

# On the path to asking whether Zip 14 and Zip 8 transport Fe<sup>2+</sup> into mitochondria: Expression of the transporters in HEK293T cells

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## Abstract

Iron is a cofactor that plays critical roles in numerous biological processes including oxygen transport, electron transport, and DNA synthesis. Even though it is necessary in nearly all eukaryotes, free iron is highly toxic because it produces free radicals. Organisms have developed pathways to import, chaperone, sequester, and export this metal ion. How iron is transported into the cell is known, but how iron is transported into the mitochondrion is not well understood. The majority of intracellular iron metabolism takes place in the mitochondria, so it's vital to understand how iron actually enters the mitochondrion. Disruption of iron homeostasis leads to either iron deficiency or iron overload and contributes to a variety of medical problems including anemia, hemochromatosis, and neurodegenerative disorders. Research over the past twenty years has led to the discovery of many molecules involved in the transport of iron. This project ultimately looks to answer the question of whether two iron transporters, **Zip 8** and **Zip 14**, are present in the outer mitochondrial membrane.

## Introduction

Mitochondria function as a focal point of iron metabolism. They are the site of heme synthesis and Iron sulfur cluster biosynthesis. Heme and iron-sulfur clusters are important for normal assembly and optimal activity of the electron transfer complexes in eukaryotic cells. Iron sulfur clusters are required for the function of proteins involved in electron transport in respiratory chain complexes, regulatory sensing, photosynthesis and DNA repair.<sup>2</sup> Therefore, Iron has to get into the mitochondria starting with the outer membrane. Recently DMT1 (Divalent Metal Transporter 1) was found in the outer mitochondrial membrane.<sup>4</sup> Whether any other transporters are present there still unknown. There is evidence supporting the idea that Zip (Zrt -and Irt-like protein) 14 and / or Zip 8 may be iron transporters present in the outer membrane of the mitochondria in addition to DMT1. Both have both been proven to transport iron across the plasma membrane.<sup>1</sup> They have both been shown to transport iron optimally at physiological pH (7-7.5), which supports the possibility these proteins are more suitable to transport iron through the outer membrane of the mitochondria. (The pH in cells is ~7 whereas DMT1 transports optimally at pH of 5.5.)

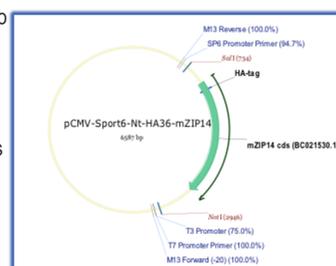
## The Questions

- Do cells transfected express **Zip 8** and / or **Zip 14**? Where are they localized?
- Are **Zip 8** and / or **Zip 14** expressed in the mitochondria?
- The **big question**: Are **Zip 8** and / or **Zip 14** present in the outer membrane of the mitochondria?

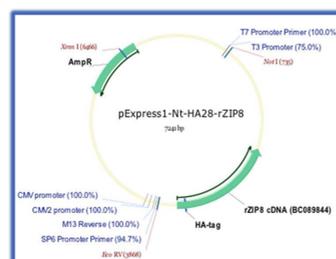
## Methods

### 1. Preparation and verification of plasmid DNA

Plasmids containing the two Zips were given to our lab. Plasmid DNA was cloned and restriction enzyme digests were done using agarose gel electrophoresis to verify fragment lengths. Both Zip 8 and Zip 14 plasmid DNA was sent to Roswell Sanger Sequencing services for verification.



Note: **Human influenza hemagglutinin (HA-tag)** is present on both plasmid vectors. This HA-tag allows for the detection of both tagged target proteins via HA-specific antibody.



