and that this contacts may lead to ubiquitination of TRIM32.

This ability to selectively modulate TRIM32 interactions suggests a regulatory role for the RAP2-TRIM32 interactions. However, it has been stated in literature that there is a poor correlation between ubiquitin ligase activity and ability to bind E2 substrates, or other downstream substrates, with high affinity (Lorick, Jensen et al. 1999). Therefore, we proceeded to investigate the impact of RAP2 on TRIM32 mediated ubiquitination.



# Chapter 5: TRIM32 TAP Identifies further Potential Interactions

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## **TRIM32-TAP**

In order to identify additional interacting proteins of TRIM32 in a non-biased proteomic approach, we performed TAP purifications of TRIM32 in several cell types. Initially, TAP-tagged TRIM32 was stably expressed in the malignant meningioma cell line IOMM-Lee in parallel with unrelated proteins which served as specificity control.

Levels of TRIM32 bait that were purified in this system were relatively low. Therefore, before sending the samples for analysis an additional TAP purification was performed in 293T cells. Transient transfection of these cells generally leads to higher expression levels and therefore higher sensitivity in TAP experiments. Furthermore, in an attempt to identify TRIM32-interacting proteins that may be regulated by RAP2, cells were co-transfected with either RAP2 V12 or a Control. This TAP purification was carried out using multiple alternative baits that served as specificity controls. After affinity purification, protein complexes were analysed by mass spectrometry by our collaborators Juan Oses and Alma Burlingame at the UCSF mass spectrometry facility.

Protein Name	Gene Name	Control 1		TRIM32	
		No Unique peptides	% Coverage	No Unique peptides	% Coverage
Control 2	-	55	71.4		
Control 3	-	28	48.2		
E3 ubiquitin-protein ligase TRIM32	TRIM32			56	77.8
14-3-3 protein zeta/delta	YWHAZ			4	17.6
14-3-3 protein epsilon	YWHAE			5	16.9
14-3-3 protein gamma	YWHAG			2	7.3
Prelamin-A/C	LMNA			13	25.9
Protein KRI1 homolog	KRI1			2	3.7

**Table 11: Peptides identified in TAP-TRIM32 purified from IOMM-LEE cells.** IOMM-LEE cells were infected with lentivirus expressing TAP-tagged TRIM32 and Control1. Protein complexes were purified as described in material and methods and mass spectrometry performed by our collaborators (Juan Oses and Alma Burlingame) at the UCSF mass spectrometry facility.

Protein Name	Gene Name	TRIM32 + control		TRIM32 + RAP2 V12	
		No Unique peptides	% Coverage	No Unique peptides	% Coverage
E3 ubiquitin-protein ligase TRIM32	TRIM32	67	81	75	84.8
14-3-3 protein zeta/delta	YWHAZ			4	16.7
14-3-3 protein epsilon	YWHAE			3	15.7
14-3-3 protein gamma	YWHAG			2	12.1
14-3-3 protein theta	YWHAQ			2	9.4
Cullin-2	CUL2	8	10.7		
Kelch domain-containing protein 10	KLHDC10	3	14.5	1	3.4
Myomesin-1	MYOM1	1	0.5		

**Table 12: Peptides identified in TAP-TRIM32 purified from 293T cells.** 293T cells were transiently cotransfected with TAP-TRIM32 and either Control wt or RAP2 V12. Protein complexes were purified as described in material and methods and mass spectrometry performed by our collaborators (Juan Oses and Alma Burlingame) at the UCSF mass spectrometry facility.

The results of the TRIM32 co-purification TAP experiment are shown in Table 11 and Table 12 for IOMM-Lee and 293T cells respectively. Very few TRIM32-interacting proteins were identified and, in most cases, the numbers of peptides found was low. This suggests the need for extra caution until further validation. Some of the appear of particular interest as highlighted below, however, due to the delay in

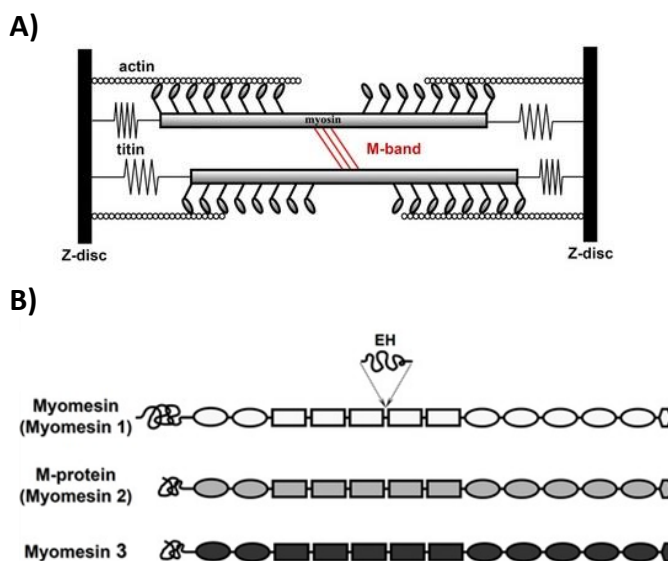
receiving the data, there was insufficient time to conduct further experiments, therefore precluding validation and further analysis.

14-3-3 proteins were identified in both purifications as TRIM32 interacting proteins. 14-3-3 proteins are dimers that function as scaffold proteins. They bind in a phosphorylation dependent manner to a variety of proteins, and have been implicated in many key cellular processes such as metabolism, protein trafficking, signal transduction, apoptosis and cell-cycle regulation (Morrison 2009). Interestingly, in HEK-293T cells, 14-3-3 were preferentially associated with TRIM32 when RAP2 was co-expressed suggesting that RAP2 may modulate TRIM32 function and/or activity by stimulating its association with 14-3-3. Recent studies by Ichimura *et al* confirmed 14-3-3 as a binding part of TRIM32, showing that each of the 14-3-3 isoforms interact with PKA phosphorylated TRIM32 in HEK293 cells (Ichimura 2014). Interestingly, it was shown that 14-3-3 binding prevented TRIM32 auto-ubiquitination as well as TRIM32 transubiquitination as well as the ability of TRIM32 to form cytoplasmic bodies. These findings suggest that 14-3-3 isoforms play a role in the modulation of TRIM32 activity as well as in maintaining the proper level of soluble TRIM32 proteins (Song *et al*, 2005; Diaz-Griffero *et al.*, 2006).

In IOMM-Lee, but not HEK-293T cells, lamin A/C was found to strongly co-purify with TRIM32. Lamins are important for the incorporation and spacing of nuclear pores, regulation of nuclear size and the shape and mechanical properties of the nucleus. They also play an important role in physically connecting the nucleus to the cytoskeleton, most likely through their interaction with SUN proteins (Sad1p, UNC-84) and nesprins. The protein complex formed by nesprins and SUN proteins is essential for intracellular force transmission, cell migration and cell polarization. Interestingly mutations in lamin A/C have been implicated in a variety of diseases including Emery–Dreifuss muscular dystrophy (EDMD), and limb-girdle muscular dystrophy type 1B (Ho and Lammerding 2012). Although the TRIM32-Lamin A/C interaction needs to be validated, considering that TRIM32 is also mutated in limb-girdle muscular dystrophy (LGMD2H), it is tempting to speculate that the TRIM32-lamin A/C interaction may be of special importance to muscle physiology and muscular dystrophy, as implied by the findings by LaBeau-DiMenna *et al*, and

Kudryashova *et al* highlighted below (LaBeau-DiMenna, Clark, et al. 2012; Kudryashova, Kudryashov et al. 2005).

In HEK-293T cells one peptide was identified for Myomesin-1. This protein is also of particular relevance because of its role in muscle physiology. Myomesin is a structural component of the M-band of the sarcomere (see Figure 29). Muscle cells are composed of tubular myofibrils which are composed of repeating sections of sarcomeres, which are composed of long, fibrous proteins that slide past each other when the muscles contract and relax. Members of the MYOM protein family act to maintain the overall structural organisation of the M-band (Pinotsis, Chatziefthimiou et al. 2012) and mutations in myomesin are associated with cardiomyopathy (Siegert, Perrot et al. 2011), symptoms often seen in muscular dystrophies. Interestingly, findings by LaBeau-DiMenna demonstrated that the TRIM32 orthologue *Thin* is localised to the Z-disc in muscle and is essential for myofibril stability in *Drosophila* (LaBeau-DiMenna, Clark, et al. 2012). Findings by Kudryashova et al have already indicated that TRIM32 can bind to the thick filament protein myosin and ubiquitinates actin (Kudryashova, Kudryashov et al. 2005) and that TRIM32 localises to Z-line proteins in mice (Kudryashova, Wu et al. 2009). Due to high conservation of these structures in animals, it is likely that TRIM32 plays a similar role in mammalian muscle cells.



**Figure 29: Sarcomere cytoskeleton and M-band protein components.** A) Scheme of the sarcomere depicting the main components of the sarcomeric cytoskeleton (M-band, Z-disk and titin). B) Myomesin (white), M-protein (gray) and myomesin-3 (dark gray) are composed of immunoglobulin-like domains (ellipses) and fibronectin type 3 domains (rectangles). From ref: (Schoenauer, Emmert et al. 2011).

Cullin-2 is a member of the cullin family of hydrophobic proteins that function as scaffolds for E3 ubiquitin ligases. They combine with RING proteins to form Cullin-

RING ubiquitin ligases (CRLs) and play an essential role in targeting proteins for ubiquitin-mediated modification. Because TRIM32 contains a RING domain, and has ubiquitin ligase activity, its co-purification with TRIM32 in HEK-293T cells is consistent with TRIM32 being a cullin-2 associated E3 ligase. Interestingly, no Cullin-2 peptides were detected when RAP2 was co-expressed suggesting that RAP2 may modulate TRIM32 association with Cullin-2.

Future work would involve validating these interactions and assessing their modulation by RAP2, as well as investigating whether they are substrates for TRIM32 ubiquitination. Also, exploring the possibility of a biological effect on the cells, such as a role on proliferation, migration, differentiation or TRIM32 localisation, would be further down the line.

# Chapter 6: Results – The Effect of TRIM32 Dependent Ubiquitination

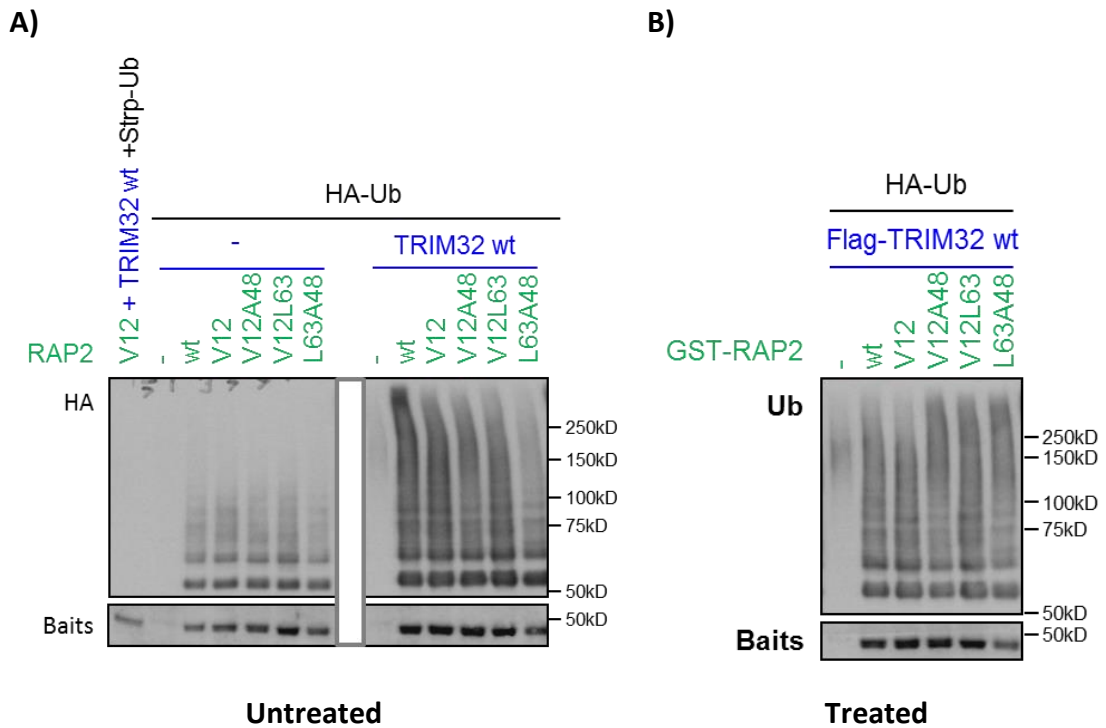
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We have shown that the RAP2-TRIM32 interaction is partially interrupted upon mutation of the TRIM32 RING domain, the site responsible for E3 ligase activity of TRIM32. Additionally, laddering seen on TRIM32 wt and P130S baits by coomassie and western blot is indicative of ubiquitination, and is absent in TRIM32 R394H, D487N and  $\Delta$ 588 mutants – the mutations that disrupt the interaction with RAP2 as shown previously. From this we can infer that the ubiquitin ligase activity is disrupted in these mutants and that the ubiquitination function of TRIM32 is somehow linked to the RAP2-TRIM32 association.

Combined with the information that RAP2 can selectively modulate TRIM32 interactions with other proteins, the key point explored below is: can we detect a RAP2 mediated effect on TRIM32 Ub activity on other interactions?

## **RAP2 is a substrate of TRIM32 Ubiquitination and mutations in RAP2 Regulate RAP2-Ubiquitination**

Prior to our investigations, it was necessary to set up and optimise the experimental system. Proteasome inhibitors are drugs that are often used in ubiquitination studies in order to reduce the degradation of ubiquitin-conjugated proteins in mammalian cells. MG-132 is a cell-permeable proteasome inhibitor and its effect was tested on HEK-293T cells by overexpressing HA-tagged Ub, RAP2 wt or RAP2 mutants, TRIM32 wt or empty control as specified on the figure (Figure 30). These cells were then either treated with control or MG-132 for 3h prior to cell lysis and incubation with glutathione beads. RAP2 proteins were pulled down by the GST tag and the effect on the signal obtained by Western blot was assessed using the HA antibody.



**Figure 30: RAP2 is a substrate of TRIM32 ubiquitination.** HEK-293T cells were co-transfected with HA-Ub (black), Flag-TRIM32 (blue), GST-RAP2 wt and mutants (green). A) Untreated cells, or B) treated with MG-132 for 3h were pulled-down using Glutathione beads. Proteins were separated using SDS-PAGE gel and transferred to PVDF membrane. Associated ubiquitin were detected using HA-antibody. Lower levels of ubiquitin were pulled down for samples that were pre-treated with MG-132 when compared to untreated samples with comparable bait levels.

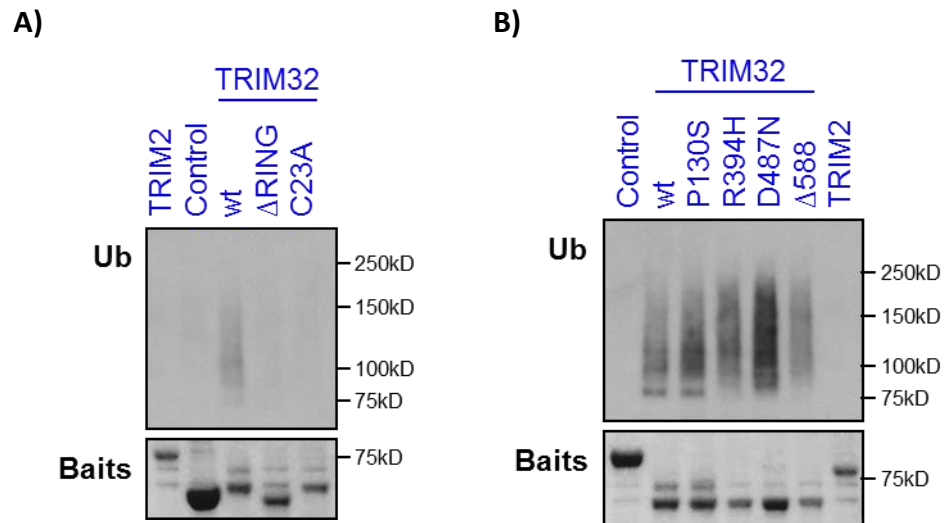
Comparing the ubiquitination levels both with and without MG-132, there was little difference and therefore subsequent experiments were conducted in the absence of MG-132.

TRIM32 enhances the ubiquitination of RAP2 (Figure 30). The RAP2 V12 and V12L63 mutants show little difference in ubiquitination levels both with and without TRIM32 (Figure 30A, lanes 7 and 13), with little detectable difference between the other mutations. Thus we looked at whether the different TRIM32 mutants would have an effect on the level of ubiquitination.

### TRIM32 mutants immunoprecipitate Ubiquitin to varying degrees

To explore the effects of the disease mutants studied previously, TRIM32 wt and mutant proteins were transiently overexpressed in HEK-293T cells. Cell lysates were immunoprecipitated and levels of auto-ubiquitination were compared with

controls. TRIM2 is from the same sub-family (VII) as TRIM32, was therefore used as a closely related control while the C23A mutation is located within the RING domain of TRIM32 and should destroy its E3 ligase activity.



**Figure 31: Effect of TRIM32 mutants on ubiquitination.** Results show variation in the levels of ubiquitination detected. Although more ubiquitin can be detected with the TRIM32 D487N mutant, bait expression is higher than the other TRIM32 constructs. The  $\Delta$ RING and C23A constructs do not show ubiquitination, likely due to the role of the RING domain in ubiquitination.

HEK-293T cells were co-transfected with HA-Ub, Flag-TRIM32 wt or mutants as indicated. Lysates were taken on day 3 and samples were immunoprecipitated using Flag- beads. Proteins were separated using SDS-PAGE gel and transferred to PVDF membrane. Protein levels were detected using HA- antibody. Unrelated controls were A) Trim2 and Shoc2 B) CIP2A.

As predicted, deleting the RING domain (Figure 31A, lane 4) or mutating the C23A residue (Figure 31A, lane 5) within the RING domain prevents TRIM32 auto-ubiquitination, as this is the site of E3 ligase activity (Figure 31A). Also, no background ubiquitination was seen in the unrelated control (Figure 31A, lane 2) or in TRIM2 (Figure 31A, lane 1), reinforcing that the ubiquitination levels detected on TRIM32 wt were due to auto-ubiquitination.

