Rab11-FIP1 interacts with Rab11-FIP5 in p53-mutant cells.

Student: Noor Irfaan Daudi
Supervisor: Dr Patricia Muller, Department of Biosciences, Durham University

Background

The p53 gene (*TP53*) is the most commonly mutated gene in human cancers, occurring in as many as 50% of tumors [1]. These mutations result in the expression of a mutant p53 protein (mutp53), which acquires gain-of-functions (GOFs) through epigenetic regulation, altering functions of other proteins and promoting expression of new transcriptional targets [2]. These GOFs allow mutp53 to exacerbate all cancer hallmarks, labelling it as an oncogene with tumorigenic roles outside of loss of wild-type p53 function [3]. Rab11-family interacting protein1 (FIP1), is able to drive the GOF effects of mutp53 by regulating endocytic recycling of proteins to the cancer cell surfaceosome [4]. Rab11-FIP1 also contributes to chemoresistance [5] and invasion [6] in p53-mutant cancer cells.

Although Rab11-FIP1 generally acts as a homodimer, preliminary data from the Muller Lab suggests that Rab11-FIP1 interacts with Rab11-FIP5, potentially forming a heterodimer. AlphaFold2 generated images of Rab11-FIP1 and Rab11-FIP5 (Figure 1) showed that their structures are highly homologous, especially at their Rab binding domains, through which Rab11-FIP1 homodimerises. Furthermore, an immunoprecipitation-mass spectrometry screen (Table 1) showed that Rab11-FIP5 was the most highly enriched protein in Rab11-FIP1's co-immunoprecipitate in R273H p53-mutant compared to that of p53-null H1299 cells. This heterodimer may contribute to Rab11-FIP1-mediated endosomal recycling and drive mutp53 GOF.

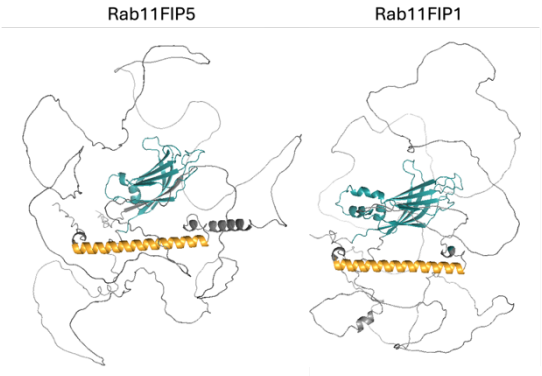


Figure 1: AlphaFold2 images of Rab11-FIP1 isoform 3 and Rab11-FIP5 isoform 2 showing the Rab-binding domains.

Table 1: Top 10 most highly enriched proteins in Rab11FIP1's co-immunoprecipitate in R273H p53 mutant-expressing, compared to p53-null, H1299 lung cancer cells

Gene	Peptides (Unique)	Sequence Coverage [%]	PEP	log_2(fold-change): p53 Mutant vs p53-Null
RAB11FIP5	21 (17)	44.9	6.30E-156	2.379
HSD17B4	17 (17)	27.2	2.13E-126	2.270
SLC12A7	12 (9)	12.2	6.02E-87	1.954
IGSF3	3 (3)	2.5	5.01E-09	1.758
PODXL2	6 (6)	9.6	4.41E-36	1.702
TCOF1	43 (43)	28.7	7.97E-211	1.530
BIRC3	5 (2)	9.4	5.91E-11	1.505
SIRPA	3 (3)	5.5	1.14E-07	1.485
TRAF2	8 (8)	16.6	1.01E-28	1.439
TRAF1	7 (7)	20.7	3.03E-102	1.408

Aims

The primary aim of this project was to determine if any interactions were taking place between Rab11-FIP1 and Rab11-FIP5. Additionally, this project aimed to examine the specificity of the antibodies used throughout the experiments, and confirm that they recognised their respective tags.

Methods used

Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) was used to determine levels of protein-protein interactions between Rab11-FIP1 and Rab11-FIP5 by isolating protein complexes from a lysate solution using an antibody specific for one of the proteins in complex. The method is summarised in Figure 2.

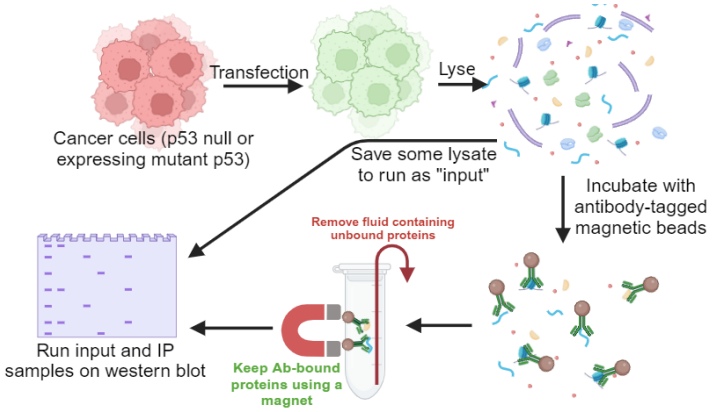


Figure 2: Summary of the Co-IP method used in this project. Figure created on BioRender.com

Immunofluorescence imaging
Immunofluorescence (IF) imaging was used to investigate the localisation and co-localisation of Rab11-FIP1 and Rab11-FIP5 in H1299 and R273H cells.