### 2. Transfection of HEK293T cells to express Zip proteins

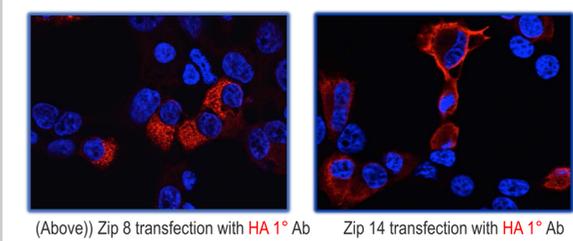
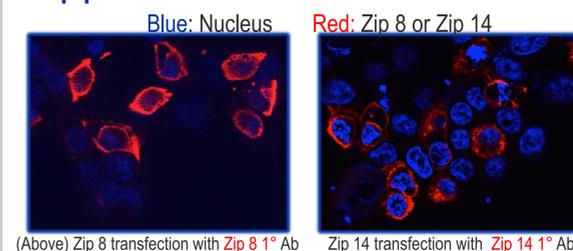
HEK 293T cell lines were cultured in DMEM + 10% fetal calf serum. After passage into twelve well plates, cells were allowed to grow 48 hours and then transfected with either Zip 8 or Zip 14 DNA using Lipofectamine reagent. Cells were allowed to recover for one day, then fixed with 4%PFA, permeabilized with 0.1% Triton and blocked with 5% BSA. Cells were then stained with ZIP8 antibody, Zip14 antibody, or HA-tag antibody followed by Anti Rabbit IgG Alexa 568, then mounted on glass slides using Prolong Gold with DAPI. Photos were taken using Zeiss Axiolmager Fluorescence microscope with 63x oil lens.

### 3. Western blotting to determine Zip 8 and Zip 14 expression in transfected HEK293T cells and isolated mitochondria

HEK293T cells were transfected as in step 2. Cellular proteins were either purified from transfected cells using standard methods or mitochondrial protein isolates were purified using Thermo Fisher Mitochondrial Isolation Kit. Proteins were then separated by SDS/PAGE and blotted onto nitrocellulose membranes. After blots were blocked in 5% nonfat milk, they were incubated overnight with a 1:1000 dilution of Zip 8, 1:100 of Zip 14, or 1:750 HA-tag antibody followed by a 1:10,000 dilution of Alexa Anti Rabbit IgG HRP antibody. For whole cells, an Actin control was used to verify protein load concentration. For mitochondria, cytochrome C was used. ECL reagents were applied according to manufacturer and then developed in a dark room.

## Results

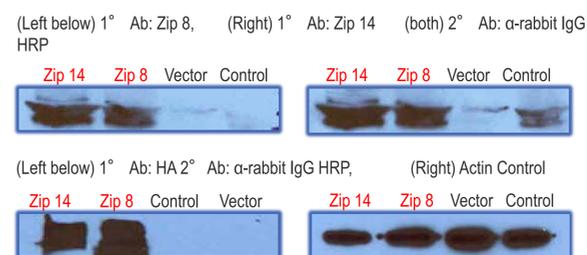
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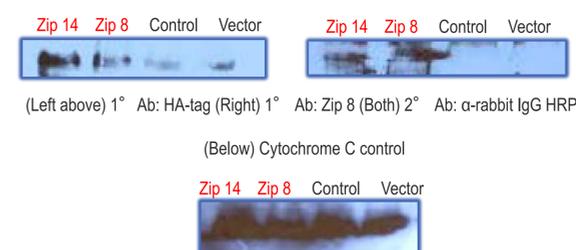
(Above) Control, 2° antibodies only; Right: Control, vector transfection

### 3. Western blotting to determine Zip 8 and Zip 14 expression in transfected HEK293T cells and isolated mitochondria

#### Western Blotting Cell Results



#### Western Blotting Mitochondria Results



## Initial Conclusions and Discussion

- Plasmid DNA is authentic.
- Transfection of Zip proteins was successful. **Zip 8** → localized throughout the cell and the membrane. **Zip 14** → localized on cell membrane
- Western blotting detects **Zip 8** and **Zip 14** in whole cell results by α-Zip and α-HA → confirmation Zip proteins are expressed
- Western blotting mitochondria → **Zip 8** and **Zip 14** are present in the mitochondria as seen by α-HA .
- It is plausible both **Zip 8** and **Zip 14** exist as oligomers as seen by the heavy bands around 250kD in all western blots. This is similar behavior to DMT1. Additional experiments and data are needed to confirm this suspicion.

## The Next Steps

- Perform additional western blotting to confirm tentative results
- Isolate mitochondria after transfection → stain as in step 2 → visualize under fluorescence microscopy → generate additional data
- Isolate mitochondria after transfection, fractionate into mitoplasts + outer membrane of mitochondria + intermembrane space → western blotting on each fraction → will show which mitochondrial fraction contains proteins

## Acknowledgments

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