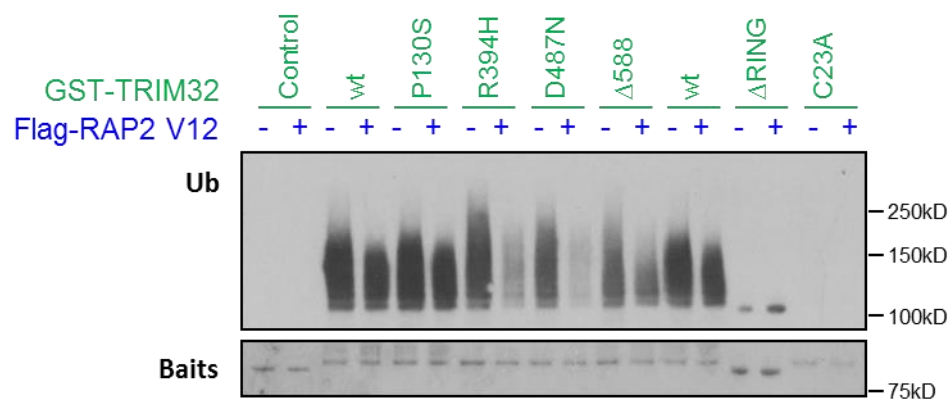
When comparing the effect of the disease mutants mentioned previously on the ubiquitin levels of TRIM32, different patterns of ubiquitination can be seen (Figure



31B). TRIM32 wt and P130S (Figure 31B, lanes 2-3) show a single band at the lower molecular weight that is not present in R394H, D487N or  $\Delta$ 588 (Figure 31B, lanes 4-6). This band correlates to the second band seen on the baits and is also indicative of mono-ubiquitination as the band shift correlates to  $\sim$ 8.5kDa. This suggests that the wildtype (wt) and BBS mutant mono-ubiquitinate TRIM32, whereas mutations from LGMD2H abrogate the mono-(auto)-ubiquitination of TRIM32. We then proceeded to explore whether RAP2 could modulate this effect, and whether this modulation was also mutation dependent.

### RAP2 V12 variably effects auto-ubiquitination of TRIM32 mutants

In order to determine whether the RAP2-TRIM32 interaction plays a role in the modulation of TRIM32-dependent ubiquitination, HEK-293T cells were transiently transfected with TRIM32 wt or mutants either with or without RAP2 V12. GST-TRIM32 was purified with glutathione beads and associated HA-ubiquitin measured with by Western blotting using an anti-HA- antibody.



**Figure 32: Effect of RAP2 V12 on TRIM32 mutant auto-ubiquitination:** Results demonstrate variation in the ubiquitination levels. The TRIM32 R394H and D487N mutants show longer ubiquitin bands, indicating more ubiquitin molecules associated with the proteins, however, the bands are less intense than the TRIM32 wt and P130S mutants. Once again, TRIM32  $\Delta$ RING and C23A mutants do not show ubiquitination.

HEK-293T cells were co-transfected with HA-Ub, GST-TRIM32 wt or mutants, with or without Flag-RAP2 V12 as specified. Lysates were taken on day 3 and samples were pulled-down using Glutathione-beads. Proteins were separated using SDS-PAGE gel and transferred to PVDF membrane. Protein levels were detected using HA- antibody. Shoc2 constructs were used as an unrelated control.

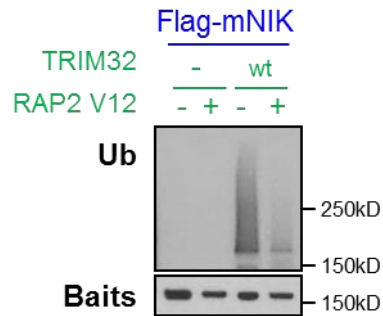
Here again, we can see auto-ubiquitination of TRIM32 but no ubiquitination in the control lane (Figure 32, lanes 1 and 2) but only when the TRIM32 RING domain was intact, i.e. ubiquitination was impaired on  $\Delta$ RING and C23A mutants (Figure 32, lanes 3-18). Looking at the remaining samples, we can again identify stronger bands in the wt and P130S mutants (lanes 3-4 and 5-6, respectively), and the weaker bands seen in R394H, D487N and  $\Delta$ 588 mutants (Figure 32; lanes 7-8, 9-10, and 11-12, respectively). In all cases, the presence of RAP2 noticeably reduced the level of ubiquitin detected, even with the mutations where we have disrupted the TRIM32-RAP2 interaction. For the R394H and D487N mutants (Figure 32, lanes 8 and 10, respectively), the TRIM32 auto-ubiquitination is almost completely reversed, while  $\Delta$ 588 shows an intermediate effect (Figure 32, lane 11). This could somehow link back to the fact that the  $\Delta$ 588 mutant disrupts the TRIM32-RAP2 interaction only partially, and not as much as the other LGMD2H mutants (Figure 27). This suggests that the RAP2 effect on the auto-ubiquitination of TRIM32 is independent of the RAP2-TRIM32 interaction that was shown previously.

Although we do not see the same pattern as Figure 31B, the GST-tag was used, adding 26kDa to the protein size. As a result, it is likely that the 8.5kDa shift from the mono-ubiquitination is no longer distinguishable from the ubiquitination smear.

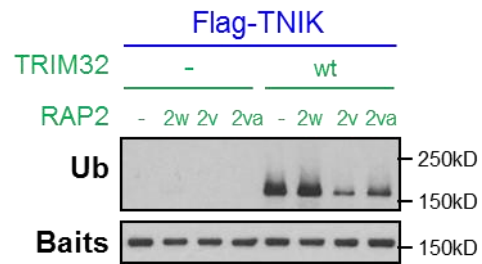
### **Effect of RAP2 on MINK, TNIK and NIK TRIM32-dependent Ubiquitination**

To test the possibility that RAP2 could modulate the ability of TRIM32 to ubiquitinate other RAP2 effectors within the same signalling complex, triple transfections were done in which the ability of TRIM32 to ubiquitinate other proteins was measured in the absence or presence of RAP2 (Figure 33).

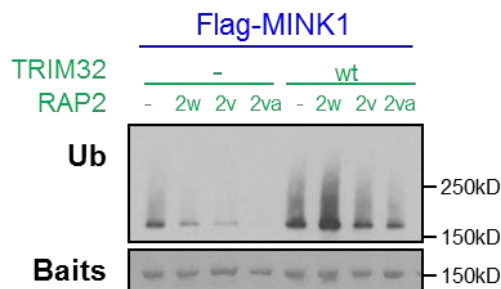
A)



B)



C)



**Figure 33: RAP2 modulates the TRIM32 dependent ubiquitination of mNIK, TNIK and MINK1.** Overexpression of TRIM32 wt and RAP2 wt, V12 (active) mutant and RAP2 V12A38 (defective effector domain) mutant, with either A) mNIK, B) TNIK, C) MINK1 were used to assess the effect on TRIM32 dependent ubiquitination of mNIK, TNIK and MINK1 respectively. A) TRIM32 is required for ubiquitination to be detected and co-expression of RAP2 V12 reduces

this effect. B) TRIM32 is required for ubiquitination to be detected. Co-expression of RAP2 wt slightly enhances the TRIM32-dependent TNIK ubiquitination, however, the expression of RAP2 V12 and RAP2 V12A38 reduces the TRIM32 dependent ubiquitination of TNIK. C) TRIM32 enhances the ubiquitination of MINK above background ubiquitination is detected.

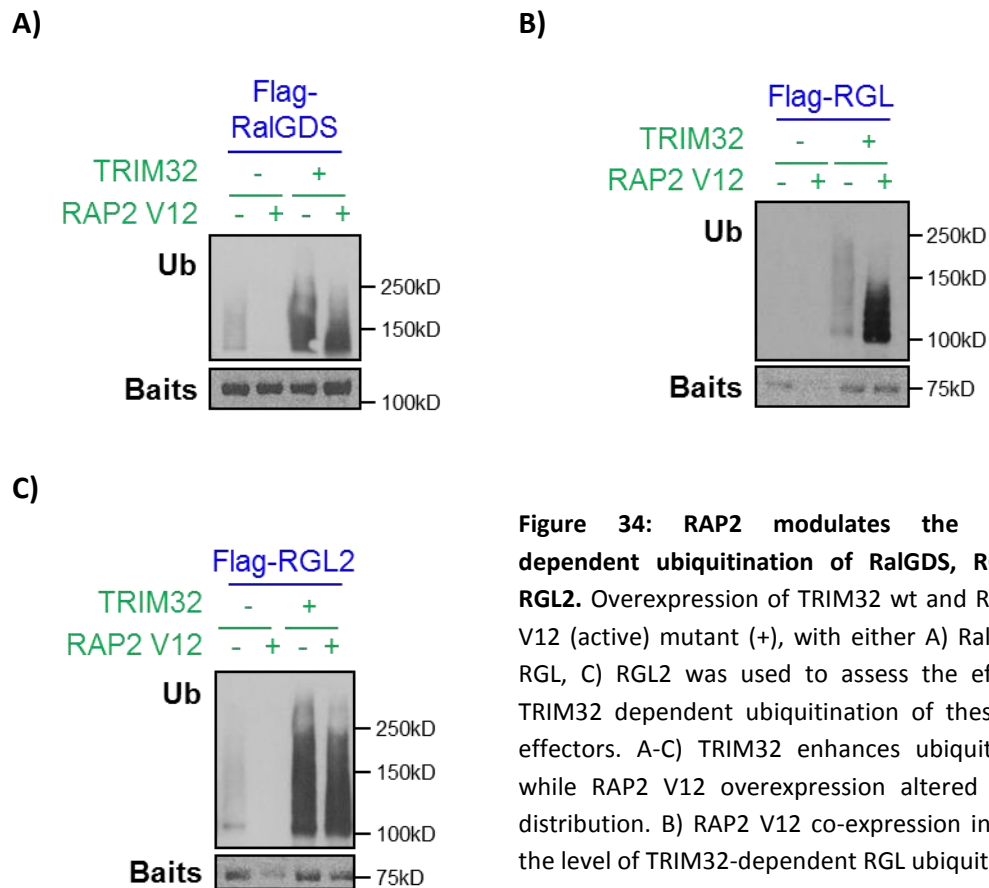
2w = RAP2 wt, 2v = RAP2 V12, 2va = RAP2 V12A38

TRIM32 stimulates and the constitutively active form of RAP2 (2v) noticeably reduced the level of TRIM32-mediated ubiquitination of NIK (RAP2 V12, Figure 33A, lane 4, lane 3), although still above the levels seen in the absence of TRIM32 (lanes 1 and 2). Apparently, NIK ubiquitination was also TRIM32 dependent (Figure 33A).

While TNIK and MINK1 ubiquitination clearly increased in the presence of TRIM32 (Figure 33B-C, lanes 5-8), the addition of RAP2 V12 reduced these ubiquitination levels (Figure 33B-C, lane 7). In clear contrast, the addition of RAP2 wt stimulates the TRIM32-mediated ubiquitination of TNIK and MINK (Figure 33B-C, lane 6), suggesting that the preferential binding of TNIK and MINK to RAP2 wt is relevant to their ubiquitination by TRIM32.

## The TRIM32 dependent ubiquitination of RalGDS, RGL and RGL2 is modulated by RAP2 V12

Subsequently, TRIM32 dependent ubiquitination of the RAP2 effectors RalGDS, RGL and RGL2 was assessed and the effect of RAP2 evaluated.



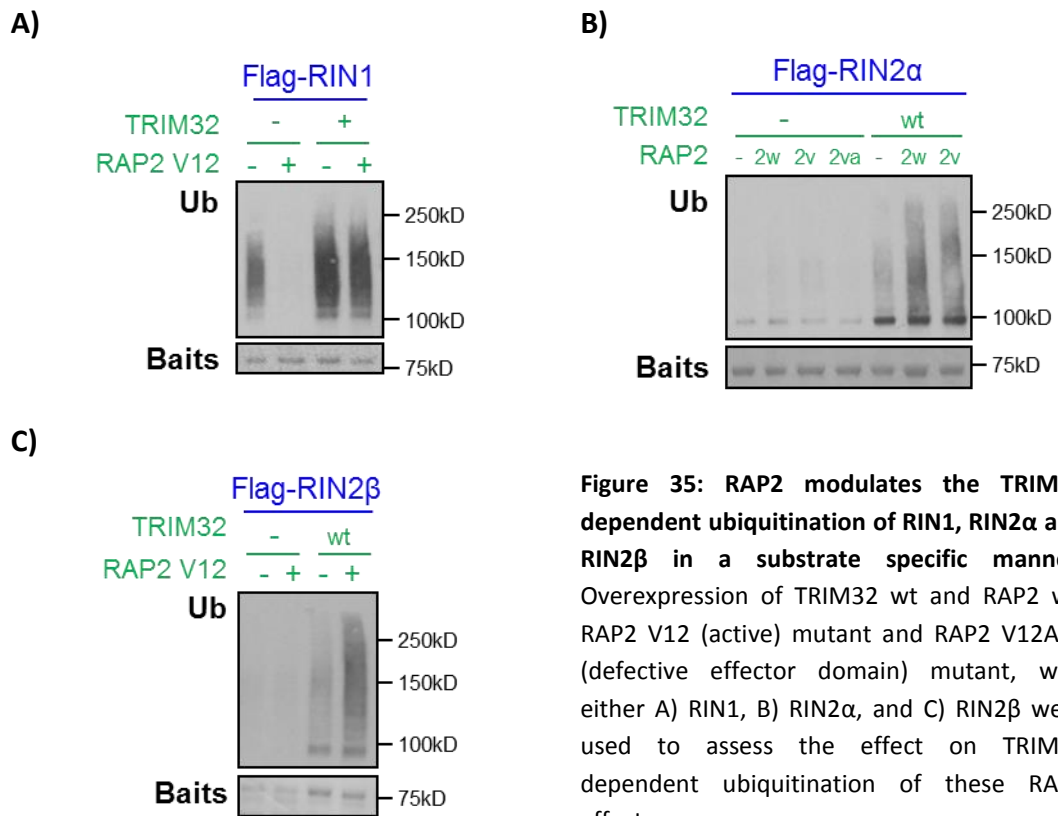
**Figure 34: RAP2 modulates the TRIM32 dependent ubiquitination of RalGDS, RGL and RGL2.** Overexpression of TRIM32 wt and RAP2 wt, V12 (active) mutant (+), with either A) RalGDS, B) RGL, C) RGL2 was used to assess the effect on TRIM32 dependent ubiquitination of these RAP2 effectors. A-C) TRIM32 enhances ubiquitination, while RAP2 V12 overexpression altered the Ub distribution. B) RAP2 V12 co-expression increased the level of TRIM32-dependent RGL ubiquitination.

The ubiquitination of RalGDS, RGL and RGL2 is stimulated in the presence of TRIM32 (Figure 34A-C, lanes 3 and 4). Addition of RAP2 V12 decreased the level of TRIM32-dependent ubiquitination of RalGDS but increased the TRIM32-dependent ubiquitination of RGL with no detectable effect on RGL2 (Figure 34, lanes 3 and 4).

## TRIM32 dependent ubiquitination of RIN1 and RIN2

Having demonstrated RIN protein interaction with both RAP2 and TRIM32; the effect of these on RIN ubiquitination was then assessed by overexpressing RIN1 or RIN2, with or without TRIM32, plus or minus various RAP2 mutants, as indicated. These samples were transiently transfected and lysates taken after 48h, and

immunoprecipitated by the Flag-tagged RIN protein. Ubiquitination levels were detected using the HA-ab, for the HA-tag on the ubiquitin protein.



**Figure 35: RAP2 modulates the TRIM32 dependent ubiquitination of RIN1, RIN2α and RIN2β in a substrate specific manner.** Overexpression of TRIM32 wt and RAP2 wt, RAP2 V12 (active) mutant and RAP2 V12A38 (defective effector domain) mutant, with either A) RIN1, B) RIN2α, and C) RIN2β were used to assess the effect on TRIM32 dependent ubiquitination of these RAP2 effectors.

A-B) TRIM32 enhanced ubiquitination, while RAP2 wt and mutants effected the distribution of the Ub band, C) TRIM32 slightly increased the ubiquitination of RIN2β which was further enhanced by co-expression of RAP2 V12.

2w = RAP2 wt, 2v = RAP2 V12, 2va = RAP2 V12A38

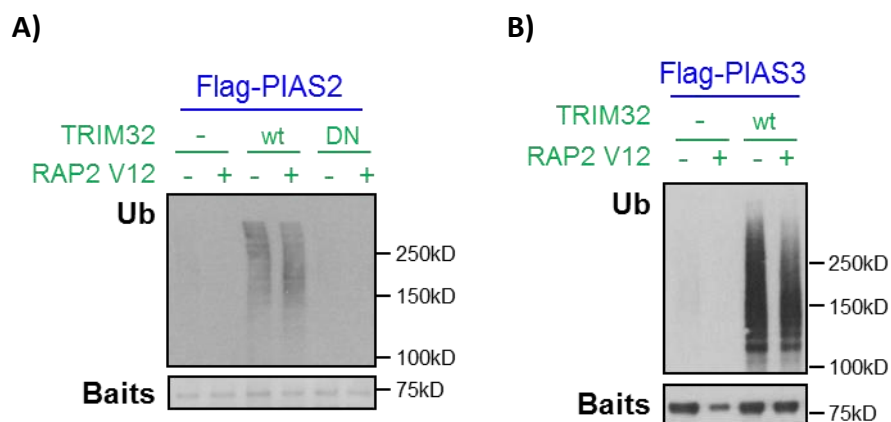
RIN1 showed some basal levels of ubiquitination in the absence of TRIM32 and RAP2 (Figure 35A, lane 1), which was abrogated by the addition of RAP2 V12 (Figure 35A, lane 2). Addition of TRIM32 wt dramatically increased (Figure 35A, lanes 3 and 4), whereas RAP2 V12 had little to no effect on the level of ubiquitination (Figure 35A, lane 4). These findings show that the TRIM32-independent ubiquitination of RIN1 is decreased by RAP2 overexpression. RAP2 expression has no effect on the TRIM32-dependent ubiquitination (Figure 35A).

RIN2α ubiquitination required TRIM32 wt (Figure 35B, lanes 5-8) and was increased in the presence of RAP2. Interestingly, despite RIN2α interaction with RAP2 being shown to occur in an activation- and effector domain dependent manner, RAP2 wt showed stronger ubiquitination of RIN2α than RAP2 V12 (Figure 35B, lane 6-7).

Similarly, RIN2 $\beta$  ubiquitination also increased in the presence of TRIM32 and RAP2 V12 (Figure 35C).

### The TRIM32 Dependent Ubiquitination of PIAS2 and PIAS3 is altered by the presence of RAP2 and is disrupted by the TRIM32 D487N mutant

The effect of TRIM32 and RAP2 on the ubiquitination of PIAS2 and PIAS3, published effectors of RAP2, was then determined by overexpressing TRIM32 wt or D487N mutant and RAP2 V12 in HEK-293T cells.

