Investigating the Interaction between Clathrin and ESCRT Machinery at the Endosome

Student: Iva Organdjieva
Supervisors: Dr. Kit Briant and Prof. Frances Brodsky

Introduction

Endocytic cargo protein degradation is a pathway which involves the formation of intraluminal vesicles (ILVs) at the endosome. This process is mediated by the endosomal sorting complex required for transport (ESCRT) machinery consisting of four protein subcomplexes (ESCRT 0-III). Charged multivesicular body protein 4b (CHMP4B) is an ESCRT-III component and is responsible for membrane deformation and scission together with the ATPase VPS4. CHMP4B is recruited after ESCRT-0 to -II and shows fast and transient dynamics at the endosome[1]. The membrane coat protein clathrin is required in this pathway for cargo accumulation at endosomes during endocytic cargo degradation. Importantly, clathrin is recruited to the endosome by the interaction between clathrin heavy chain and ESCRT-0 component HRS and is required for ESCRT-0 dissociation. Wenzel, E.M et al have shown that in HRS mutants, the ESCRT-0 component is hyperstabilised at the endosomal membrane and alters CHMP4B dynamics, recruitment, and strength of interaction[1]. Recently, CC2D1A, a master regulator of signalling pathways, was identified by the Brodsky lab as a specific interacting protein of clathrin light chain isoform CLCa (unpublished). CC2D1A also directly interacts with CHMP4B, providing a possible new link between clathrin and ESCRTs. Mice knockout studies have shown that genetic defects in CC2D1A are implicated in autism spectrum disorder (ASD)[2]. Therefore, we hypothesise that the interaction between CLCa and CC2D1A regulates ESCRT assembly at the endosome by indirectly interacting and recruiting the ESCRT-III component CHMP4B. Consequently, the hypothesis explores how the CLCa-CC2D1A interaction at the endosome can affect cargo degradation and normal neuronal differentiation, which may be affected in ASD.

Aims of the project

The overall aim of the project is to explore the potential link between the ESCRT and clathrin pathways at the endosome by investigating the CLCa-CC2D1A and CLCa-CHMP4B interaction and dynamics in the formation of ILVs during receptor and cargo degradation.

Aims:

1. Generate stable Hela cell lines expressing fluorescently-tagged CLCa, CHMP4B, and/or CC2D1A proteins by viral transfection
2. Perform immunofluorescence to confirm protein expression and localisation at the endosome of GFP-CC2D1A or CHMP4B-L-GFP relative to mCherry-CLCa and the endosomal marker EEA1
3. Perform real time live-cell imaging to determine the kinetics of CHMP4B and CC2D1A recruitment to the endosome after EGF-647 treatment by tracking GFP-CC2D1A or CHMP4B-L-GFP relative to mCherry-CLCa

Description of work

Molecular biology: PCR-based plasmid construction and Hi-Fi assembly was used to ligate the fluorescently-tagged protein sequences into a pNG72 backbone obtained from the Martin-Serrano Lab. The PCR products were transformed into *E.coli* DH5a cells. The plasmids were isolated and sent for DNA sequencing to confirm the ligation.

Cell culture: Gag-Pol, VSVG and tagged protein plasmids were transfected into HEK293 cells for viral production. The viral particles were used to generate stable HeLa cell lines (mCherry-CLCa, CHMP4B-L-GFP, GFP-CC2D1A). Stable mCherry-CLCa cells were transfected with CHMP4B-L-GFP and GFP-CC2D1A separately. The cell lines were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin in a humidified incubator at 37°C with 5% CO2.

Western blotting: Cell lysates were separated by SDS-PAGE and analysed by immunoblotting. The proteins were immunodetected using anti-GFP and anti-mCherry antibodies before and after FACS.

Immunofluorescence: Cells were fixed with 3% PFA for immunostaining. The cells were stained before and after FACS for the endosomal marker EEA1 and the CHC antibody X22. Before FACS, the cells were co-stained for anti-GFP and anti-CC2D1A antibodies. The images were processed using ImageJ.

