The Endocytic Pattern of Metal Ion Transporter ZIP14 and Its Regulation by Zinc

Student: Muyang Duan
Supervisor: Dr Katherine Bowers

Introduction

Zrt- and Irt-like proteins (ZIP) are a family of plasma membrane solute carriers responsible for the intake of several doubly charged ions such as iron, manganese and zinc. ZIP14, which possesses eight transmembrane domains, is globally expressed in various mammalian tissues, with the highest expression in the intestine, liver and kidney. Mutations in ZIP14 result in multiple ion homeostasis-related conditions, including early-onset Parkinsonism induced by magnesium overload and hypozincemia, a mechanism of extracellular zinc depletion in response to pathogen-afflicted inflammation [1]. The cell surface level of ZIP14 largely dictates the extent of ion uptake, which makes it of great research interest. The Bowers lab has previously shown that ZIP14 is internalised in response to exposure to the ions it transports (unpublished). This strongly suggests that metal ions (specifically zinc and manganese) stimulate ZIP14 endocytosis.

Aims, Methods and Results

The overarching goal was to elucidate the change in ZIP14 endocytosis in response to zinc exposure. Multiple experiments needed to be carried out to investigate this.

1. Quantifying cell surface levels of ZIP14 over time to assess the rate of ZIP14 endocytosis by flow cytometry:
   A stable ZIP14-HA HeLa cell line was used. The initial experiments including looking at ZIP14-HA vs HeLa cells (no HA tag) were successful, demonstrating cell surface ZIP14 in the former (Figure 1).
2. Confirming localisation of ZIP14 in recycling endosomes:
   The localisation of ZIP14 in recycling endosomes implies ZIP14's recycling back to the cell surface following endocytosis. I looked into the extent of ZIP14's colocalisation with the transferrin receptor (TfR, recycling marker). Our results show a clear colocalisation (Figure 2), aiding further investigation into ZIP14 recycling. I also became familiar with the immunofluorescence protocol, equipping me with the essential skills for further investigations.

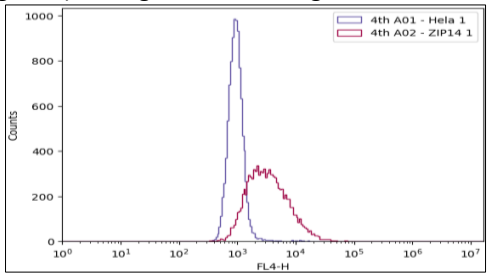

Figure 1. Flow cytometry results of Hela (blue) and ZIP14 (red) cells samples. A significant peak at FL4-H (channel for 647nm fluorescence-conjugated antibodies used) around 10^4 is present in ZIP14 but not Hela cells (Figure made with FlowCal) [2].

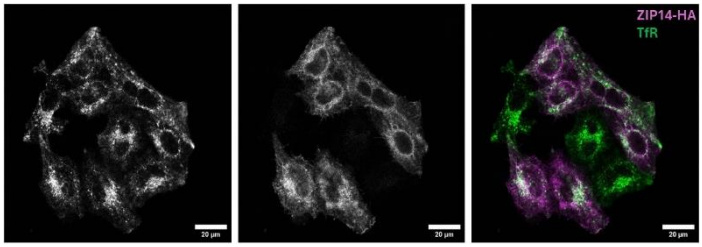

Figure 2. Immunofluorescence staining of transferrin (left) and HA-tagged ZIP14 (both internal and cell surface) (centre). White areas in the merged image represent their colocalisations.

3. Comparing the rate of ZIP14 endocytosis in the presence and absence of Zn^{2+}:

This can be investigated both qualitatively and quantitatively by internalisation and subsequent colocalisation of ZIP14, first with EEA1 (early endosome marker) and then CD63 (late endosome and lysosome marker) using immunofluorescence staining.