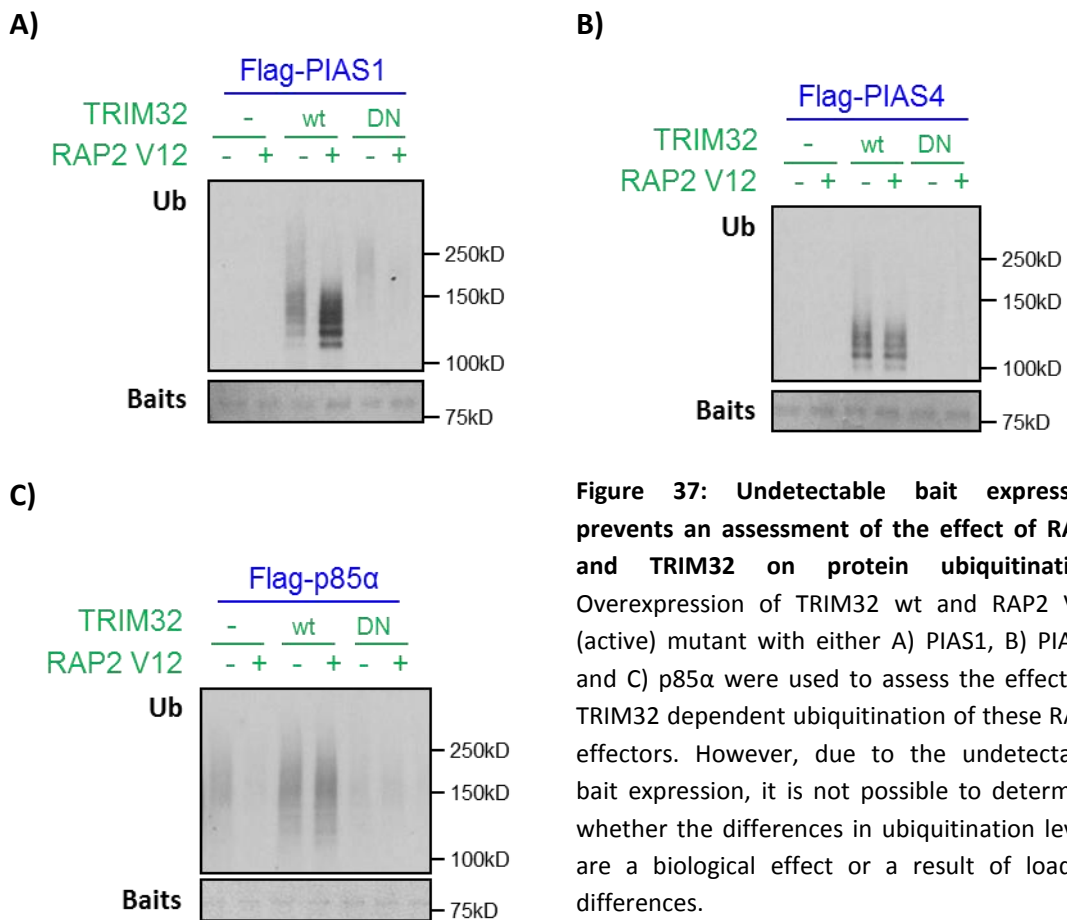


**Figure 36: RAP2 alters the TRIM32 dependent ubiquitination of PIAS1 and PIAS2.** Overexpression of TRIM32 wt and RAP2 wt, V12 (active) mutant and RAP2 V12A38 (defective effector domain) mutant, with either A) PIAS2, B) PIAS3 were used to assess the effect on TRIM32 dependent ubiquitination of these RAP2 effectors. A-B) TRIM32 is required for ubiquitination to be detected, while RAP2 V12 effects the distribution of the Ub band. A) Overexpression with the TRIM32 D487N mutant does not result in ubiquitination of PIAS2 (data not available for PIAS3). 2w = RAP2 wt, 2v = RAP2 V12, 2va = RAP2 V12A38, DN = TRIM32 D487N mutant

In PIAS2 and PIAS3 the presence of TRIM32 wt increases the level of ubiquitination of the respective proteins (Figure 36, lanes 3 and 4). In PIAS2 this effect is abrogated when TRIM32 D487N is used (Figure 36, lanes 5 and 6) (data not available for PIAS3). This suggests that TRIM32 D487N is unable to induce TRIM32-dependent ubiquitination of PIAS2. RAP2 V12 had little effect on the ubiquitination of PIAS2 or PIAS3 (Figure 36A-B).

## Inconclusive data on the effect of RAP2 and TRIM32 on protein ubiquitination

Due to previous data (shown above) and information derived from the literature, several other interactions were also tested to assess the effect of RAP2 on their TRIM32 dependent ubiquitination. Unfortunately due to bait issues (Figure 37) and problems with reproducibility (Figure 38), we were unable to draw conclusions from this data, but these have been included below.



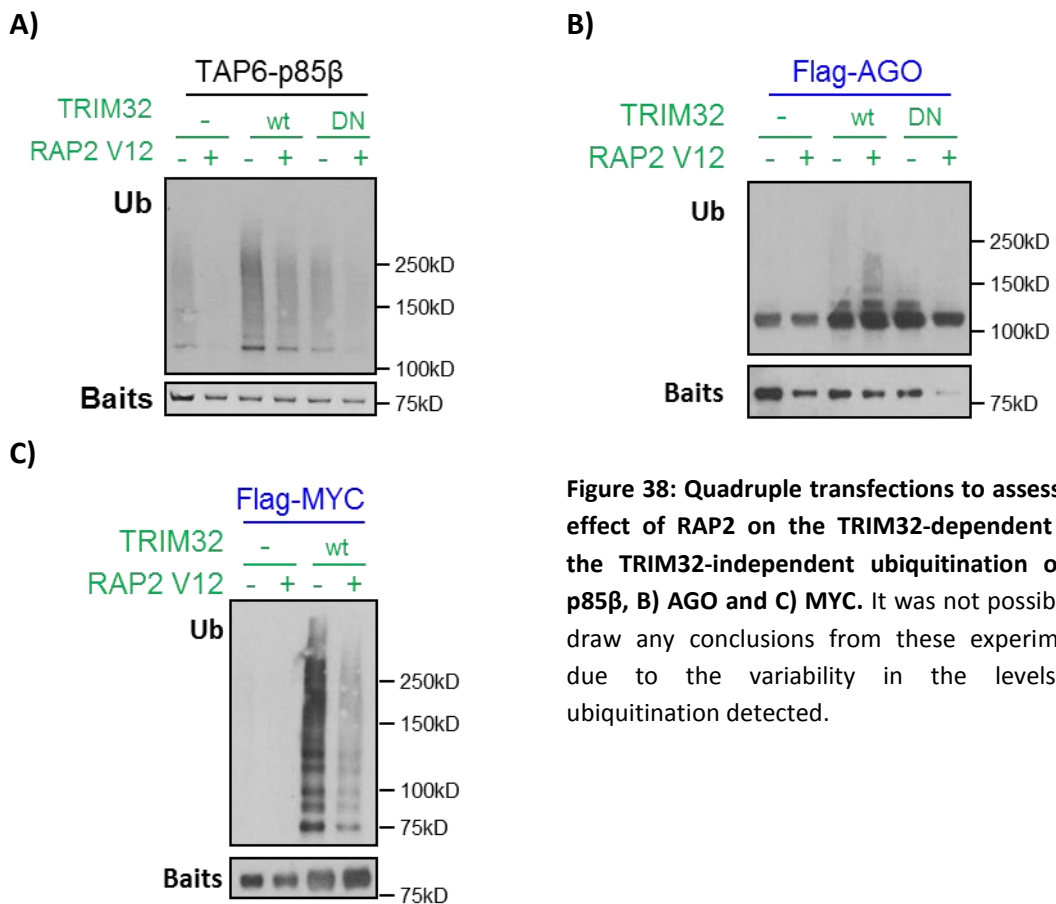
**Figure 37: Undetectable bait expression prevents an assessment of the effect of RAP2 and TRIM32 on protein ubiquitination.** Overexpression of TRIM32 wt and RAP2 V12 (active) mutant with either A) PIAS1, B) PIAS4, and C) p85α were used to assess the effect on TRIM32 dependent ubiquitination of these RAP2 effectors. However, due to the undetectable bait expression, it is not possible to determine whether the differences in ubiquitination levels are a biological effect or a result of loading differences.

DN = TRIM32 D487N mutant

Overexpression of TRIM32 wt and RAP2 V12 (active) mutant with either A) PIAS1, B) PIAS4, and C) p85α were used to assess the effect on TRIM32 dependent ubiquitination of these RAP2 effectors (Figure 37). However, due to the undetectable bait expression, it is not possible to determine whether the differences in ubiquitination levels are a biological effect or a result of loading differences.

Furthermore, an assessment of the effect of TRIM32 on the ubiquitination of p85β, AGO and MYC also yielded inconclusive results due to the variability in the

ubiquitination detection patterns (Figure 38), suggesting that the ubiquitination of these proteins is very sensitive to variation.

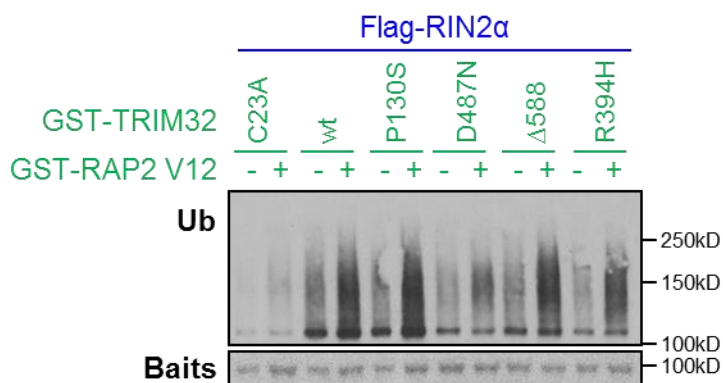


**Figure 38: Quadruple transfections to assess the effect of RAP2 on the TRIM32-dependent and the TRIM32-independent ubiquitination of A) p85 $\beta$ , B) AGO and C) MYC.** It was not possible to draw any conclusions from these experiments due to the variability in the levels of ubiquitination detected.

### Effect of TRIM32 mutations on RIN2 $\alpha$ Ubiquitination

Subsequently, the effect of different TRIM32 mutants on RIN2 $\alpha$  ubiquitination levels was assessed. RAP2 V12 was also tested to ascertain whether it modulates the TRIM32 dependent ubiquitination of RIN2 $\alpha$  and whether this modulation is affected by the TRIM32 mutants.





**Figure 39: RAP2 modulates the TRIM32 mutant ubiquitination of RIN2 $\alpha$ .** Overexpression of TRIM32 wt and mutants, with or without RAP2 V12, with RIN2 $\alpha$ , was used to assess the effect of RAP2 V12 on TRIM32 mutant ubiquitination of RIN2 $\alpha$ .

+ = RAP2 V12, C23A = disrupts RING domain E3 ligase activity, P130S = mutation in BBS, D487N,  $\Delta$ 588, R394H = LGMD2H mutations.

Again, not surprisingly, we see that the disruption of the RING domain via a C23A mutation disrupted the TRIM32-dependent ubiquitination of RIN2 $\alpha$  (Figure 39, lanes 1-2). The addition of TRIM32 increased RIN2 $\alpha$  ubiquitination, with a greater effect seen with the wt than the disease mutants (Figure 39, lanes 3-12). This effect is further enhanced in the presence of RAP2 V12 (Figure 39, lanes 4, 6, 8, 10 and 12), by varying degrees depending on the mutant, suggesting that the TRIM32-dependent ubiquitination of RIN2 $\alpha$  is enhanced by RAP2 V12. Notably, the RAP2 V12-dependent increase in ubiquitination is abrogated when the TRIM32 D487N mutation is present and, to a lesser extent, is partially disrupted in the presence of the R394H and  $\Delta$ 588 mutations (Figure 39, lanes 7-12).

## Summary

We demonstrated that TRIM32 enhances the level of RAP2 ubiquitination, indicating that as a result of its direct interaction with TRIM32 RAP2 activity could also be regulated by TRIM32. RAP2 ubiquitination by Nedd4-1 has been previously shown to control the function of RAP2A by perturbing interactions between GTP-bound RAP2A and downstream targets (Kawabe, Neeb et al. 2010), this suggests that the TRIM32 ubiquitination of RAP2 may be regulating it's downstream effects on the putative effectors we have identified above.

Our data suggests that the TRIM32 disease mutantations affect the degree to which TRIM32-dependenent ubiquitination occurs, demonstrated by the varying degrees to which ubiquitin is immunoprecipitated by these overexpressed constructs. The

TRIM32 wt and BBS mutant show TRIM32 ubiquitination at a lower band by western blot, possibly mono-ubiquitination, this is not detected in the LGMD2H mutants. Additionally, GST-coomassie we detected laddering that correlated to ubiquitination. This could be mono-, di- and tri- ubiquitination or mono-ubiquitination at multiple sites, although the nature of the ubiquitination was not investigated further.

TNIK and MINK1 ubiquitination clearly increased in the presence of TRIM32 (Figure 33), and the additional expression of the RAP2 V12 reduced these ubiquitination levels. However, the addition of RAP2 wt stimulates the ubiquitination of TNIK and MINK even further. TNIK and MINK binding to RAP2 wt is much stronger than to RAP2 V12, and RAP2 wt enhances the ubiquitination of TNIK and MINK. This suggests that the preferential binding of TNIK and MINK to RAP2 wt is relevant to their ubiquitination state and the inability or weak binding to RAP2 V12 reduces the ubiquitination of TNIK and MINK, possibly due to RAP2 targeting the ubiquitination to another effector with a higher affinity to RAP2 V12. The ubiquitination of RaIGDS, RGL and RGL2 all required the presence of TRIM32, while RAP2 V12 enhanced the ubiquitination of RGL (Figure 34). As RaIGDS, RGL and RGL2 all interact more strongly with RAP2 V12 than wt, this supports the possibility that stronger interaction with RAP2 enhances the ubiquitination of protein.

Having demonstrated RIN protein interaction with both RAP2 and TRIM32; the effect of these on RIN ubiquitination was then assessed. RIN1 showed some basal levels of ubiquitination in the absence of TRIM32 and RAP2 which were no longer detected on the overexpression of the RAP2 V12 construct. Addition of TRIM32 wt dramatically increased, whereas RAP2 V12 decreased the level of ubiquitination (Figure 35).

RIN2 $\alpha$  ubiquitination required TRIM32 wt and was increased in the presence of RAP2. RAP2 wt showed stronger ubiquitination of RIN2 $\alpha$  than RAP2 V12, despite RIN2 $\alpha$  interaction with RAP2 being shown to occur in an activation- and effector domain dependent manner. Similarly, RIN2 $\beta$  ubiquitination also increased in the presence of TRIM32 and RAP2 V12 (Figure 35).

The overexpression of TRIM32 was shown to increase RIN2 $\alpha$  ubiquitination (Figure 39), with a greater effect seen with the wt than the disease mutants (Figure 39). This effect was further enhanced in the presence of RAP2 V12 (Figure 39), by varying degrees depending on the mutant. This suggests that the TRIM32 and RAP2-dependent increase in ubiquitination levels is affected and/or disrupted by the TRIM32 mutants. Notably, the RAP2 V12-dependent increase in ubiquitination is partially disrupted when the TRIM32 D487N mutant is used (Figure 39), which would be expected as we have previously shown that it no longer interacts with RAP2 V12 (Figure 39).

In PIAS2 and PIAS3 the presence of TRIM32 wt increases the level of ubiquitination of the respective proteins. In PIAS2 this effect is abrogated when TRIM32 D487N is used (data not available for PIAS3). This suggests that TRIM32 D487N is unable to induce ubiquitination of PIAS2, possibly linked to its inability to interact with RAP2 V12 (Figure 36).

For some of the experimental systems (e.g. Figure 38) there was a lot of variations in the expression levels seen and the effect of RAP2 on the TRIM32 modulated ubiquitination. This could be a result of many factors such as experimental sensitivity. In order to reduce the variation, upscaling the experiment to use more sample could reduce the margin for error and thus any variability that may have been introduced into the system.

# Chapter 7: The Effect of TRIM32 and RAP2 on Cellular Kinetics

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## Localisation

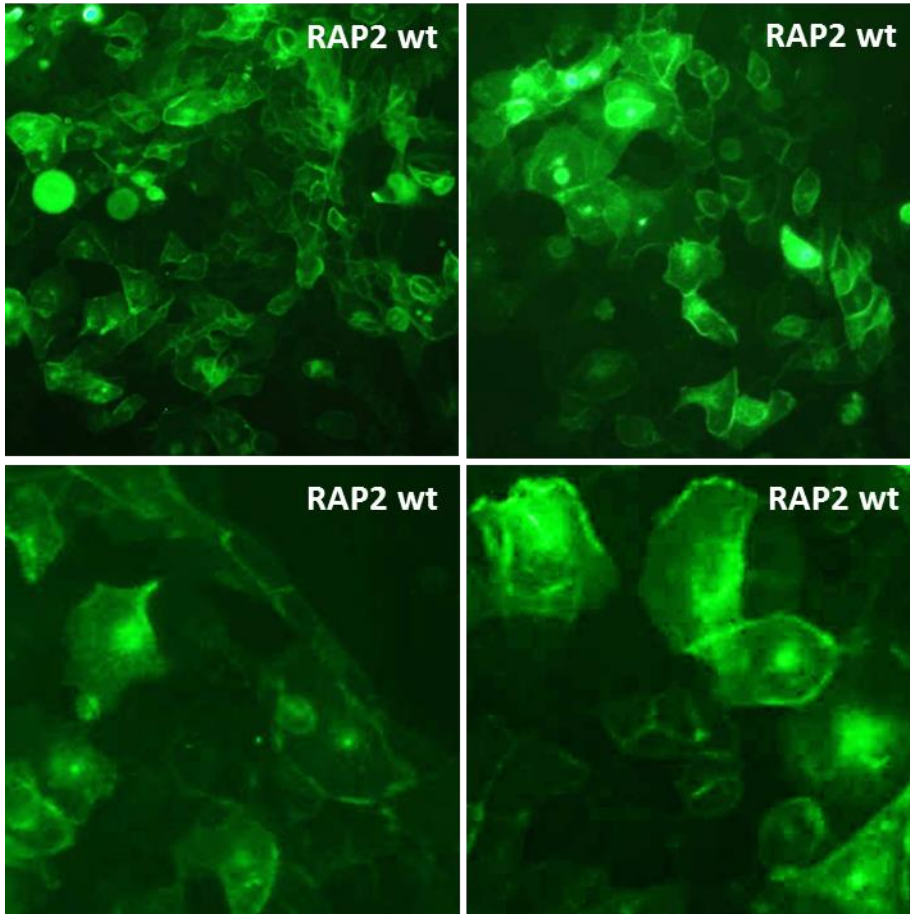
In the literature, RAP2 has been shown to localise primarily in the recycling endosomes, as well as the Golgi and plasma membrane (Uechi, Bayarjargal et al. 2009; Gloerich, ten Klooster et al. 2012), whereas TRIM32 localisation has been demonstrated in cytoplasmic bodies, often around the nucleus (Locke, Tinsley et al. 2009).

RAS family GTPases are known to regulate the function of at least some of their effectors by recruiting them, upon activation, to specific membrane compartments and/or signalling complexes. For example, H/K/NRAS activate RAF kinases by recruiting them to the plasma membrane microdomains where other activating steps then take place (McKay and Morrison 2007). Similarly, MRAS recruits the SHOC2-PP1 complex to regulate the activity of RAF kinases at specialised signalling complexes (Rodriguez-Viciano, Oses-Prieto et al. 2006). We speculated that by analogy with other GTPases of the family, RAP2 may recruit TRIM32 to specific complexes at some membrane compartment upon activation. In order to test this, we first set out to investigate the localisation of both RAP2 and TRIM32 in several cell types to set the stage for potential co-localisation studies.