FACS: the single-transfected cell lines were sorted into medium and high expressing batches. The double-transfected cell lines were sorted into a high-expressing batch. The cells were sorted using the Sony MA900 Cell Sorter.

Live cell imaging was done using the sorted cell cultures by two different methods:

1. Cells were cooled on ice and cell surface EGF receptors labelled with cold EGF media (200ng/ul). After 20 minutes on ice, the EGF was swapped with warm Fluorobrite media to initiate endocytosis. The cells were imaged live by confocal microscopy, with 1 image recorded every 3 seconds for 25 minutes. 2. Pre-warmed EGF media was added to the cells and incubated at 37℃ for 2 minutes. The EGF media was swapped with Fluorobrite and the cells were recorded by confocal microscope for 25 minutes as previously.

Results and outcomes

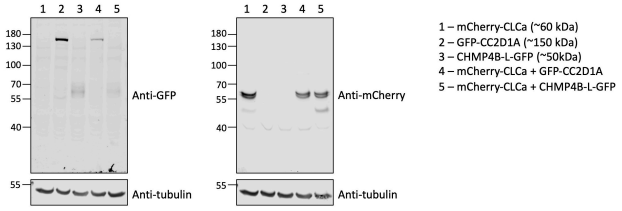


Figure 1. **Immunoblotting for GFP and mCherry proteins in the stable HeLa cell lines before FACS. 3, 5)** Very low expression of CHMP4B-L-GFP in both single-transfected and double-transfected cells. **4)** Low expression of GFP-

CC2D1A in double-transfected cells. **4-5)** Low expression of mCherry-CLCa in double-transfected cells

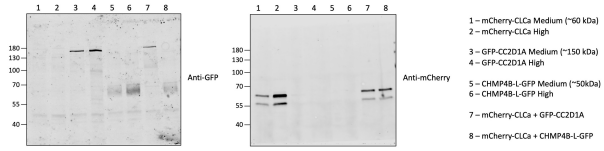


Figure 2. **Immunoblotting for GFP and mCherry proteins in stable HeLa cell lines after FACS. 1-2, 7-8)** Increased expression of mCherry-CLCa in all sorted cells. **5-6)** Relatively increased expression of CHMP4B-L-GFP in single-transfected cells. **7-8)** Expression of GFP-tagged proteins in double-transfected cells remained low.

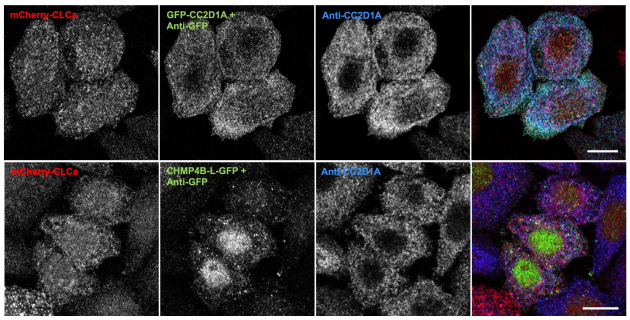


Figure 3. **Immunofluorescence images of mCherry-CLCa + GFP-CC2D1A and mCherry-CLCa + CHMP4B-L-GFP cells stained with anti-CC2D1A and anti-GFP**

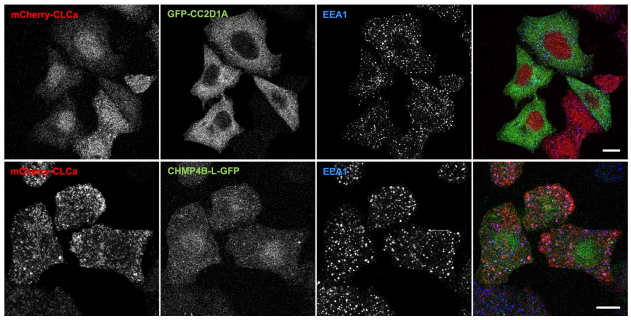


Figure 4. **Immunofluorescence images of mCherry-CLCa + GFP-CC2D1A and mCherry-CLCa + CHMP4B-L-GFP cells stained with endosomal marker EEA1**