Western blotting
Western blotting was performed to detect and compare levels of specific proteins in a cell lysate. Membranes were imaged with a LI- COR Odyssey 9260 Fluorescence Imager and Image Studio using the 700nm and 800nm laser.

Confocal imaging
IF coverslips were imaged with a Zeiss 800 Laser Scanning Confocal Microscope (LSCM) with Airyscan, using the 20x Zeiss Plan-Apochromat air lens (NA=0.8) and 63x Zeiss Plan-Apochromat oil lens (NA=1.4). In another set of images, a z-stack of the entire cells in view was taken (30-60 slices, depending on the size of the cells), which was done at a faster scan speed, and so produced images with a compromised S/N in order to speed up the experiment.

Threshold analysis of confocal images
The brightness and contrast of images acquired from the Zeiss 800 were adjusted using ImageJ, and thresholds were generated for all 3 (red, green and blue) channels. Regions of interest were drawn around the cells of interest, and the % area of red, green and yellow thresholds were compared, quantifying the proportion of red-tagged proteins within the cytoplasm that colocalise with green-tagged proteins.

Results and Discussion
Antibody Specificity Testing

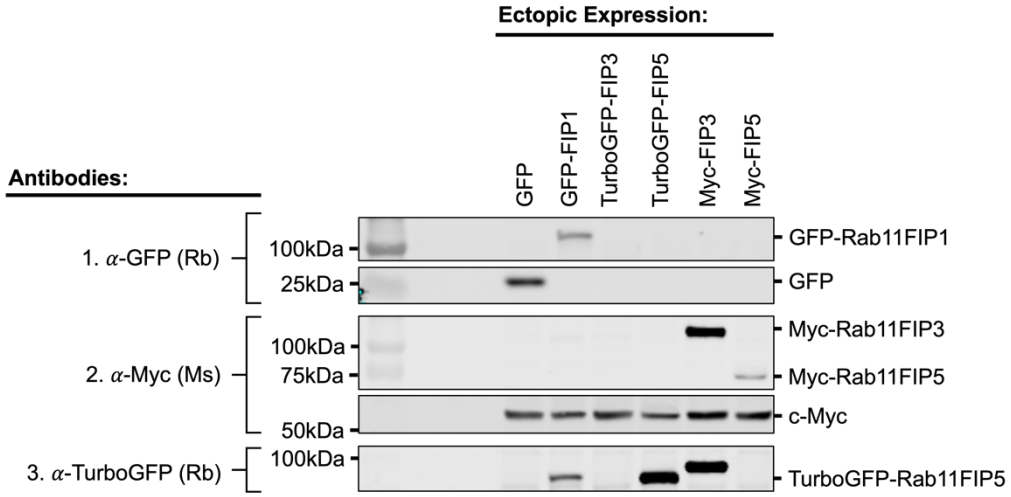
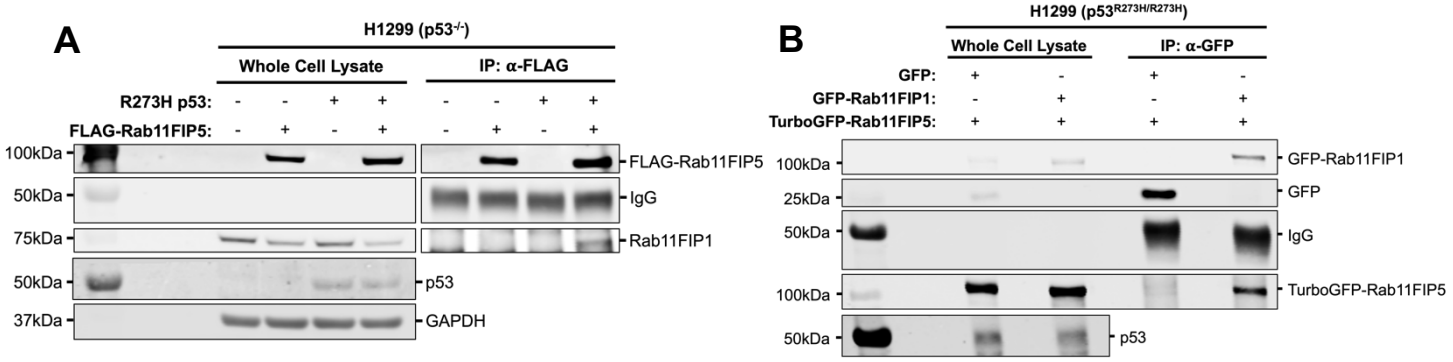


Figure 3: Western blot showing the results of antibody specificity testing.