## RAP2 localisation in HMLE and U2OS cells

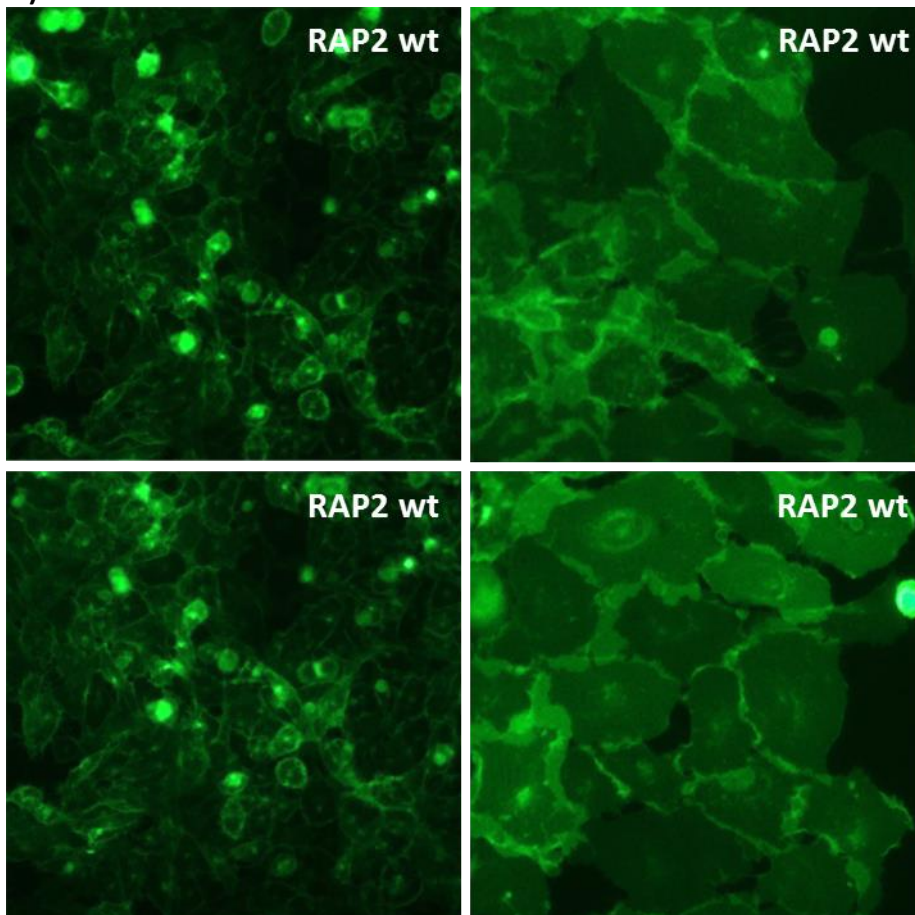
By transducing the cells with overexpressed YFP-tagged proteins and fixing cells as described in the methods section, the localisation of RAP2 and TRIM32 was examined in various cell lines. Initial experiments were conducted in human mammary epithelial HMLE cells (Elenbaas, Spirio et al. 2001) (Figure 40).

**A) HMLE**



**Figure 40: RAP2 wt localisation in HMLE and U2OS cells.** Cells were transduced with YFP-tagged RAP2 as described in the methods section and images were taken of live cells immediately after selection. In both HMLE and U2OS cells, RAP2 localises predominantly at the plasma membrane and appear enriched at areas of cell-cell contact. RAP2 = green. A) HMLE, B) U2OS.

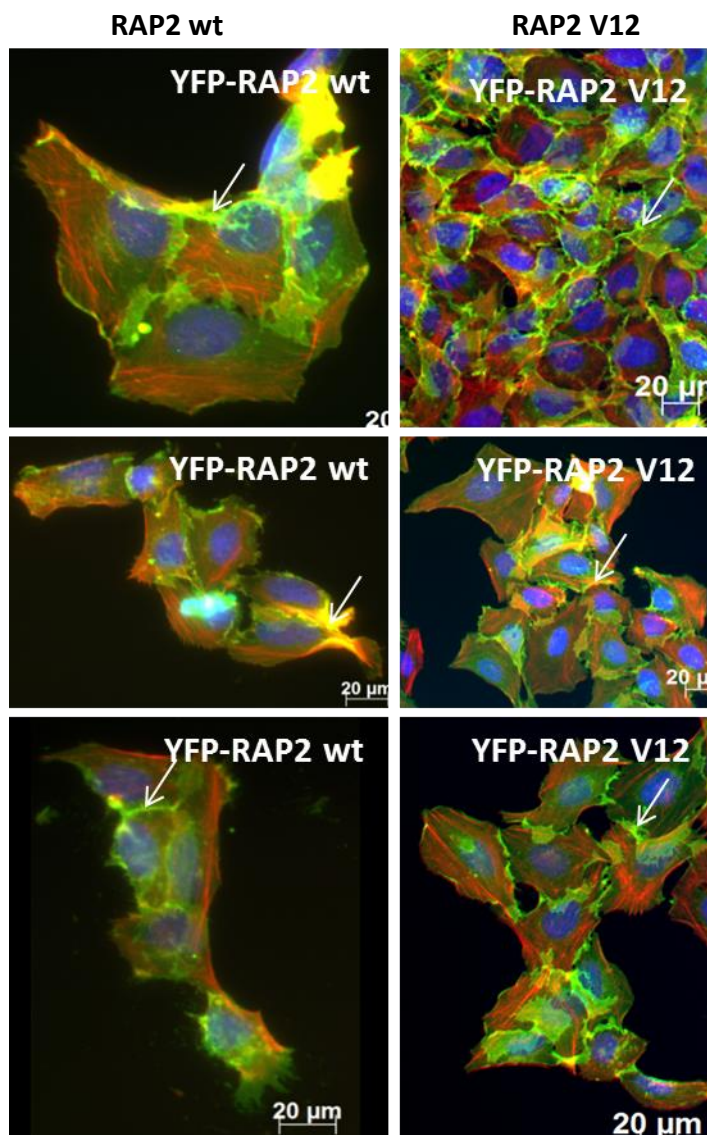
**B) U2OS**



YFP-RAP2 localises predominantly at the plasma membrane and appears enriched at areas of cell-cell contact in both HMLE cells and U2OS cells (Figure 40A-B). This appearance of a higher concentration of RAP2 may also be a result of seeing the more plasma membrane being visualised from above. This is in clear contrast to the results of Uechi et al showing predominant endosomal and Golgi localisation in COS-1 (monkey kidney tissue) cells (Uechi, Bayarjargal et al. 2009), suggesting the possibility of tissue dependent localisation.

### RAP2 wt and mutant localisation in U2OS osteosarcoma cells

The localisation of YFP-RAP2 wt and mutant proteins was assessed in fixed U2OS osteosarcoma cell lines that were costained for actin (Phalloidin-red) and Hoechst nuclear staining (Figure 41).

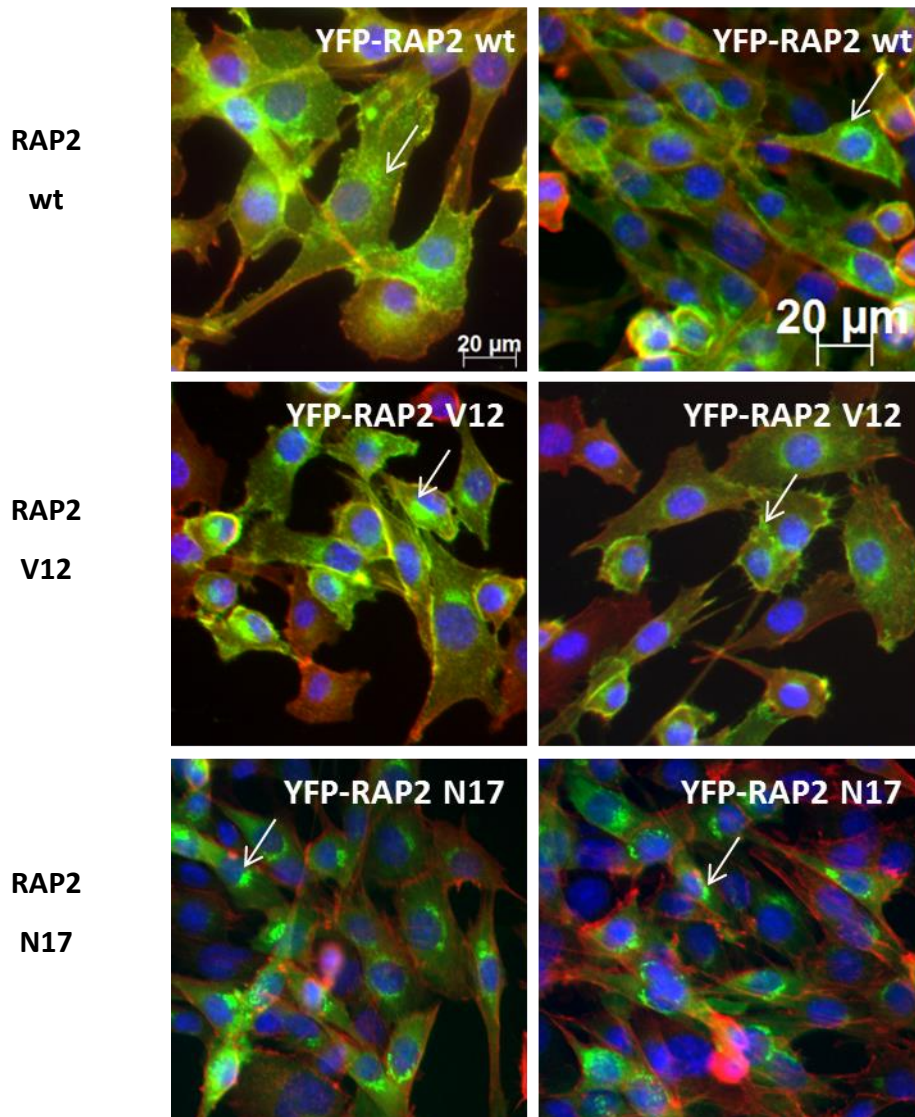


**Figure 41: Localisation of RAP2 and TRIM32 in U2OS cells.** U2OS cells were transduced with YFP-tagged overexpression constructs (green) and subsequently fixed and immunostained with Hoechst (blue) and Phalloidin (red). RAP2 expression is highest at areas of cell-cell contact.

Looking at the U2OS RAP2 wt and V12 staining, we can see higher concentrations of RAP2 at areas of cell-cell contact, indicating localisation to the cell membrane. Contrary to the HMLEs, no perinuclear staining is seen (Figure 41).

### RAP2 localisation in mouse myoblast cells

Due to the suggested involvement of the RAP2-TRIM32 interaction in LGMD2H, the localisation of RAP2 was investigated in the C2C12 mouse myoblast cell line.

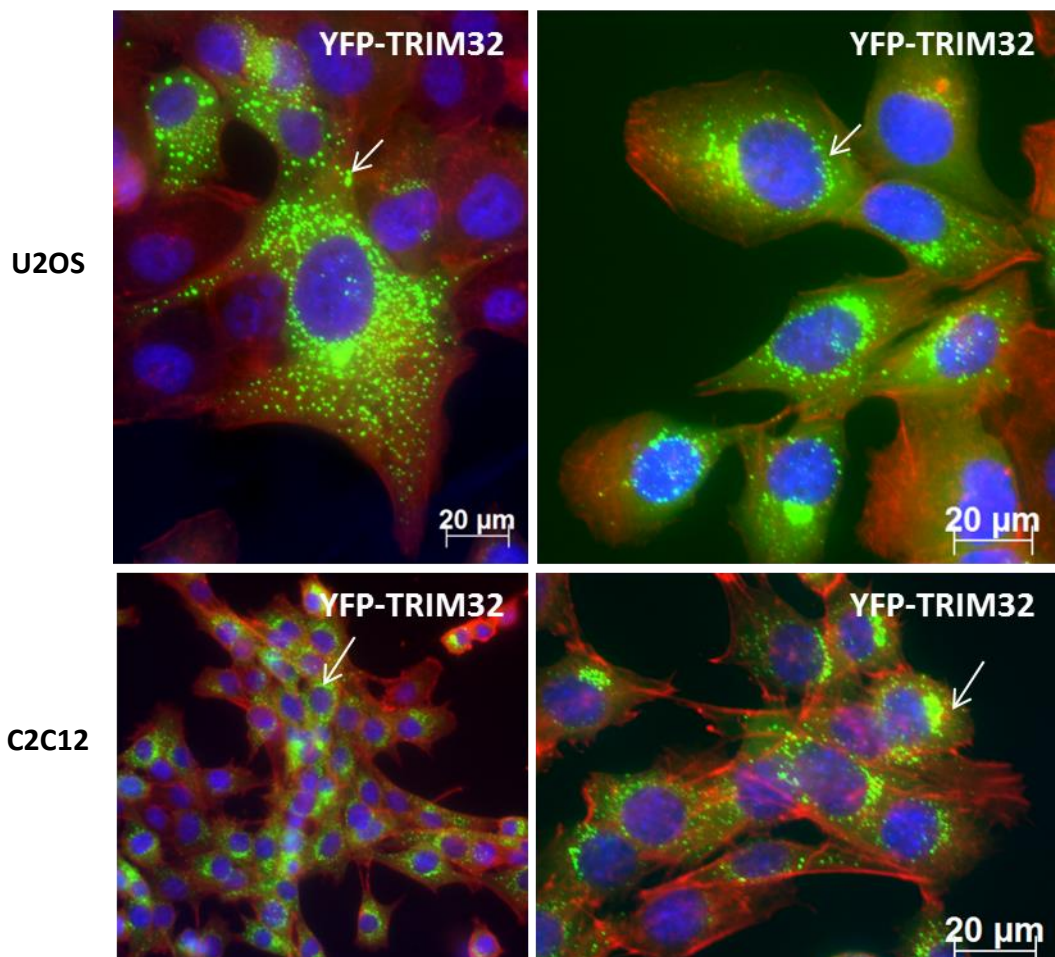


**Figure 42: RAP2 localisation in mouse myoblast cell line.** C2C12 cells were transduced with YFP-tagged protein using lentivirus and fixed after selection. RAP2 wt and V12 localises to areas of cell-cell contact as before, but higher levels are also expressed in the perinuclear region. RAP2 = green, Hoechst = Blue, Phalloidin = Red.

In the C2C12 cells, YFP-RAP2 wt and V12 localises to the plasma membrane in some cells as seen with U2OS and HMLE cells. However in this cell type much more prominent perinuclear staining consistent with Golgi/ER localisation is observed, although due to lack of time it was not possible to perform Golgi/ER counterstains to confirm this. Distinct dots and/or vesicles are seen in some cells which is consistent with the endosomal localisation reported by Uechi et al (Uechi, Bayarjargal et al. 2009; Gloerich, ten Klooster et al. 2012).

### TRIM32 localisation in osteosarcoma and myoblast cells

In addition to RAP2 localisation, the localisation of TRIM32 wt cells was determined in osteosarcoma and myoblast cells.



**Figure 43: TRIM32 localisation in U2OS cells and C2C12 cells.** U2OS and C2C12 cells, as indicated, were infected with YFP-tagged TRIM32 lentivirus, and cells were fixed after selection. TRIM32 staining is predominantly in the cytosol, around the nucleus. TRIM32 = Green, Hoechst = Blue, Phalloidin = Red.



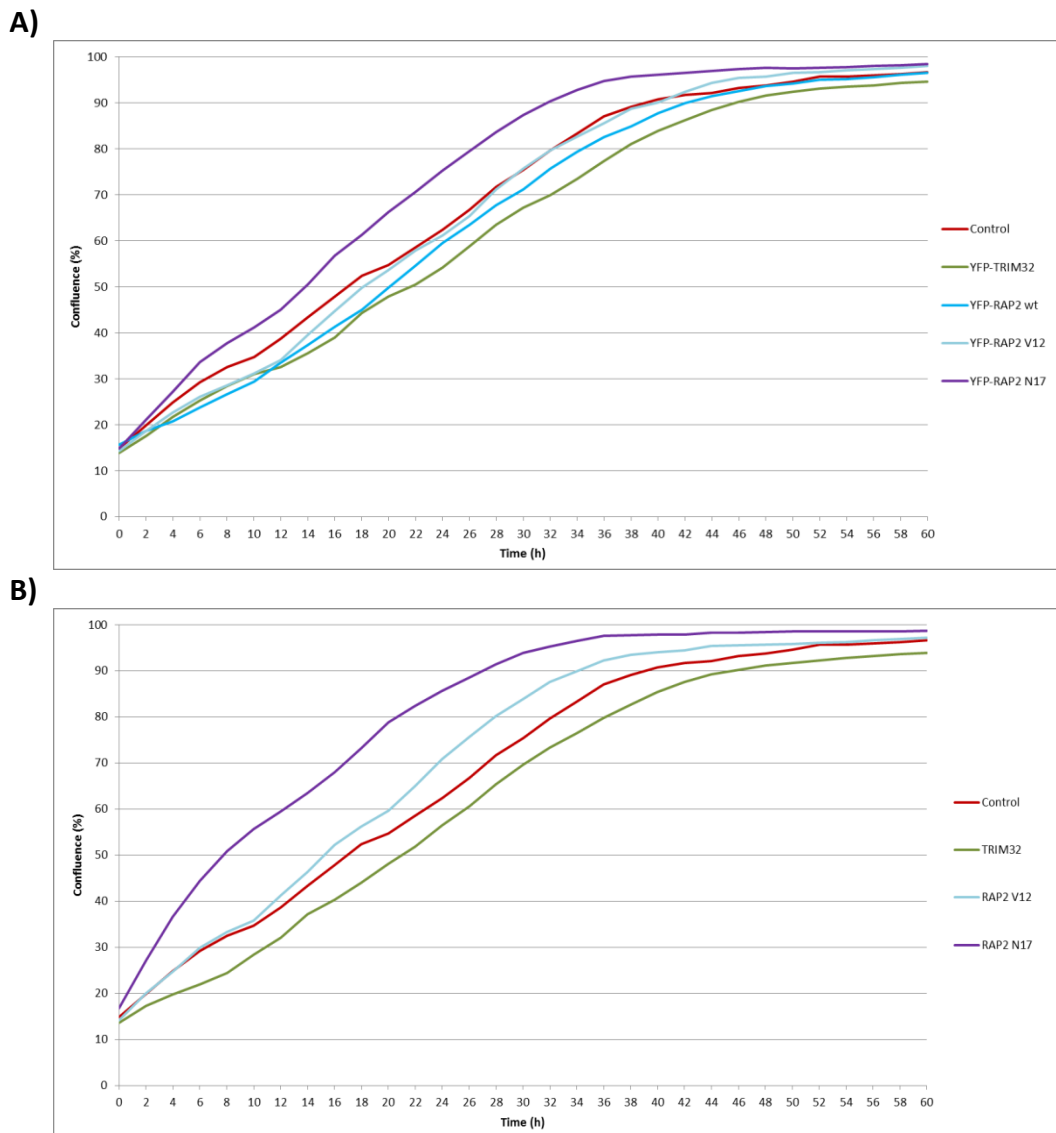
In both U2OS and C2C12 cells, TRIM32 shows punctate staining in the cytosol, mainly clustering around the nucleus (Figure 43). This is consistent with the reported localisation of TRIM32 in cytoplasmic bodies, often around the nucleus (Locke, Tinsley et al. 2009).