Immunofluorescence of fixed cells confirmed the localisation of CLCa, CC2D1A and CHMP4B at the endosome. Colocalisation between the tagged proteins was more challenging to determine due to the varying expression between the GFP and mCherry-tagged proteins. Staining with anti-CC2D1A and anti-GFP enabled us to amplify the GFP signal and confirm the localisation of the weakly expressed CC2D1A. The use of FACS enabled us to obtain cells with higher expression of the proteins. The images in Figure 4 show several puncta of colocalisation between CLCa with CC2D1A and CHMP4B at the endosome (EEA1).

By tracking EGF-647, we obtained videos from live cell imaging that show several points of interaction between CHMP4B and CC2D1A with CLCa (data not shown). These results confirmed that our methods for tracking the interaction in live cells were successful. For analysis, we would require higher quality videos on more specialised live-cell imaging microscopes to accurately quantify the dynamics of CHMP4B and CC2D1A, which is the most challenging step.

Departures from the original aim

We were not able to knockdown CLCa in the stable cell lines and perform live cell imaging on these cells due to time limitation.

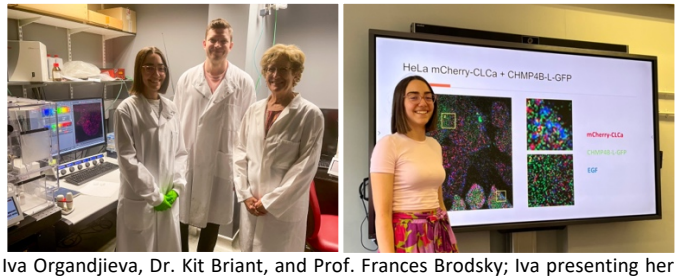
Future directions for the project

The obtained results provide a solid starting point for this project and have covered our major aims. The project is still ongoing and Dr. Briant is currently focusing on improving live cell imaging with these cells. Knockdown of CLCa will also be performed. These cells will be recorded using a specialized confocal microscope for live cell imaging, which allows easier and more accurate quantification of protein dynamics.

Value of the studentship

To the student: The studentship provided me with the opportunity to gain first-hand experience of biomedical research in academia. I gained skills in molecular cloning, HiFi plasmid assembly, PCR, transfection of plasmid DNA, bioinformatical skills in primer design, and cell biology skills in culturing mammalian cell lines, Western blotting, and FACS. Additionally, performing confocal microscopy is an extremely valuable skill, which I can apply to my final year project. In the Brodsky Lab, my confidence was boosted and I improved my collaborative and organizational skills. The technical and transferable skills I was equipped with during this studentship are invaluable to my future studies and potential career in research. My hope is that this research will also contribute to new discoveries related to neuronal development and ASD.

To the lab: The cell lines developed during this project will be of great use to the lab to determine the role of the CC2D1A – CLCa on CHMP4B dynamics, which is a key question for our group. Following this studentship, Dr. Briant has given these cells to collaborators with a specialism in live cell microscopy who will test the cells on several microscopes to assess which will be the best system for quantifiable live cell microscopy.



Iva Organdjieva, Dr. Kit Briant, and Prof. Frances Brodsky; Iva presenting her work to the lab members

References

1. Wenzel, E.M., Schultz, S.W., Schink, K.O. et al. Concerted ESCRT and clathrin recruitment waves define the timing and morphology of intraluminal vesicle formation. Nat Commun 9,2932 (2018). https://doi.org/10.1038/s41467-018-05345-8
2. Reiff T, Baeumers M, Tibbe C, Klein T. Unravelling of Hidden Secrets: The Tumour Suppressor Lethal (2) Giant Discs (Lgd)/CC2D1, Notch Signalling and Cancer. Adv Exp Med Biol. (2021) 1287:31-46. doi: 10.1007/978-3-030-55031-8_3. PMID: 33034024.