

## Lily Huang

### Biochemical Society Summer Studentship 2011

## Role of Rab11-family interacting proteins in influenza virus assembly and budding

### Background and Aims:

This project investigated the roles of FIP3 and FIP4 in Influenza A viral budding.

The cellular RAB11 pathway has been shown to play a role in both transporting Influenza A genome from the cell nucleus to the plasma membrane and in virion assembly/pinching off at the plasma membrane (Amorim *et al.*, 2011; Bruce *et al.*, 2010). RAB11 effectors, such as RAB11-family interacting proteins (FIPs), interact with cytoskeletal motors to mediate RAB11's role in membrane and vesicular transport (Horgan & McCaffrey, 2009). We investigated two such effectors, FIP3 and FIP4.

Infection of 293T cells with the PR8 strain of influenza A caused the redistribution and eventual disappearance of Rab11-FIP3 (Bruce, unpublished observations). This loss of FIP3 could be due to degradation by the virus or due to FIP3 incorporation into virus particles for export from the cell. siRNA depletion experiments showed that FIP3 was required in 293T cells for formation of viral filaments but not for spherical virions (Bruce *et al.*, 2010). However, such depletion did not significantly change virus titer (Bruce *et al.*, 2010), thus suggesting that FIP3 was being degraded by the virus. My summer project focused on 1) whether FIP3 behaved similarly in infected A549 cells and on 2) discerning the mechanism of FIP3 disappearance over the time course of infection.

### Description of Work:

The majority of experiments consisted of immunofluorescence (IF) or Western Blot (WB) analysis of infected cells.

#### siRNA Depletions:

- 293T and A549 cell types were seeded in 24 well plates overnight, then transfected with siRNA using the Dharmafect transfection reagent protocol.
- 3 days later, cells were infected with PR8 at an MOI of 10 and fixed with formaldehyde at 16 h.p.i.

#### Immunofluorescence (IF):

- Plasmid constructs for GFP and mCherry tagged proteins were grown up using the MIDI prep procedure.
- 293T and A549 cell types were seeded on glass coverslips overnight, then transfected using the previously grown up plasmids.
- 24 hours later, the cells were infected with PR8 at an MOI of 10 (filament experiments used Udorn virus instead).
- Infected cells were fixed, usually at 16 h.p.i., and then treated with primary and secondary antibodies to visualize proteins of interest.
- The glass coverslips were fixed to glass slides with ProLong Gold Solution and examined under the SP5 confocal microscope.

#### Western Blot (WB):

- Cells of interest were harvested into eppendorf tubes, where they were pelleted and washed with PBS.
- PBS was replaced by Laemmli's Buffer, and solutions were boiled for 8 min.
- Loading and stacking gels were prepared.
- Protein samples and a protein ladder were loaded into the gel and run at constant voltage.
- The gel was transferred onto a nitrocellulose membrane and stained with primary antibodies overnight.
- The membrane was visualized for protein quantification using secondary antibodies.

### Results:

Western blot results showed that siRNA depletion of FIP3 in both A549 and 293T cells were successful. Moreover, PR8 infection led to the disappearance of FIP3 in both cell types by 16 h.p.i., as seen in the "NT-inf" lanes of Fig 1. The gel was co-stained for tubulin as a control for loading error, and the "GFP-FIP3 Total 1uL" lane was run as a positive control for the FIP3 band.

Fig 2 showed that, unlike 293T cells, A549 cells infected with a filamentous virion (Udorn) and depleted of FIP3 can still make filaments.

Fig 3 showed that FIP3 and FIP4 depletion in A549's do not seem to significantly decrease virus titer. However, these results were clouded by the fact that Rab11 depletion, which has been previously shown to decrease virus titer by 10-100 times and should have acted as a positive control, also failed to significantly decrease titer. For more conclusive results, this experiment should be repeated.

SEM experiments showed that FIP3 depletion in A549's infected with PR8 did not greatly change virus morphology (Fig 4). An abundance of spherical virions were seen budding from the cell surface both in cells treated with non-targeting (NT) siRNA and FIP3 siRNA. In fact, patches of FIP3 depleted cells may have shown a greater number of spherical virions in the budding process than NT treated cells did, creating a crowded appearance. However, there were no gross defects in virion formation visible by SEM, consistent with titer data showing that depletion had no significant effects on viral growth (Fig 3).