## **Proliferation, Motility and Differentiation**

The literature has stated that increased TRIM32 expression can lead to an increase in proliferation, transformation and cell motility via Abi in Hep2 cells (Kano, Miyajima et al. 2008) and that an increase in RAP2 expression can decrease cell spreading in HEK-293T cells (Uechi, Bayarjargal et al. 2009). Additionally, mutations in TRIM32 found in LGMD2H, a disease involving the degeneration of muscles, disrupt the TRIM32 interaction with RAP2. In order to better understand the role of this interaction on a cell type more relevant to muscle biology, we focused on the C2C12 mouse myoblast. C2C12 cells are a subclone (produced by Blau, Pavlath et al. 1985) of the C2 mouse myoblast cell line established by D. Yaffe and O Saxel (Yaffe and Saxel 1977). C2 cells originate from 2-month-old normal mouse thigh muscle, 70h after crush injury. The C2C12 cell line differentiates rapidly, forming contractile myotubes and producing characteristic muscle proteins.

## **Effect of RAP2 and TRIM32 on proliferation of C2C12 cells**

Lentiviral transduction was used to stably overexpress constructs of interest in C2C12 cells (as described in the methods section) and cells were manually counted and seeded in duplicate in 24 well plates. These were then placed into the IncuCyte FLR Live Cell Imaging System (Essen Bioscience) that can be used to follow kinetic cell proliferation, quantify cell migration and record changes in cell morphology. The IncuCyte system allows you to place a microscope inside your incubator, enabling around-the-clock kinetic imaging without the need to move and disturb cells from the controlled environment. It uses a proprietary imaging methodology to acquire phase-contrast images in multi-well plates and a confluence-based algorithm as a surrogate for cell number. Readings were taken measuring percentage confluence at regular intervals until plates reached confluence.



**Figure 44: Effect of RAP2 and TRIM32 on proliferation of C2C12 cells.** C2C12 cells were infected with the indicated lentiviral constructs and growth curves generated using the IncuCyte system, Pictures were taken every 2h with each data point being a composite of 4 different images. A and B show independent experiments. In both instances RAP2 N17 overexpression leads to increased cell proliferation. TRIM32, RAP2 wt and V12 did not show any consistent effect on the proliferation of C2C12 cells.

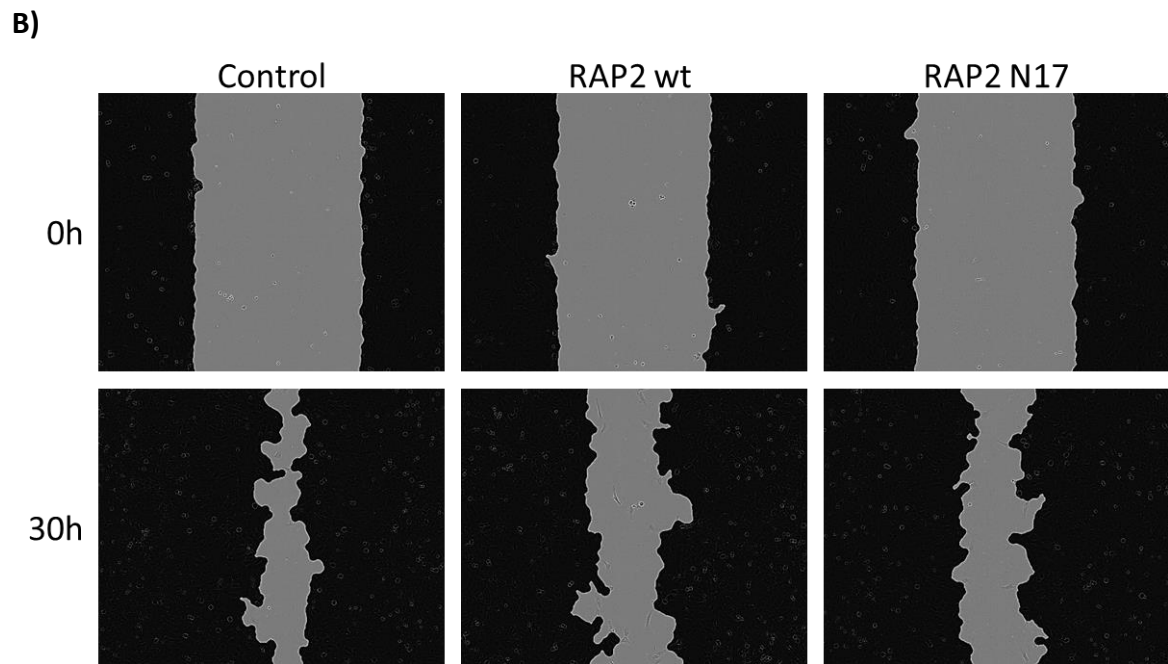
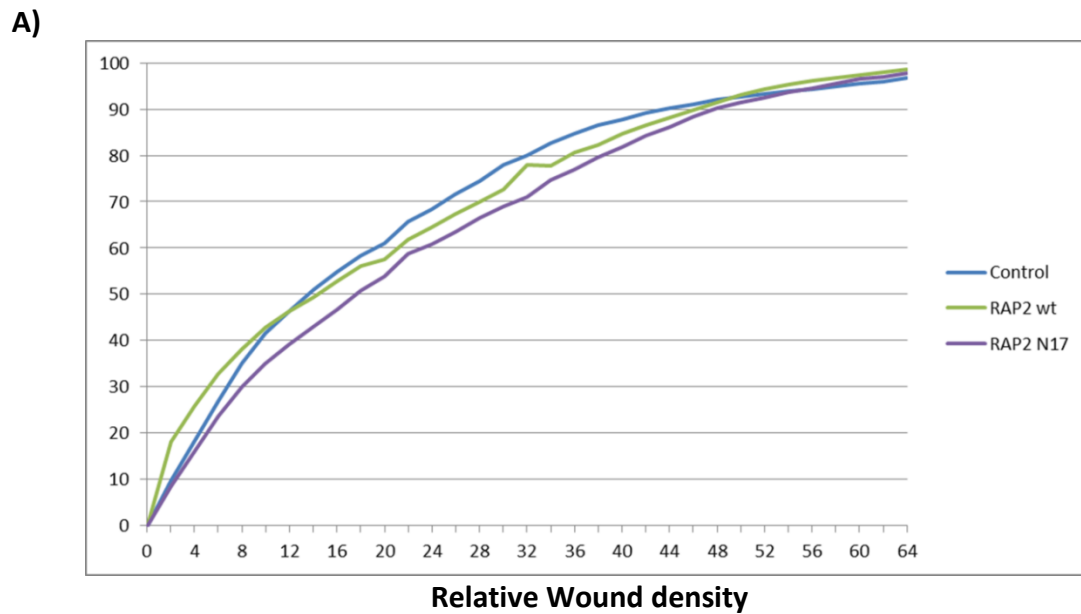
Figure 44A and 1B show the data from independent experiments for C2C12 cells with control and TRIM32 wt, RAP2 wt, RAP2 V12 and RAP2 N17 overexpression. Looking at the initial slope of the curve, it appears as though there may be a slight increase on the rate of proliferation when RAP2 N17 is overexpressed, indicating that RAP2 plays a role in regulating proliferation. Although our preliminary experiments do not show an effect of TRIM32 on proliferation, Kano et al. have

demonstrated that overexpression of TRIM32 in NIH3T3 cells using retroviral transduction significantly increased cell growth as compared to mock transfected and mutant TRIM32 S651A (Kano, Miyajima et al. 2008). NIH3T3 cells naturally express TRIM32. It has been shown in the literature that TRIM32 S651A mutant is unable to bind 14-3-3, whereas it has been identified as a potential TRIM32 effector in our more recent TAP experiments (data above). The work of Ichimura et al., confirmed that its overexpression could not illicit the cell growth activity, suggesting that 14-3-3 binding is important for regulating the function of TRIM32 (Ichimura 2014).

### **Overexpression of RAP2 has no effect on U2OS wound healing**

In order to assess the effect of overexpressed RAP2 on migration, we used stably infected cells, as described in the methods section. These cells were then grown to confluence and scratched using a wound-maker tool (Essen Biosciences) designed to generate consistent and precise wounds on multiwell plates (Figure 45).

Looking at the effect of overexpressed RAP2 wt and RAP2 N17 on wound closure on the plotted data (Figure 45A) there does not appear to be a difference on the rate of migration. Similarly, comparing the wound mask from 30 h, a similar level of closure can be seen (Figure 45, middle row). This is visualised more clearly by the 'final mask' (Figure 45B, bottom row). Unfortunately the data is not available for TRIM32.



**Figure 45: Effects on migration.** Confluent wells of U2OS cells infected with control, RAP2 wt or RAP2 N17 were scratched and wound closure tracked using the Incucyte system. The Incucyte comes with a wound-making tool to generate consistent and precise wounds on multiwell plates. By computing the initial wound mask and using a similar methodology as for kinetic growth assays, the Incucyte tracks and quantifies wound closure. No difference in migration was seen in the U2OS cell line.

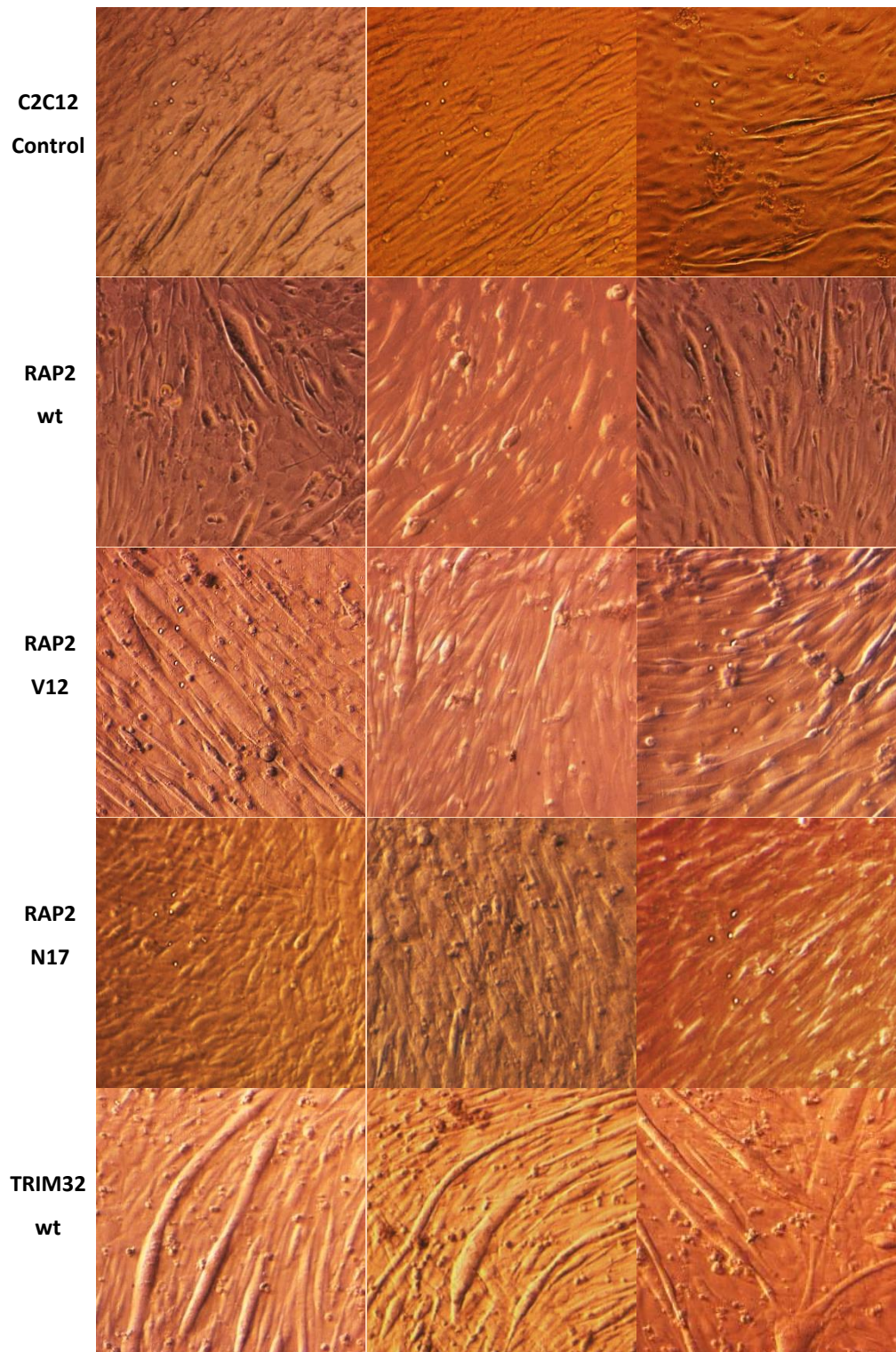
## Effect of RAP2 and TRIM32 wt overexpression on myogenic differentiation

Skeletal muscle differentiation is a highly coordinated multistep process in which mononucleated myoblasts first withdraw from the cell cycle in response to extracellular cues, differentiate into post-mitotic myocytes (early differentiation), and subsequently fuse into multinucleated myotubes (late differentiation) which finally bundle to form mature muscle fibres (terminal differentiation). This process is elaborately controlled by various regulatory factors. Given that the RAP2-TRIM32 interaction is linked to LGMD2H, where muscle weakness and atrophy is caused by mutations of TRIM32, and that TRIM32 is involved in the regulation of differentiation and self-renewal in neural progenitor cells and that in the absence of TRIM32 myogenic differentiation is disrupted (Schwamborn, Berezikov et al. 2009; Nicklas, Otto et al. 2012), we wanted to assess whether an effect can be detected on the differentiation of muscle cells when overexpressing these proteins.

To determine whether RAP2 and TRIM32 play a role in myogenic differentiation, we used lentiviral transduction of Trim32, RAP2 wt, RAP2 V12 (active) and RAP2 N17 (dominant-negative) constructs and monitored morphological differences during cell differentiation (Figure 46).

Upon the differentiation of C2C12 control cells, we can see that the cells have begun to fuse together to form thick, elongated tubules, distinct from the surrounding packed cells (Figure 46, row 1). On longer incubation with the fusion media, more of these can be seen.

Little effect was seen upon overexpression of RAP2 wt and V12 (Figure 46, row 2 and row 3), although there are occasional shorter, fatter cells that can be seen such as in the middle panel, which appear to be cells that are differentiating but unable to elongate. Strikingly RAP2 N17 overexpression results in a dramatic decrease in the number of myotubes observed. Cells appear flat and have lost their elongated shape (Figure 46, row 4).



**Figure 46: RAP2 N17 inhibits differentiation of C2C12 cells.** C2C12 myoblasts were transduced with control, RAP2 wt or TRIM32 wt. These were selected using puromycin selection and reseeded upon complete selection and changed to fusion media. Top row – C2C12 cells show formation of myotubes. Second and third row – RAP2 wt and V12 cells show normal myotube formation combined with the formation of shorter myotubes. Forth row – RAP2 N17 overexpression inhibits microtubule formation. Bottom row – TRIM32 wt overexpression may enhance microtubule formation.

Slightly enhanced muscle cell fusion was observed with exogenous TRIM32 (Figure 46, row 5). Although it would be necessary to quantify this in order to be certain whether this difference is significant, this supports previous findings that TRIM32 inhibition inhibits myogenic differentiation (Schwamborn, Berezikov et al. 2009; Nicklas, Otto et al. 2012).

## Summary

RAP2 localisation varies in different cell lines. In HMLE and U2OS cells the localisation is predominantly in areas of cell-cell contact whereas in C2C12 cells localisation is both at the plasma membrane and perinuclear region, possibly the Golgi. Interestingly, disruption of RAP2 activation through the dominant-negative mutant (N17) results in stronger perinuclear/Golgi localisation suggesting that the localisation of RAP2 and its trafficking between Golgi and plasma membrane may be linked to its activation state.

TRIM32 in U2OS and C2C12 gives a punctate staining consistent with vesicular localisation, concentrating to a region near the nucleus consistent with Golgi staining. From which it can be inferred that TRIM32 is either being transported or involved in transport, and is being processed in the Golgi. It is, of course possible that high levels of overexpression are resulting in the overexpressed protein being aggregated in vesicles. In order to overcome that it would be necessary to test different vector promoters in order to see whether there is a difference between high level and low level expression of TRIM32 or look at immunofluorescence with endogenous TRIM32.

In C2C12 myoblast cells RAP2, in addition to the plasma membrane localisation seen with U2OS and HMLE cells, gave a very distinct Golgi and vesicular staining that was not observed in other cell types such as U2OS and HMLE cells. This suggests that RAP2 may localise to different membrane compartments in a cell-type (and likely context) dependent manner.

Because of lack of time I was unable to successfully perform co-expression and co-localisation studies and test for example the possibility that active RAP2 may translocate TRIM32 to the plasma membrane in HMLE or U2OS cells.

In C2C12 cells both RAP2 and TRIM32 localise to dots concentrated around the Golgi. Interestingly, this Golgi-like localisation is more prominent in the case of RAP2 N17 suggesting the activation cycle of RAP2 may be involved in its transport between the plasma membrane and other endomembrane compartments. Although because of lack of time I was unable to perform the experiments to see if RAP2 and TRIM32 co-localise in C2C12 cells, these cell-type specific observations are consistent with a specific role for the RAP2-TRIM32 interaction in muscle cell biology.

RAP2 N17 consistently demonstrates an increase in proliferation and inhibits differentiation of C2C12 cells. This would suggest that RAP2 plays a role in reducing proliferation and that this effect is reversed on introduction of the RAP2 dominant-negative construct, perhaps sequestering the GEFs of the endogenous RAP2 and thereby abrogating endogenous RAP2 activation. Use of si- or shRNA knockdown of RAP2 would confirm this. More recent studies by Cohen et al. also suggest that over-expression of TRIM32 induces or enhances muscle atrophy and that this is linked, in part, to the expression and interaction with Plakoglobin (Cohen, et al. 2014). Further experiments to assess the implications of this protein in our cell line both individually and in combination with TRIM32 and/or RAP2 could provide a further understanding of the developmental and degredational implications of our interaction.

Overexpressing TRIM32 also appears to decrease proliferation slightly, although this is contrary to the results of (Kano, Miyajima et al. 2008) in NIH3T3 cells, where they showed a marked increase in proliferation on TRIM32 overexpression. Nevertheless, it is possible for TRIM32 to show different effects in different cell lines, especially in cell lines of different origins.

No effect was seen on migration with overexpression of RAP2, despite what is stated in the literature (Uechi, Bayarjargal et al. 2009). However, Uechi *et al* used COS-1 cells which are from monkey kidney tissue, and this discrepancy could be a result of cell-type specificity. Therefore, repeating this experiment within our muscle cell model could yield further interesting results. Similarly, the role of



TRIM32 in cell motility has also been published in the literature and would be worth investigating in our model (Kano, Miyajima et al. 2008).