As shown in Figure 3, the GFP and Turbo-GFP antibodies specifically recognised GFP- and Turbo-GFP-tagged proteins. However, the Myc antibody recognised endogenous c-Myc as well, which is highly expressed in our cell lines of interest. This meant that the Myc antibody may not be as selective as the GFP and Turbo-GFP antibodies, and, therefore, was not used in following experiments.

Rab11-FIP1 Co-immunoprecipitates with Rab11-FIP5 upon ectopic and endogenous mutant p53 expression



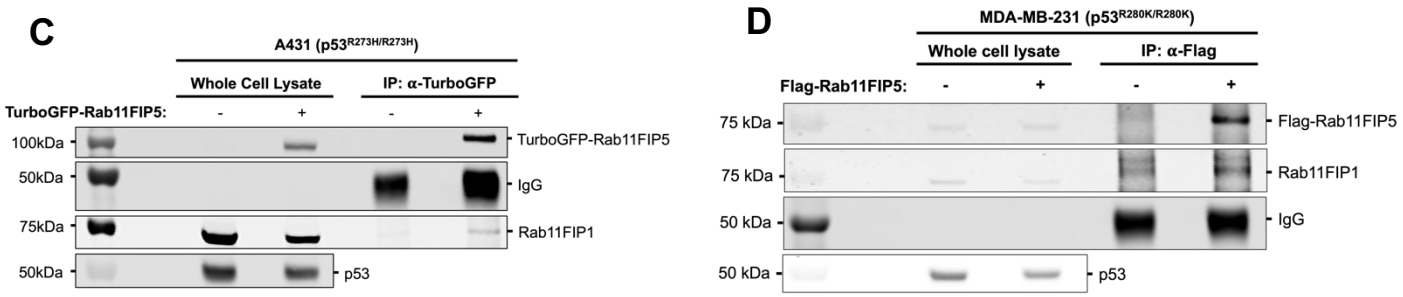


Figure 4: Co-IP experiments to investigate interactions between Rab11-FIP1 and Rab11-FIP5. A-B - GFP- or Flag-IP experiments run in p53-null H1299 and p53-mutant R273H cells, transfected with **(A)** Flag-tagged Rab11-FIP5 and **(B)** GFP-tagged Rab11-FIP1 and Turbo-GFP-tagged Rab11-FIP5. **C-D -** Turbo-GFP- or Flag-IP experiments run in A431 or MDA cells transfected with **(C)** Turbo-GFP-tagged Rab11-FIP5 and **(D)** Flag-tagged Rab11-FIP5. Pulldown of Rab-11FIP1 was examined in all panels. A431 is an endogenously R273H p53 mutant epidermoid carcinoma cell line, while MDA-MB-213 is endogenously R280K p53 mutant breast cancer cell line.

Co-IP studies were performed to attempt to verify that Rab11-FIP1 interacts with Rab11-FIP5, as shown in the mass spectroscopy screen in Table 1. H1299 cells are an immortalised cell line originally isolated from the NSCLC carcinoma of a 43-year old male. As shown in Figure 4, Rab11-FIP1 was pulled down with Rab11-FIP5 in R273H cells, which ectopically express mutp53, and A431 and MDA cells, which endogenously express mutp53, but not in H1299 cells. This demonstrates that Rab11-FIP1 and Rab11-FIP5 do interact in a mutp53-dependent manner.