Cells expressing ZIP14 were first incubated with anti-HA antibodies on ice where the protein trafficking processes were suspended. The cells were subsequently warmed up to 37 °C in the presence or absence of 200μM zinc sulfate for 5, 10, 30 or 90 minutes before being fixed with paraformaldehyde to permanently halt all cellular activities. Afterwards, cells were permeabilised, stained with anti-EEA1 or CD63, followed by anti-mouse and anti-rat fluorescently-labelled secondary antibodies, before eventually being mounted onto slides for confocal microscopy.

ZIP14 internalisation can be observed from the images generated. In addition, the exposure of Zn^{2+} ions resulted in a significant change in the endocytic pattern (Figure 3). In the presence of Zn^{2+} ions,

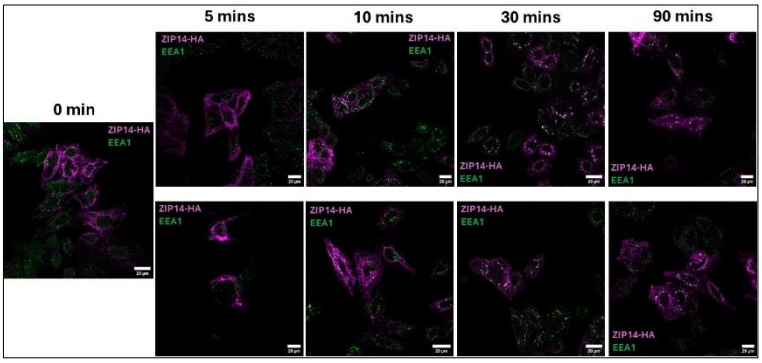

Figure 3. Change in ZIP14-EEA1 colocalisation patterns in the **presence** (top row) and **absence** (bottom row) of Zn^{2+}. Anti-HA is shown in magenta, anti-EEA1 in green and overlaps in white.

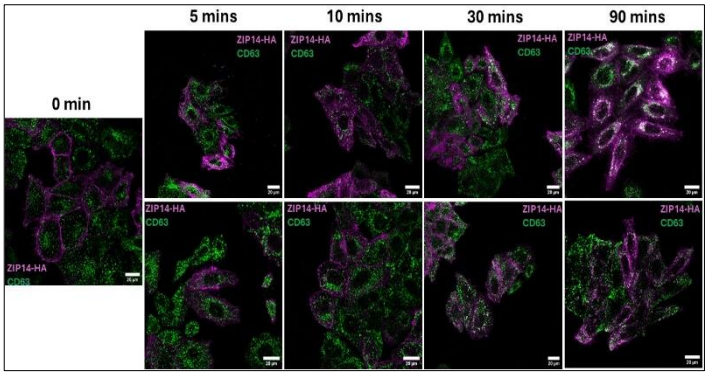

Figure 4. Change in ZIP14-CD63 colocalisation patterns in the **presence** (top row) and **absence** (bottom row) of Zn^{2+}. Anti-HA is shown in magenta, anti-CD63 in green and overlaps in white.

significant colocalisation with EEA1 (white areas) can be observed after 10 minutes, and a dramatic change in cell surface ZIP14 signal is seen after 30 minutes. The second staining attempt was subsequently carried out with different time points (0, 5, 10, 30 and 90 minutes) and CD63 introduced (Figure 4). The endocytic pattern under a single condition (with or without Zn^{2+}) was consistent with that in the first attempt, indicating that ZIP14 is internalised and that the rate of internalisation is faster in the presence of zinc.

Moreover, the extent of ZIP14 and EEA1/CD63 colocalisation was quantified to assess Zn^{2+}'s effect (Figure 5). ZIP14 colocalised more with EEA1 at 10 and 30 minutes of internalisation in the presence of zinc, implying that additional Zn^{2+} increased the rate of ZIP14 endocytosis. After 90 minutes with zinc, the colocalization with EEA decreases, presumably as ZIP14 moves out of early endosomes. Without additional zinc, colocalization with EEA1 continues to increase over 90 minutes, consistent with a slower endocytosis rate. Colocalization of CD63 with ZIP14-HA increases with internalisation time both with and without additional Zn^{2+}, with higher colocalization scores in the presence of zinc. These data provide clear evidence that additional zinc increases the rate of ZIP14 endocytosis.