# Chapter 8: Discussion of the TRIM32-RAP2 Interaction Studies

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## **Validating and Characterising RAP2-TRIM32 interactions**

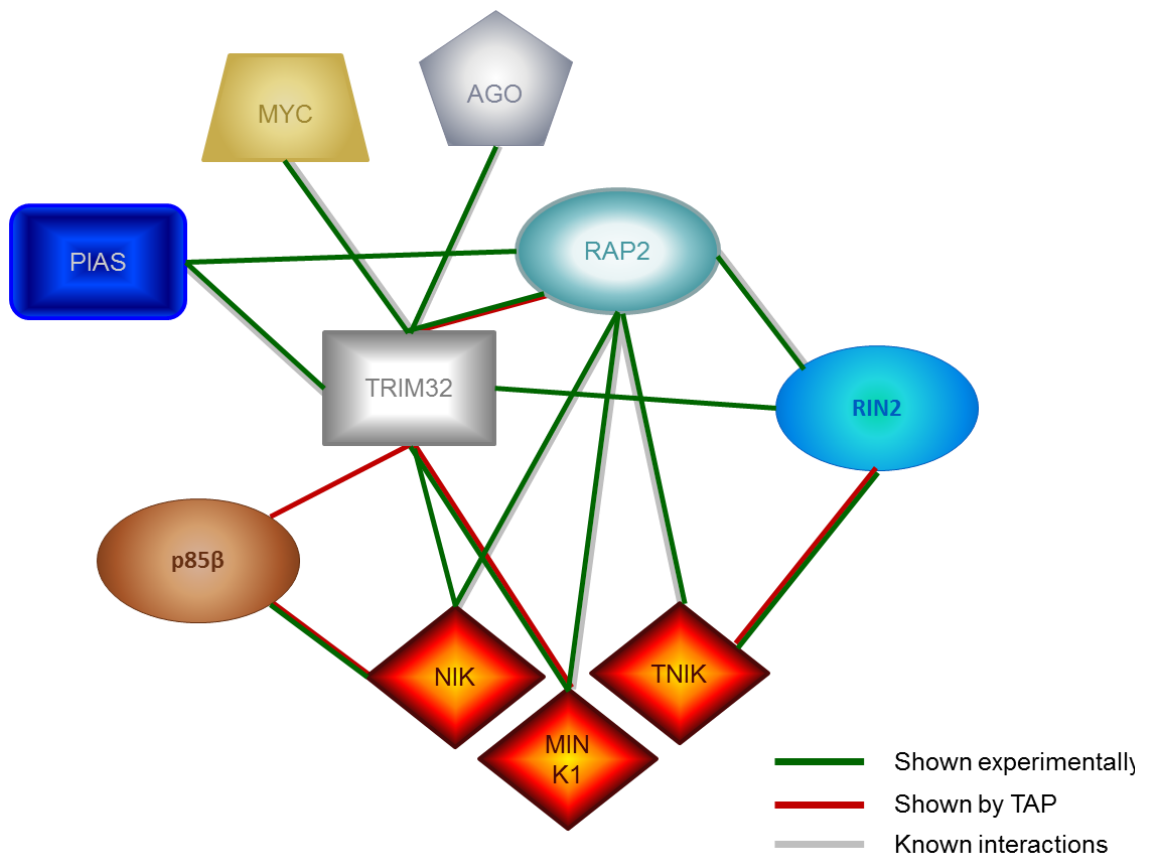
In this work we have identified several novel putative binding partners for the protein RAP2. Of these, TRIM32 was selected for further investigation and this putative interaction was confirmed by assessing the association of endogenous TRIM32 with overexpressed mutant and wt RAP2 proteins in HEK-293T cell lines. TRIM32 was shown to interact specifically in a RAP2 activation- and effector domain-dependent manner. The data presented in Figure 20, demonstrates this specificity by comparing the interaction with closely related RAS family members that did not interact with TRIM32. Several other interactions that were identified in the RAP2-TAP assay were also validated in a similar manner. Of these VPS35, a scaffold protein in the cargo complex, was found to interact specifically with RAP2 in an activation but non-effector domain-dependent manner suggesting that VPS35 is not an effector of RAP2 but could be involved upstream of the RAP2. Although this interaction was not pursued further, subsequent data indicate that it may play a role in the RAP2-TRIM32 interaction, discussed below.

Previously published RAP2 interactions with NIK, TNIK and MINK were also validated as above. However, contrary to what has been published in the literature (Taira, Umikawa et al. 2004; Nonaka, Takei et al. 2008), we saw preferential binding of these factors to the wild-type RAP2 when compared to the active RAP2 V12 and RAP2 L63 mutants. These findings suggest that the interaction may not be activation-dependent although it is effector domain-dependent as the A38 mutant disrupts the interaction. This could be due to preferential binding to the GDP bound conformation of RAP2 when compared to the GTP-bound. Alternatively, the RAP2 V12 mutation may affect the interaction in a Guanine nucleotide independent manner. Nonanka *et al* used glutathione-Sepharose resin carrying GTPγS-bound, GDP-bound, or nucleotide-free forms of GST-RAP2A for their assays, which could explain the difference between the interaction data obtained. Furthermore, when specific TRIM32 mutants, which have been previously implicated in LGMD2H, were

tested (Schoser, Frosk et al. 2005; Saccone, Palmieri et al. 2008; Borg, Stucka et al. 2009; Cossee, Lagier-Tourenne et al. 2009) the RAP2-TRIM32 interaction was disrupted whereas the binding of these mutants to MINK was not. This strongly suggests that the RAP2-TRIM32 interaction is functionally involved in the pathology of this disease. Despite demonstrating that RAP2 interacts with C-terminus of TRIM32, we have also shown that mutations in the N-terminal RING domain that abrogate the E3 ligase activity of TRIM32 disrupt its interaction with RAP2 but not with MINK (Figure 26). Again, this is interesting as it implies that although RAP2 is binding to the C-terminus of TRIM32, the RING domain activity is necessary for the interaction between RAP2 and TRIM32. As the effector domain-dependent nature of the interaction suggests that RAP2 functions upstream of TRIM32, it is possible that RAP2 controls downstream effects of TRIM32, such as ubiquitination. It has also been shown that RING E3 activity can be controlled by binding partners, e.g. Cand1 binds to Cullins and sequesters them in an inactive state (Liu, Furukawa et al. 2002; Zheng, Yang et al. 2002).

### **RAP2 modulation of TRIM32 interactions**

The experimental data obtained in Chapter 4: Assessing the Effect of TRIM32 mutants demonstrate that RAP2 and TRIM32 share a number of binding partners, a schematic of these putative interactions is shown in Figure 47. While RAP2 V12 does not appear to affect the TRIM32 interaction with PIAS4, RIN2 or MINK, the NIK-TRIM32 interaction was slightly inhibited in the presence of RAP2 V12 while RAP2 wt abrogated the binding of NIK to TRIM32. As MINK, TNIK and NIK interact more strongly to RAP2 wt than the V12 mutant, it is possible that a similar disruption occurs in MINK and TNIK. Therefore, to follow up on this finding, future investigations would need to look at whether MINK and TNIK interaction with TRIM32 can be modulated by RAP2 wt.



**Figure 47: Schematic of RAP2 and TRIM32 interactions.** Demonstrating a clear overlap in the binding partners of RAP2 and TRIM32. Green = shown experimentally, Red = shown by TAP, Grey = shown in literature.

## Ubiquitination

### LGMD2H mutations disrupt TRIM32-dependent Ubiquitination

This ability to selectively modulate the binding of TRIM32 to other proteins suggests a regulatory role for the RAP2. We demonstrated that TRIM32 enhances the ubiquitination of RAP2, ubiquitinating RAP2 wt to a greater degree than the active mutants RAP2 V12 and V12L63. Additionally, blocking the RAP2 effector domain via the RAP2 V12A38 or RAP2 L63A38 mutations decrease the TRIM32-dependent ubiquitination of RAP2 when compared to the levels of ubiquitination seen with RAP2 wt (Figure 30). We suggest that the ubiquitination activity of TRIM32 is relevant to LGMD2H as the characteristic laddering pattern seen on the TRIM32 wt and P130S baits is indicative of endogenous ubiquitination, as determined by western blot, is absent in TRIM32 R394H, D487N and  $\Delta$ 588 mutants which are

associated with the disease. These LGMD2H disease mutants also disrupt the TRIM32 interaction with RAP2. From this we can infer that the ubiquitin pathway is disrupted by these mutants and that the ubiquitination function of TRIM32 may be linked to in the RAP2-TRIM32 association which is linked to LGMD2H. Therefore, we proceeded to examine the impact of RAP2 on TRIM32 mediated ubiquitination.

### **Auto-ubiquitination of TRIM32**

E3 ligases have the ability to auto-ubiquitinate through their intrinsic E3 ligase activity, which can lead to self- degradation via the proteasome (Bell, Malyukova et al. 2012; Xie, Avello et al. 2012). Similarly, we have shown exogenous auto-ubiquitination of TRIM32, with those mutations associated with LGMD2H altering the degree of auto-ubiquitination. The TRIM32 wt and BBS mutants show a single ubiquitinating band below the ubiquitin smear, which correlates approximately to mono- or di-ubiquitination of TRIM32, whereas upon the introduction of the LGMD2H mutations, R394H, D487N and  $\Delta$ 588, this band is no longer detectable. When observing the effect of RAP2 on the auto-ubiquitination of TRIM32, it was observed that in all cases the presence of RAP2 noticeably reduced the level of ubiquitin detected, even with the mutations where we have disrupted the TRIM32-RAP2 interaction. For the R394H and D487N mutants (Figure 32), the TRIM32 auto-ubiquitination is almost completely reversed, while  $\Delta$ 588 shows an intermediate effect (Figure 32, lane 11). This could somehow link back to the fact that the  $\Delta$ 588 mutant disrupts the TRIM32-RAP2 interaction only partially, and not as much as the other LGMD2H mutants (Figure 27). This suggests that the RAP2 effect on the auto-ubiquitination of TRIM32 is independent of the RAP2-TRIM32 interaction that was shown previously.

### **Ubiquitination of TNIK, NIK and MINK**

It has recently been shown that RAP2 forms a complex with TNIK and NEDD4-1 that leads to the ubiquitination of RAP2, thereby inhibiting it's function and regulating neurite growth and arborisation in mammalian neurons (Kawabe, Neeb et al. 2010). While Kawabe *et al* did not see any ubiquitination of TNIK, we have shown that RAP2 can modulate the E3 ligase activity of TRIM32 towards MINK, TNIK and NIK. Although the ubiquitination of TNIK and MINK1 clearly increased in the presence of TRIM32 and upon the addition of RAP2 V12, the addition of RAP2 wt increases the

ubiquitination of TNIK and MINK even further. This suggests that the preferential binding of TNIK and MINK to RAP2 wt may influence their ubiquitination state.

### **PIAS ubiquitination by TRIM32**

We started to investigate other pathways that may be aberrant in LGMD2H using the TRIM32 mutants by testing the ubiquitination status of other published binding partners of TRIM32. PIAS family members function as small ubiquitin-like modifier (SUMO) proteins and their interaction with RAP2 has been published (Hershko and Ciechanover 1998; Hay 2005). When looking at the ability of TRIM32 to ubiquitinate PIAS2 and PIAS3, we have shown that TRIM32 wt increases the ubiquitination of the respective proteins. In PIAS2 this effect is abrogated when TRIM32 D487N is used (data not available for PIAS3). This suggests that TRIM32 D487N is unable to induce ubiquitination of PIAS2, suggesting a link to LGMD2H, the muscular dystrophy within which this mutation is prevalent.

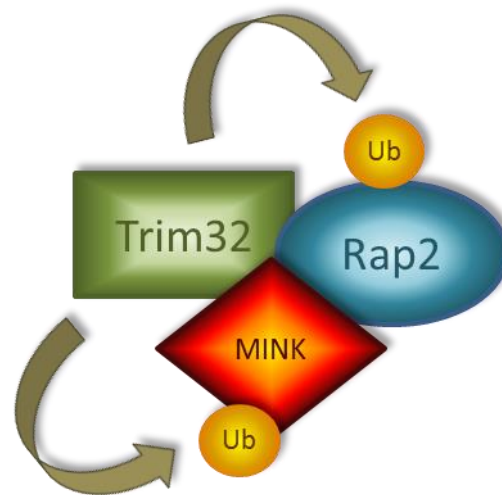
### **RIN protein ubiquitination by TRIM32**

RIN proteins are close relatives of RAS and have been shown to play a role in mediating neuronal differentiation (Hoshino, Yoshimori et al. 2005). Having reproduced the RIN interaction with RAP2 and demonstrated a novel interaction with TRIM32 (Figure 21, Figure 28); the effect of these on RIN ubiquitination was then assessed. RIN1 showed some basal levels of ubiquitination in the absence of TRIM32 and RAP2 which were abrogated by the addition of RAP2 V12. TRIM32 wt dramatically increased the ubiquitination of RIN when compared to an empty control, whereas RAP2 V12 had no effect on level of TRIM32-dependent ubiquitination.

RIN2 ubiquitination also increased on overexpression of TRIM32 wt in HEK-293T cells. Despite the RIN2 interaction with RAP2 occurring in an activation- and effector domain dependent manner, RAP2 wt showed greater TRIM32-dependent ubiquitination activity towards RIN2 when compared to RAP2 V12. Also, when TRIM32 LGMD2H disease mutants, D487N, R394H,  $\Delta$ 588 are overexpressed, RIN2 ubiquitination is only slightly increased when compared to non-TRIM32 transfected control, and although RAP2 V12 still increases RIN2 ubiquitination in the presence

of the LGMD2H mutants, this effect is much more subtle than that which is seen with TRIM32 wt and the P130S BBS mutant.

From all these findings, we suggest a model whereby RAP2 recruits TRIM32 and effectors (MINK in the example below) and brings them into proximity in order that TRIM32 can ubiquitinate both the RAP2 and the effector but this interaction is not responsible for the modulation of TRIM32 auto-ubiquitination (Figure 48). While we have provided experimental data to demonstrate these interactions and effects on ubiquitination, we have not shown conclusively that these interactions are direct, it is possible that other intermediate proteins are involved in enabling the recruitment of these proteins.



**Figure 48: RAP2 may modulate TRIM32 dependent ubiquitination of downstream targets.** We propose a model that RAP2 enhances TRIM32 dependent ubiquitination of MINK by acting as a bridge to bring the two proteins to close proximity, resulting in MINK and RAP2 ubiquitination.

In order to get a better understanding of the type of ubiquitination taking place, ie mono-, di- or poly- ubiquitination, it would be useful to take advantage of selective ubiquitin mutants that can only bind to specific residues, e.g. K48 or K63 (kind gift from P.Meier), that we may understand whether these proteins are being targeted for degradation or activation for downstream effects or any number of cellular processes.

## Localisation

RAS family GTPases are known to regulate the function of at least some of their effectors by recruiting them, upon activation, to specific membrane compartments and/or signalling complexes. For example, H/K/NRAS activate RAF kinases by recruiting them to the plasma membrane microdomains where other activating steps then take place (McKay and Morrison 2007). Similarly, MRAS recruits the SHOC2-PP1 complex to regulate the activity of RAF kinases at specialised signalling complexes (Rodriguez-Viciano, Oses-Prieto et al. 2006). We speculated that by analogy with other GTPases of the family, RAP2, upon activation, may recruit TRIM32 to specific complexes at some membrane compartment. In order to test this, we first set out to investigate the localisation of both RAP2 and TRIM32 in several cell types to set the stage for potential co-localisation studies.

TRIM32 transduced in U2OS and C2C12 cells localises to punctate vesicles, accumulating to a region near the nucleus, possibly to the Golgi. From which it can be inferred that TRIM32 is either being transported or involved in transport from endosomes to the trans-Golgi network, and is being processed in the Golgi. It is, of course possible that overexpression of TRIM32 is resulting in the exogenous protein being aggregated in vesicles. In order to investigate this it would be necessary to test different vector promoters or inducible promoters in order to determine if the effect of increasing levels of TRIM32 alters localisation, or to use immunofluorescence to determine the localisation of endogenous TRIM32.

Exogenous RAP2 localisation varies in different cell lines. In U2OS cells the protein is predominantly found in areas of cell-cell contact whereas in HMLEs and C2C12 cells localisation is both in regions of cell-cell contact and in the area surrounding the nucleus, possibly the Golgi. However, in the C2C12 cells, the dominant-negative RAP2 N17 mutant demonstrates a cytosolic and nuclear distribution and was not detected in areas of cell-cell contact.

With putative Golgi localisation for TRIM32 and RAP2 seen in C2C12 cells, overexpressing RAP2 wt and mutant proteins, or use of si- or shRNA to abrogate RAP2 expression could disrupt the localisation of TRIM32, which would indicate that RAP2 is responsible for TRIM32 localisation. Conversely, looking at the TRIM32



disease mutants previously used, would allow us to determine whether it is TRIM32 that is sequestering the RAP2 protein in order to determine its localisation. Additionally, testing the mutants in the RING domain that disrupt TRIM32's E3 ligase activity would allow us to establish whether this is a ubiquitination dependent effect.

Using Golgi counter-stain to demonstrate whether TRIM32 and RAP2 are co-localised to the Golgi, as well as looking at the localisation of some proteins known to be involved in retrograde transport of proteins from endosomes to the trans-Golgi network in order to determine whether they have an effect on the localisation of TRIM32 or RAP2, e.g. VPS35 and VPS29. VPS35 is a component of the retromer complex and is involved in retrograde transport of proteins from endosomes to the trans-Golgi network, areas to which we have seen RAP2 and TRIM32 localisation. Therefore, by examining whether co-localisation of VPS35 with RAP2 or TRIM32 is occurring, or whether the knockdown of VPS35 disrupts the localisation of RAP2 or TRIM32 to the Golgi, than this would support the involvement of RAP2 or TRIM32 in transport, either as mediators or as being transported.

## **Proliferation and Migration**

TRIM32 mediates the ubiquitination of Abi2, resulting in enhanced proliferation and cell motility in HEK-293T and NIH-3T3 cells (Kano, Miyajima et al. 2008). Although in our model, we did not see a significant effect of TRIM32 on proliferation or motility, through further investigation into the biological role of TRIM32 by inhibiting TRIM32 using sh- or siRNA, or by overexpressing the LGMD2H disease mutants, R394H, D487N and  $\Delta$ 588, or disrupting the E3 ligase activity of TRIM32 by mutating the RING domain, we may observe an effect on proliferation or motility. Nevertheless, the dominant-negative RAP2 N17 increased the proliferation of C2C12 cells, implying that endogenous RAP2 inhibits proliferation. Again, the use of si- or shRNA to knockdown RAP2 expression would corroborate this, and, as RAP2 exists in three different isoforms, would enable us to discover if this is an isoform specific effect on proliferation.