Rab11-FIP1 co-localises with Rab11-FIP5 in p53-mutant cells

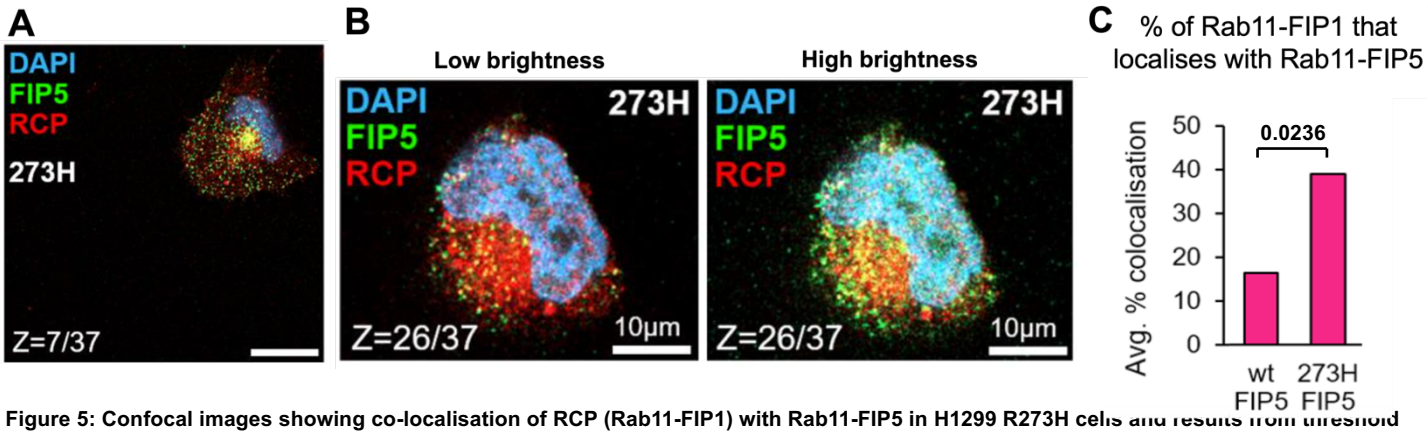


Figure 5: Confocal images showing co-localisation of RCP (Rab11-FIP1) with Rab11-FIP5 in H1299 R273H cells and results from threshold analysis of Rab11-FIP1 and Rab11-FIP5 co-localisation. A - Cytoplasmic and perinuclear localisation of Rab11-FIP1 and Rab11-FIP5. **B -** Comparison of Rab11-FIP1 and Rab11-FIP5 localisation at the same height within the cell, but with the brightness of each channel adjusted in ImageJ. **C -** Percentage of Rab11-FIP1 that co-localised with Rab11-FIP5 in every layer of z-stack throughout entire cells in view. Measurements were taken from one image, with 5-20 cells in view per bar. Scale bars are 20μm unless indicated otherwise.

The co-localisation of Rab11-FIP1 and Rab11-FIP5 was examined to determine if they are found in similar regions of the cell. If they are found in similar areas, this may further confirm the formation of heterodimers between these proteins. It was observed that Rab11-FIP1 is most strongly concentrated at the apex of the cell, and that Rab11-FIP5 also gathers here to some extent (Figure 5A).

However, as demonstrated by the differing images of the same cell in Figure 5B, reliable visual comparisons cannot be made based on qualitative observations, as this depends on the extent of TurboGFP-FIP transfection, immunohistochemistry for the green and red channels respectively, and the brightness of each channel when viewing images in ImageJ. Therefore, quantitative analysis of the area of fluorescent signals throughout the cells was used to determine the extent of co-localisation (Figure 5C). This revealed that Rab11-FIP1 co-localised significantly more with Rab11-FIP5 in p53-mutant R273H cells that p53-null H1299 cells.

Taken together, these results suggest that Rab11-FIP1 interacts with Rab11-FIP5 in p53-mutant cancer cells (both in cell lines that are ectopically expressing p53-mutant genes, and those with endogenous mutations in their p53 genes), as were able to pull down either Rab11-FIP1 or Rab11FIP5 to a greater extent in p53-mutant cells than p53 null cells in co-IP experiments. Additionally, similar localisation of these proteins and their homologous structures suggest that they may form a heterodimer that may be involved in mediating the GOF effects of Rab11-FIP1.

Future Directions

The novel interaction described in this report will be used by the Muller Lab as part of their research to implicate p53 mutations in increased chemoresistance and invasion of cancer cells. Next steps for them are to confirm the formation of a heterodimer between Rab11-FIP1 and Rab11-FIP5, and determine the exact role of this heterodimer, including its possible

interaction with Rab11-FIP1 cargoes. This may also reveal a completely novel set of oncogenic effects that can be therapeutically targeted.

Departures from Original Plan

Although I prepared the IF coverslips correctly, following relevant protocols, I could not take my own images again as our confocal microscope was being serviced for the duration of my studentship. Therefore, since my experiment was a repeat of one done by a previous Master's student of the Muller Lab, her images are used in the final report.

Acknowledgements/Value of Studentship

To Student

This studentship has been an incredible opportunity, provided by the Biochemical Society, to gain wet-lab research experience in a field that I now wish to pursue in the future. I would like to extend my sincere thanks to Dr Muller and Matthew Morris, a PhD student of the Muller Lab who supervised me; their advice and expertise were invaluable throughout my project. Through this studentship, I've been able to carry out techniques beyond the remit of an undergraduate degree.

To Lab

During her time in my lab, Noor produced publishable figures on Rab11-FIP1 interacting with Rab11-FIP5. This will be important for my future grant proposal to BBSRC and could also feature in a publication in which we want to explore how interactions in the Rab11-FIP family contribute to invasion and chemoresistance of mutant-p53 cancer cells. Noor has interacted well with members in the lab, and presented her data clearly during lab meetings, offering several insights into her experiments.

References

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