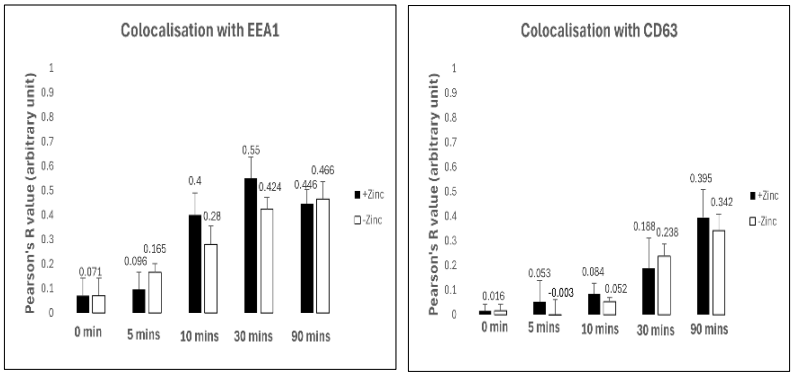

Figure 5. Colocalisation scores of ZIP14-EEA1 and ZIP14-CD63. Data at 5 minutes without Zn^{2+} may be slightly anomalous.

References

1. Aydemir and Cousins, (2018), *J Nutr.*, **148**:174-184
2. Castillo-Hair et al., (2016), *ACS Synth. Biol.*, **5**:774-780
3. Mechanobiology Institute NUS Singapore. URL: https://www.mbi.nus.edu.sg/mbinfo/what-is-clathrin-mediated-endocytosis/
4. Liuzzi et al., (2005), *PNAS*, **102**:6843-6848

Deviation from Plan and Troubleshooting

Difficulties were encountered when quantifying cell surface ZIP14 levels using flow cytometry. Specifically, initial results suggested dominance of debris instead of intact cells in the samples. To address this issue, various aspects of the protocol, such as cell pellet resuspension techniques, centrifugation speed and the use of the flow cytometer, were revisited and challenged. Eventually, the combination of examining the events detected when running pure water on the flow cytometer to assess its competence and more refined sample preparation led to results of higher quality.

Future Directions of Project

1. Repeating the endocytosis colocalisation experiment: We should generate larger data sets and confirm zinc's effect on the rate of endocytosis. We should also add longer time points to investigate ZIP14's fate after being internalised (eg. degradation).
2. Elucidating the effects of other ions: Based on preliminary results, manganese ions also alter ZIP14 cell surface levels. Whether manganese ions exhibit similar endocytosis-accelerating properties is of interest.

Values of Studentship

Apart from the many wet lab skills I acquired, I accessed fluorescence imaging and practised confocal microscopy, inspiring me to dive deeper into this field. I also gained insights into fundamental research into ion metabolism, further fostering my interest in it. Moreover, the motivating and supportive research environment created by the Bowers/Brodsky lab taught me not only invaluable scientific expertise but also ways to form a harmonious relationship with both myself and people around me.

Dr Katherine Bowers said, "It was a pleasure to have Muyang with us for 6 weeks this summer. She was able to show that the addition of zinc to the cells increased the rate of ZIP14 endocytosis. This is an important result and provides the basis for our future studies of the mechanism of ZIP14 endocytosis and endosomal trafficking. Thank you to the Biochemical Society for providing us with this opportunity."

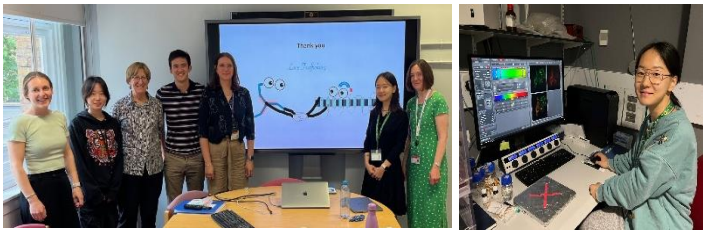

Figure 6. Left: After project presentation at the Bowers/Brodsky lab meeting. (Figure on screen modified from [3,4]). Right: Muyang operating confocal microscope.