## Differentiation

TRIM32 is expressed in both proliferating and differentiating satellite cell progeny and the absence of TRIM32 disrupts myogenic differentiation (Nicklas, Otto et al. 2012). Additionally, TRIM32 has been shown to induce neuronal differentiation by ubiquitinating MYC and targeting it for degradation (Schwamborn, Berezikov et al. 2009). Furthermore, c-MYC has been demonstrated to block the action of Myo-D and Myogenin-mediated skeletal muscle stem cell differentiation (Miner and Wold 1991) and TRIM32 reverses the c-MYC-mediated block on differentiation by targeting c-MYC for degradation (Nicklas, Otto et al. 2012).

TRIM32 appears to increase the differentiation of C2C12 cells used in our model. Additionally, the dominant-negative RAP2 N17 mutant consistently demonstrates an inhibition on the differentiation of C2C12 cells, indicating that abrogating RAP2 activity inhibits myotube differentiation. Combined, this indicates that both TRIM32 and RAP2 are required for the differentiation of skeletal muscle cells. As RAP2 N17 is the dominant-negative mutant for RAP2, it would be interesting to look at whether inhibiting RAP2 by knocking down by sh- or siRNA, we can obtain a similar result. It is important to bear in mind that there are three different isoforms for RAP2. While it is possible that there may be an isoform specific effect of RAP2 on proliferation and differentiation, it would also be of interest to knockdown all three.

In order to assess whether the subtle increase in differentiation identified for TRIM32 is in fact significant (Figure 46), it would be useful to repeat the experiment using fluorescent constructs which would then enable us to count the number of fluorescent-differentiated cells under each condition. Using myotube analysis, counting the myotube nuclei, by staining the nucleus, would also allow an assessment of the number of multinucleated cells. Alternatively, looking at the effect of TRIM32 knock down by sh- or siRNA to see whether differentiation is inhibited as in the paper (Schwamborn, Berezikov et al. 2009; Nicklas, Otto et al. 2012).

Interestingly, a recent paper in *Molecular and Cellular Biology* has demonstrated that NIK, a putative RAP2 and TRIM32 binding partner, attenuates the differentiation of C2C12 cells via its kinase activity in a Myf5 dependent manner,

silencing NIK expression using siRNA or expression of a NIK kinase-inactive mutant enhanced the differentiation of C2C12 myoblasts. NIK suppression results in elevated Myf5 expression and enhanced differentiation of C2C12 cells (Wang, Amano et al. 2012). A further study of some of the other RAP2 and TRIM32 binding partners that we have identified, and their effect on the differentiation of myoblasts by examining the expression of muscle differentiation markers such as Myf5, MyoD, myogenin and MRF4, would enable us to elucidate whether these novel interactions can also have an effect on the differentiation of C2C12 cells. Looking at transducing the cells with lentivirus at different stages of differentiation to identify at which stages RAP2 and TRIM32 may play a role.

It would also be of additional interest to observe whether the TRIM32 mutants that we have previously demonstrated are unable to bind to RAP2 would have an effect on the differentiation of the cells similar to that seen when RAP2 activity is abrogated. A further look at the mutants that disrupt the E3 ligase activity of TRIM32, such as  $\Delta$ RING and C23A, and a study of the effect of 14-3-3 would also inform us as to whether the TRIM32 ubiquitin activity plays a role.

## Summary

Having demonstrated a novel interaction between RAP2 and TRIM32 that is linked to the disease LGMD2H, we then investigated the biological implications of this interaction. We propose that these proteins are involved in the proliferation and differentiation of myoblasts and that the E3 ligase activity may play a role. As suggested by current literature, the modulation of this activity is likely to be linked to several of the targets identified in our final TAP experiments, e.g. 14-3-3 as demonstrated by Ichimura et al. (Ichimura 2014). Through further investigation we hope to achieve a better understanding of the pathology of the LGMD2H disease as well as a deeper understanding of other possible implications of TRIM32 on neurodevelopmental phenotypes as suggested by the work of Lionel et al (Lionel, et al. 2014).

# Chapter 9: Validation of the TAP-NOEY2 data

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## Identification of potential NOEY2 interactions

Tandem affinity purification (TAP) is an unbiased approach that allows identification of protein interactions.

In order to identify novel interacting proteins of NOEY2 that may shed light on its function, we had performed independent TAP purifications using TAP-tagged NOEY2. We identified PKA regulatory 1A (-R1 $\alpha$ /PRKAR1A) and catalytic (-cat/PRKACA) subunits that co-purified specifically with NOEY2 but not other baits, including other RAS family members (Table 13).

ID	Unique Peptides	% Coverage
DIRAS3/NOEY2	26	46.3
PRKAR1A	2	7.9
PRKACA	1	2.6

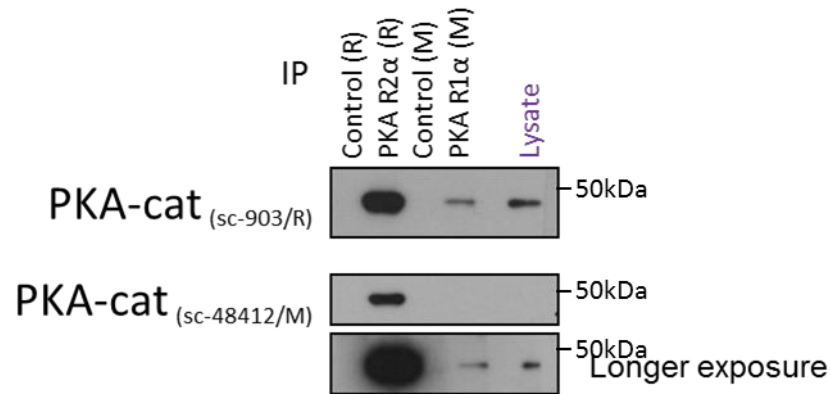
**Table 13: Novel NOEY2 interactions identified by TAP screen in HEK-293T cells.** NOEY2 was identified to interact with PRKAR1A and catalytic subunits.

NOEY2 has tumour suppressor properties and PRKAR1A can also function as a tumour suppressor, however, the mechanism by which they act is not known. Therefore, the potential regulation of PRKAR1A by NOEY2 could provide a possible molecular mechanism for the tumour suppressor properties of NOEY2. This was considered an exciting preliminary finding that was selected for subsequent validation and characterization experiments.

## Antibody Characterisation

Various commercially available antibodies against NOEY2, PRKAR1A, PRKAR2A and PRKACA proteins were ordered for use in subsequent experiments, and these were characterised, and their specificity tested prior to any further experiments being conducted. The ability of the antibodies to specifically immunoprecipitate was determined following the protocol described in the Methods section. Initially, PRKAR1A and PRKAR2A antibodies were tested for their ability to

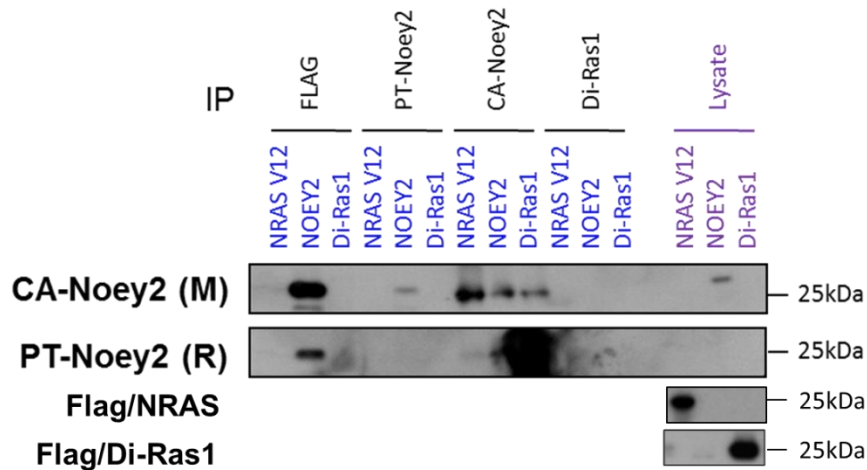
immunoprecipitate PRKACA, where two different PRKACA antibodies were compared (Figure 49).



**Figure 49: PRKAR2A and PRKAR1A antibodies are able to immunoprecipitate endogenous PRKACA.** HEK-293T lysates were incubated with Protein A or G beads and the indicated antibody for up to 4h. Lysates were then washed and run on an SDS-PAGE gel. The level of protein was detected by western blot. Rabbit and Mouse SHOC2 antibodies were used as unrelated control antibodies for immunoprecipitation.

Testing the ability of the PRKAR2A and PRKAR1A antibodies to immunoprecipitate endogenous PRKACA, we can see that both immunoprecipitate PRKACA. More PRKACA is found associated with PRKAR2A than PRKAR1A although from this experiment we cannot say whether that is only a reflection of the relative levels of expression of PRKAR2A vs PRKAR1A in this cell line resulting in more capacity to pull down the PRKACA protein. Also from this experiment it was apparent that the PRKACA sc-903 antibody had higher sensitivity in western blot. In summary; R2A and R1A antibodies IP endogenous protein (Figure 49).

In order to compare the ability of a panel of commercially available antibodies to immunoprecipitate and detect by western blot NOEY2 an experiment was set up where Flag-tagged NOEY2 was overexpressed in 293T cells and FLAG beads used as a positive control (**Error! Reference source not found.**).

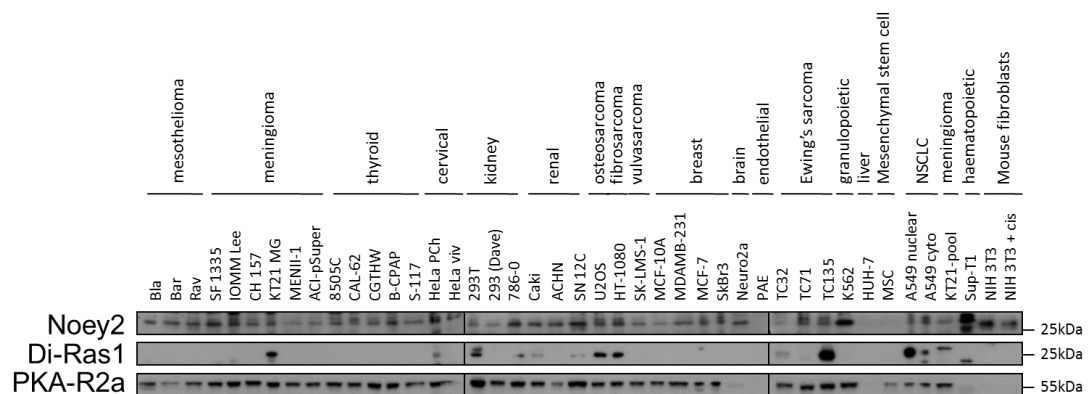


**Figure 50: NOEY2 antibody is able to immunoprecipitate overexpressed Flag-NOEY2.** HEK-293T lysates with overexpressed Flag-NOEY2, NRAS-V12 or Di-Ras1 as indicated, were split and incubated with Protein A or G beads and the indicated antibody for up to 4h. Lysates were then washed and run on an SDS-PAGE gel. The level of protein pulled-down was detected using western blot. NRAS V12 and DIRAS1 were used as unrelated and closely related controls respectively and the level of NOEY2 expression is shown on the righthand lanes.

HEK-293T cells were transfected with Flag-NOEY2, Flag-NRAS V12 or Di-Ras1 as construct of interest, unrelated and closely related background controls respectively. Each lysate was then divided into 4 tubes and these were then immunoprecipitated using either Flag- beads, PT-NOEY2 antibody, CA-NOEY2 antibody or Di-Ras1 antibody as indicated above (**Error! Reference source not found.**). Samples were then run on western blot, and interactions detected using CA- or PT-NOEY2 antibodies as indicated. Flag-bead immunoprecipitated NOEY2 can be detected with both NOEY2 antibodies, (CA more sensitive than PT). Additionally, on immunoprecipitation with the PT antibody, we can then detect a band using the CA antibody, suggesting that the PT antibody is able to immunoprecipitate NOEY2. Immunoprecipitation by the CA-NOEY2 antibody yielded a lot of background (**Error! Reference source not found.**). Di-Ras1 immunoprecipitation was used as a negative control and no interaction was detected in these lanes. In brief, both antibodies can detect NOEY2 and the PT/R antibody can immunoprecipitate although with very poor efficiency as compared to the Flag-antibody positive control. Furthermore, the PT/R antibody can only immunoprecipitate the overexpressed protein (Flag-NOEY2 transfected lanes) but not the endogenous NOEY2 (data not shown). After validating that the antibodies can specifically recognise NOEY2 (but not other

related GTPases) the CA antibody was used to measure NOEY2 expression levels in a panel of cell lines derived from multiple tissue and tumour types.

Lysates were taken from cell lines derived from different tissues and tumour types (as indicated, Figure 51) in order to assess the endogenous NOEY2 expression levels. Based on reported observations that loss of NOEY2 expression is linked to tumour progression in some tumours, we were hoping to find cell lines that had lost expression (Yu, Xu et al. 1999; Hisatomi, Nagao et al. 2002). Cells were grown to 90% confluence and lysed using PBS-M lysis buffer. Samples were prepared and gels run and transferred as described in the methods section and the membrane was probed with the NOEY2 antibody.



**Figure 51: NOEY2 is expressed in a panel of cell lines.** Lysates were taken of various cell lines, as specified, and run on SDS-PAGE gels. Expression levels were assessed using NOEY2 antibody with PRKAR2A used as a loading control. NOEY2 expression can be detected in all cell lines except mesenchymal stem cells (MSC).

PRKAR2A is an ubiquitously expressed protein and was used as our loading control. However we detected NOEY2 expression everywhere except mesenchymal stem cells (MSC), in other lanes where there is no NOEY2 the PRKAR2A loading control has no band, so it is likely a loading effect. Interestingly K562 and Sup T1, both haematopoietic suspension cells, show stronger bands. K562 was shown to express DIRAS3 according to microarray data on “gnf atlas”.

## Generation of constructs

To further explore protein-protein interactions, overexpression of a battery of tagged constructs (Table 14) was generated as described in the Methods section.

These genes were ligated to ENTR Gateway vector (Gateway®, Invitrogen) and mutagenesis was performed and verified by sequencing where appropriate. Gateway technology was then used to transfer the inserts into different Gateway-compatible tagged- destination vectors for expression in mammalian cells. The resulting tagged proteins (Flag-, GST-, Myc-, TAP6 tags) were used in subsequent experiments.

Gene	Mutations status	Properties
NOEY2	wt	Wild-type (wt)
	ΔN	N-terminal extension deleted
	T69S	Effector domain
	E71G	Effector domain
	N72D, T73S	Effector domain
	Y74C	Effector domain
	C227S	CAAX box
	A45V46	GTP-binding/Possibly activating
	K93A, G95Q	GTP-binding/Possibly activating
NOEY3	wt	Wt
NOEY4	wt	Wt
PRKAR1A	wt	Wt
	N	N-terminal truncation (aa 1-135 )
	C	C-terminal truncation (aa 136-382 )
PRKAR1B	wt	Wt
PRKAR2A	wt	Wt
PRKAR2B	wt	Wt
PRKACA	wt	Wt

**Table 14: List of Constructs generated.** Shown above, the different constructs generated. For each protein, various mutations were generated using the primers listed.

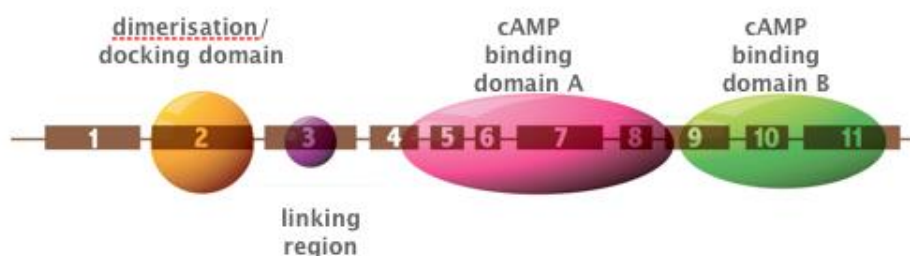
The N-terminal extension in NOEY2 (Figure 52) has been shown to confer some of its tumour suppressor properties (Luo, Fang et al. 2003). The mutants T69S, E71G, N72D, T73S, Y74C were selected as they align with the RAS effector domains. C227S





# PRKAR1A gene

Encoding regulatory subunit type 1 $\alpha$  of Protein Kinase A (PKA)

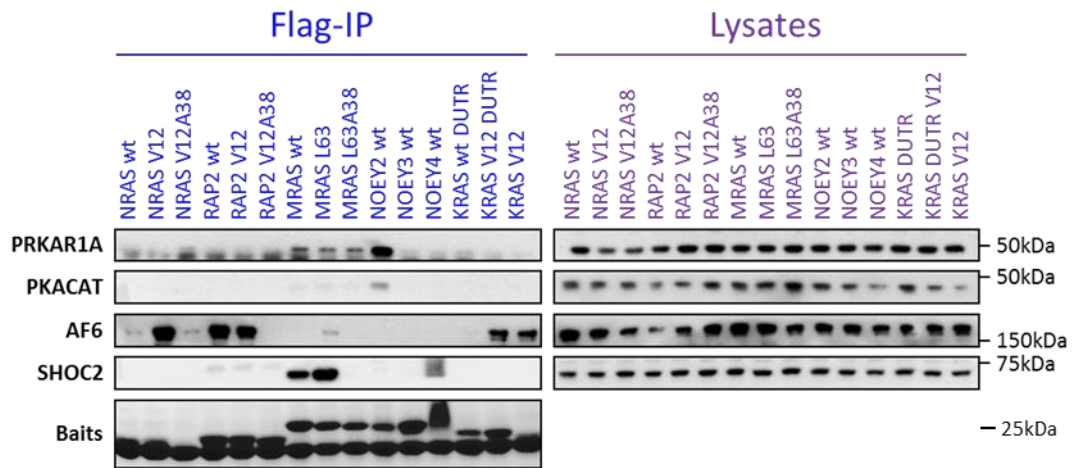


**Figure 53: PRKAR1A structure.** PRKAR1A consists of a dimerization/docking domain (orange), linking region (purple) and cAMP binding domain A and B (pink and green, respectively). Upon cAMP binding, the inactive holoenzyme dissociates into a dimer of regulatory subunits bound to four cAMP and two free monomeric catalytic subunits which can then proceed to activate downstream effectors.

Figure from <http://prkar1a.nichd.nih.gov/hmdb/prkar1a.html>

## Validation of NOEY2 interactions

In order to validate and characterise the interactions detected by TAP (Table 13), Flag-tagged NOEY2 (lane 10, Figure 54) and RFG controls were overexpressed in HEK-293T cells and subsequently immunoprecipitated with Flag-beads. Samples were run on SDS-PAGE and interacting endogenous proteins detected using specific antibodies.



**Figure 54: Endogenous PRKAR1A and PRKACAT interact with FLAG-NOEY2.** HEK-293T cells were transfected with Flag-RFG. Lysates were taken on day 3 and immunoprecipitated using Flag- beads. Proteins were separated using SDS-PAGE gel and probed with the indicated antibodies.

Endogenous PRKAR1A and PRKACA were found to co-immunoprecipitate specifically with NOEY2 but not NOEY3, NOEY4 nor NRAS (Figure 54). The band seen on MRAS immunoprecipitates does not appear to be activation or effector domain dependent, and appears most likely to be background binding. This demonstrates the potential for the antibody to bind unspecifically. As equivalent NOEY2 mutants that discriminate between activation/effector domain dependent binding vs unspecific binding were not known, a series of NOEY2 constructs were generated to attempt to find mutations to that effect, in order to disrupt the NOEY2 interaction with PRAKR1A and PRKACA and confirm specificity.