Overexpression of FIP3 by GFP-FIP3 transfection seemed to disrupt the normal viral NP pattern, as seen at 10 h.p.i. in A549's. FIP3IE is a mutant form of FIP3 that does not bind Rab11. NP in untransfected cells and in GFP-FIP3IE transfected cells formed cytoplasmic aggregates, whereas NP in GFP-FIP3 transfected cells appeared diffuse (Fig 5). This result further supported the theory that FIP3 degradation was specifically orchestrated by the virus to prevent disruption of normal viral functions. Moreover, the experiment suggested that FIP3 disruption of viral functions occurred via Rab11, since the Rab11-binding mutant did not seem to disrupt normal NP patterns.

MG-132 is a proteasomal inhibitor that prevents ubiquitin-mediated degradation. In western blots, MG-132-treated, infected cells indeed seemed to retain a FIP3 band (Fig 6), suggesting that proteasomal inhibition prevented FIP3 degradation, and thus supporting the degradation theory of FIP3 disappearance. However, proteasomal inhibition may merely prevent ubiquitin recycling, which could, in theory, prevent FIP3 trafficking and subsequent incorporation into virions.

IF experiments were conducted to determine whether or not GFP-tagged FIP3 was packaged into viral filaments to be transported out of the cell. A549's were transfected with GFP-FIP3, infected with filamentous virus (Udorn), and stained with anti-H3 antibodies to visualize the viral filaments. Unfortunately, the resulting images were not of a high enough resolution to conclusively show whether or not FIP3 entered the viral filaments (Fig 7).

Staining GFP-FIP3/4 transfected cells with anti-M2 antibodies showed co-localization of FIP3/4 with viral M2 (Fig 8, 9). This finding was somewhat unexpected as FIP3 is thought to reside in recycling endosomes while M2 traffics through the Golgi apparatus.

## Future Directions:

Going forwards, we should rerun the plaque assays to measure virus titer in FIP3 depleted cells.

Staining with an anti-trans Golgi network (TGN) antibody in conjunction with anti-FIP3 and anti-M2 antibodies could help elucidate whether FIP3 and M2 are truly co-localizing in IF experiments.

The role of FIP3 in viral filament formation still eludes us. It seems to be required for 293T filament formation, but not for A549 filament formation. This may be due to differences in cell origin or to differences in endogenous FIP3 levels.

## Departures from Plans:

Originally, we had planned on investigating FIP3's interaction with Arf5/6 to modulate the actin cytoskeleton and the effect of this interaction on viral filament formation. However, additional time was needed to trouble shoot detection of FIP3 by Western Blot, so we chose to focus on a single component of the pathway instead.

## Value of the Studentship:

Working in the lab has exposed me to novel procedures and protocols that I would not have encountered otherwise. The experience was incredibly collaborative yet independent. Supervisors and lab mates were always available to guide me through my thought processes and to answer my questions, but I was also allowed to plan my own experiments and to follow my own schedules. I found my vacation studentship both intellectually challenging and rewarding.

The Biochemical Society studentship provided to Lily allowed her to investigate the role of FIP3/FIP4 in influenza A virus infection. This project was technically difficult, given the lack of good quality anti-sera and the toxicity of over-expressed protein but her persistence and enthusiasm allowed us to confirm previous results as well as making several new observations. Lily was a pleasure to work with, integrating well into the lab atmosphere and quickly learning new techniques. Her work has added considerably to this project, and provides important preliminary data for future work.

## References:

**Amorim, M. J., Bruce, E. A., Read, E. K., Foeglein, A., Mahen, R., Stuart, A. D. & Digard, P. (2011).** A Rab11- and microtubule-dependent mechanism for cytoplasmic transport of influenza A virus viral RNA. *J Virol* **85**, 4143-4156.

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- Horgan, C. P. & McCaffrey, M. W. (2009).** The dynamic Rab11-FIPs. *Biochem Soc Trans* **37**, 1032-1036.