AF6, a promiscuous RAS family effector, can be shown to strongly co-immunoprecipitate with RAP2, NRAS and MRAS in a manner that is strongly disrupted by the A38 effector domain mutations (Figure 54). In the case of NRAS and MRAS, the interaction takes place preferentially with the activating V12 mutant (lane 2, lane 14-15 respectively). In contrast however, no difference can be seen between wild type and V12 versions (lane 4, lane 5) of RAP2 in its ability to interact with AF6. This could be a reflection of the possibility, that unlike other RAS family GTPases, the wild type RAP2 protein may already be predominantly GTP-bound in the cell in some contexts.

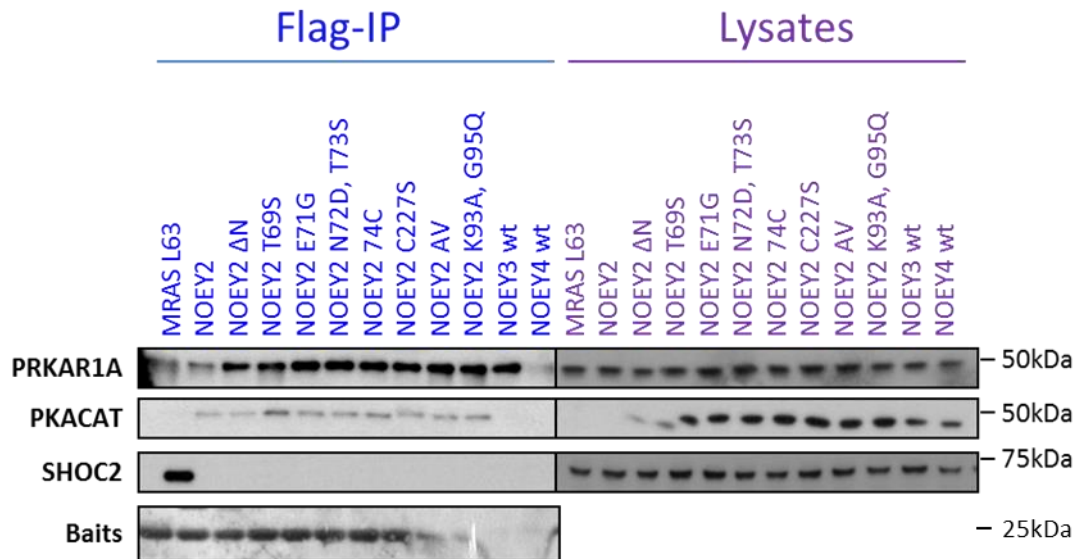
SHOC2 behaves as specific MRAS effector, with demonstrably stronger MRAS L63 (lane 8) binding than wt (lane 7), and disrupted A38 binding (lane 9), and no

interaction with any of the other RFGs. There does appear to be background binding to NOEY4 (lane 12).

## **NOEY2 mutants**

To further characterize the specificity of the interaction of NOEY2 with PRKAR1A and PRKACA and demonstrate that the interaction could indeed be disrupted, and was not unspecific, a panel of NOEY2 mutants were generated based on sequence alignments and similarities with other RFG mutants that disrupt binding with their respective down-stream effectors. Other constructs were produced based on the literature identifying features of NOEY2, such as the N-terminal extension, that could be of interest.

The A38 mutation in the effector domain of H/K/NRAS oncoproteins leads to full loss-of-function and is known to disrupt the interaction of RAS proteins with all known effectors. However, other substitutions within the RAS effector domain are known to create partial loss-of-function mutants by differentially disrupting the ability to interact with some effectors but not others. HRAS T35S for example can no longer interact with RALGDS or PI3K, while still being able to interact with RAF kinases and activate the RAF-ERK pathway (although with decreased efficiency)(White, Nicolette et al. 1995; Rodriguez-Viciano, Warne et al. 1997). These partial loss-of-function mutants have provided useful experimental tools to assess the contribution of the different RAS effector pathways to the known biological effects regulated by RAS. As similarly disruptive mutations were not known for NOEY2, mutations were generated in the GTP-binding domain and effector domains in order to test whether the equivalent substitutions in NOEY2 could differentially disrupt the interaction with PRKAR1A and/or PRKACA. Additionally, the role of the NOEY2 N-terminal extension in tumourigenesis has been published in the literature (Luo, Fang et al. 2003), thus we included the N-terminal deletion mutant in our panel.

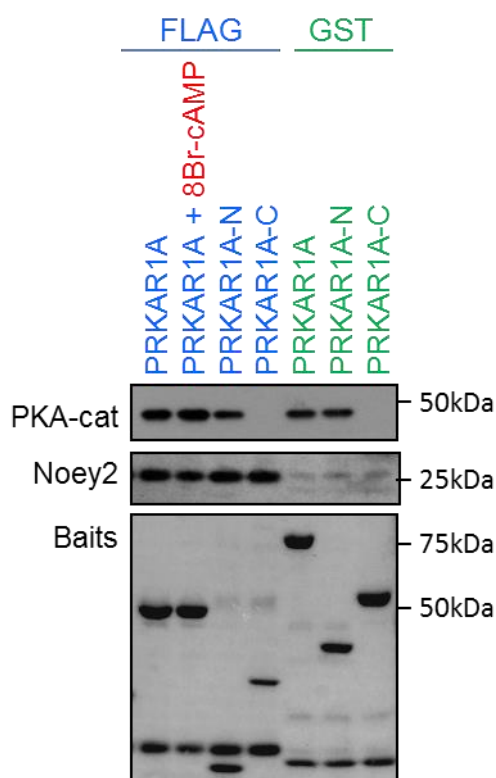


**Figure 55: Endogenous PRKACA and PRKAR1A show unspecific binding to NOEY2 and RFGs respectively.** HEK-293T cells were transfected with Flag-NOEY2 mutants and RFGs controls. Lysates were taken on day 3 and immunoprecipitated samples were run on SDS PAGE gel and transferred to PVDF membrane. Protein levels were detected using endogenous antibody. All the NOEY2 mutants show binding to PRKACA and all the RFGs bind to PRKAR1A consistent with unspecific binding.

Endogenous PRKACA and PRKAR1A were found to co-immunoprecipitate with all the NOEY2 mutants (Figure 55). Contrary to previous experiments, some PRKAR1A interaction could also be detected associating with the MRAS, NOEY3 and NOEY4 controls. This suggests a level of background binding, rendering it difficult to assess whether the NOEY2 interaction is consistent and specific.

### Mapping N vs C

We also attempted to map the interaction using a different approach, looking at where NOEY2 binds on PRKAR1A. For this, truncation mutants (N-terminal or C-terminal) were generated as described previously with both Flag- and GST-tags. These were overexpressed in HEK-293T cells and immunoprecipitated or pulled-down using Flag- or GST- beads respectively. Endogenous PRKACA and NOEY2 interactions were then detected with previously used antibodies (Figure 56).



**Figure 56: Overexpressing full length or truncated (N-terminal or C-terminal) PRKAR1A and probing for endogenous PRKACA and NOEY2 to map their interaction sites.** PKACA interaction with PRKAR1A is only disrupted with the C-truncation, not the N, and does not appear to be affected by treatment with 8Br-cAMP. This is seen with both the Flag- and GST-beads. The Noey2 interaction with PRKAR1A does not appear to be disrupted by any of the mutations when immunoprecipitated with the Flag- beads, however no interaction is seen with the GST- beads. It is possible that the Flag-bands are non-specific background interactions.

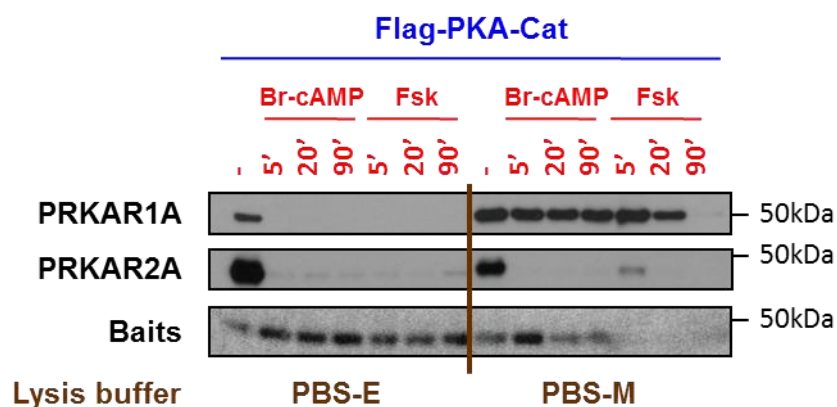
Endogenous PRKACA interacts with the N-, but not the C-terminal domain of PRKAR1A when using either Flag or GST-constructs (Figure 56) consistent with PRKACA binding to the N-terminal region of PKAR1A. However when probing for NOEY2, it is detected with both the PRKAR1A-N and -C terminal truncation mutant. This seems highly unlikely and suggests (in combination with other experiments) that NOEY2 may just be very 'sticky' and bind non-specifically to some proteins.

## Drug conditions

Prior to those later results questioning the specificity of the NOEY2 interactions and based on the original promising results experiments were performed to study how NOEY2 could regulate PKA activity. NOEY2 could be bind to PRKAR1A and thereby stimulate or inhibit PRKAR1A binding or dissociation to PRKACA subunit. This modulation could be either direct or indirect. In other words, NOEY2 could stimulate dissociation of PRKAR1A or it could inhibit dissociation induced by PKA activators. In order to test this we set out to optimize an experimental system measuring the dissociation of the regulatory subunits from PRKACA and stimulating the dissociation using drug treatments over time. By identifying the lowest dose of

drug that induces dissociation and looking at a time course of dissociation/reassociation, it would then be possible to assess whether NOEY2 inhibits dissociation or does not completely block dissociation but only delays it. Because RFGs (including NOEY2) need  $Mg^{2+}$  to bind to the guanine nucleotide and be in an active state, different lysis conditions were also tested, comparing the use of PBS-M (in the presence of  $Mg^{2+}$ ) and PBS-E (in the presence of EDTA, i.e. no  $Mg^{2+}$ ) (Paduch, Jelen et al. 2001).

HEK-293T cells were transfected with Flag-PRKACA and after 48h treated with either 8-Br-cAMP (lanes 2-4 and lanes 9-11, Figure 57) or Forskolin (lanes 5-7 and 12-14, Figure 57) as indicated. Lysis was with either PBS-E (EDTA, lanes 1-7, Figure 57) or PBS-M ( $Mg^{2+}$ , lanes 8-14, Figure 57) and samples were immunoprecipitated with Flag-antibody and probed for PRKAR1A or PRKAR2A as indicated (Figure 57).



**Figure 57: Optimising drug treatment conditions by comparing Br-cAMP to forskolin (Fsk) over time, lysing in the presence of EDTA or  $Mg^{2+}$ .** HEK-293T cells were transfected with Flag-PRKACA and after 48h treated with either 8-Br-cAMP or Forskolin as indicated above. Lysis was with either PBS-E (EDTA) or PBS-M ( $Mg^{2+}$ ) and samples were immunoprecipitated with Flag-antibody and probed for PRKAR1A or PRKAR2A as indicated. PRKAR1A no longer associates with PRKACA when treated with 8-Br-cAMP and Forskolin under PBS-E lysis conditions, whereas it can be immunoprecipitated by PRKACA under PBS-M lysis and IP conditions.

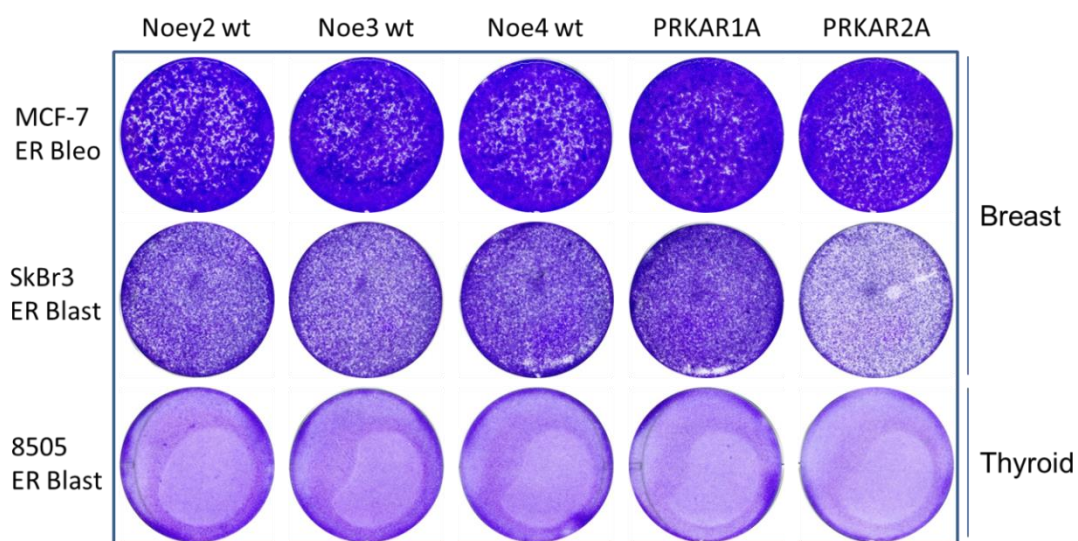
We found different behaviour of PRKAR1A and PRKAR2A immunoprecipitation with PRKACA when the lysis buffer had  $Mg^{2+}$  vs EDTA. PRKAR1A and PRKAR2A are not detected in PRKACA immunoprecipitates after 8-Br-cAMP or forskolin treatment at each time point tested under PBS-E lysis conditions.

Under PBS-M lysis and immunoprecipitation conditions, PRKR1A is immunoprecipitated by PRKACA with both 8-Br-cAMP and forskolin treatment at each time point tested, except 90' Forskolin treatment although this appears to be a bait issue. Under PBS-M lysis and immunoprecipitation conditions, PRKR2A dissociation with Br-cAMP and forskolin at each time point tested except with 5' Forskolin treatment where only partial dissociation is seen. This suggests that although 8-Br-cAMP and forskolin can potentially stimulate dissociation of both PRKR1A and PRKR2A from PRKACA, PRKR1A may be able to reassociate with PRKACA after cell lysis when Mg<sup>2+</sup> is present.

### **Colony Formation**

As mentioned previously, it has been demonstrated in the literature that NOEY2 expression is lost in breast and ovarian tumours, and that introduction of NOEY2 can inhibit the growth of transformed cells that have lost expression of the endogenous gene (Yu, Xu et al. 1999). Therefore, we tested the proliferation effects of NOEY2 expression in a panel of cell lines. Cells were infected with lenti- or retrovirus and selection was introduced to ensure only successfully transduced cells remain. Once selected, cells were seeded at equal density in 6 well plates and grown until a control (GFP) well reaches confluence. The colony formation of each sample was then compared visually as below (Figure 58). An overview of the data obtained is shown in Table 15.





**Figure 58: Colony formation assays showed no reproducible effect of Noey2 or PRKAR1A on cell proliferation.** Multiple cell lines were infected with PRKAR1A, PRKAR2A, Noey2 and closely related Di-Ras family members Noe3 and Noe4 as negative controls. No consistent effect was seen on colony growth, even in cell lines such as SkBr3 which the literature had shown to be affected.

No inhibitory effects of either NOEY2 or PRKAR1A were seen on the proliferation rate of multiple cell lines tested and therefore I could not reproduce any of the published observations of the inhibitory effects of NOEY1 and PRKAR1A on proliferation (Yu, Xu et al. 1999).

Source	Cell line	Effect on proliferation	Tested
Human ovarian carcinoma	Hey	No effect	4 times
	Skov3	No effect	3 times
	A2780	No effect	2 times
Human breast carcinoma	SkBr3	No effect	4 times
	MCF7	No effect	1 time
Thyroid carcinoma	8505	No effect	3 times
Fibrosarcoma	HT1080	No effect	2 times

**Table 15: Summary of data obtained through colony formation assays.** Despite testing multiple times in multiple cell lines, including those used in the literature, no effect was seen upon the overexpression of NOEY2.

## Chapter 10: Discussion – NOEY2

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Although the initial interaction data seem to confirm the TAP results and showed promise, the Noey2 interaction with PRKAR1A was not consistently reproducible, with the distinct possibility that it was an unspecific interaction as a result of NOEY2 being sticky. Additionally, we had problems reproducing published data of the tumour suppressor properties of both NOEY2 and PRKAR1A – I assessed the Noey2 and PRKAR1A effect on proliferation/colony formation on a panel of cell lines selected both from amongst those shown to be affected in the literature as well as some other closely related cell lines but was unable to reproduce any inhibitory effect on proliferation despite multiple attempts.

Several differences in approach exist between literature (Yu, Xu et al. 1999) and those shown above, namely the use of transfection as opposed to transduction, respectively. Yu, Xu et al used the transient transfection of the NOEY2 constructs and seeded the cells with selection medium after 48h. As a result, those cells seeded were newly transfected, however not selected, so that non-transfected cells were also included in the wells. It is therefore a possibility that the colony formation assays shown are indicative of the transfection efficiency of certain constructs rather than representative of the effect of each construct.

Conversely, in the experimental data included above, cells were transduced using a viral vector and selected prior to seeding. This delayed the seeding of the cells, however all cells that were seeded consisted of the construct of interest. Although transfected cells had been assessed for expression levels, due to time constraints and issues with initial interaction data, this had not been carried out for the virally induced cells so the data cannot confirm expression of the intended construct. However, the visible fluorescence and antibiotic resistance (both in the construct, original transfected cell lines and confirmed in the transduced cells) does suggest a strong probability of the construct of interest being present. Furthermore, the assessment by (Yu, Xu et al. 1999) involved seeding the cells at very low density, allowing them to grow for two weeks and counting the number of colonies that had formed. In our experiments we seeded more cells per dish and observed the

confluence/coverage of the cells once the controls had reached confluence. It is possible that the inhibitory effect of our constructs would have been more apparent if the cells were left to grow for longer and had our initial interaction data proved more robust, further experiments would have then been conducted in order to attempt to reproduce the findings reported in the literature.

Nevertheless, as a result of the difficulties in reproducing the initial interaction data, the NOEY2 project was abandoned and I went on to study the RAP2 interaction that constitutes the majority of this thesis.

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## Additional resources

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<sup>i</sup> <http://www.cellzome.com/>

<sup>ii</sup> Ross, Allison; PL Beales, J Hill (2008). [The Clinical, Molecular, and Functional Genetics of Bardet–Biedl Syndrome, in \*Genetics of Obesity Syndromes\*. Oxford University Press. ISBN 978-0-19-530016-1.](#)

<sup>iii</sup> [May 2006 report to Congress](#) on Implementation of the [MD CARE Act](#), as submitted by Department of Health and Human Service's [National Institutes of Health](#)

<sup>iv</sup> Duchenne Muscular Dystrophy; Emery AE, Muntoni F. Oxford University press, 2003