Glimpse into the Dimerization, Distribution and Interaction Dynamics of ATP7A using Super Resolution Imaging

Aparna Calindi, Meng-Hsuan Wen, Dr.Tai-Yen Chen

Department of Chemistry, University of Houston

INTRODUCTION

Copper plays a critical role in human metabolism as a cofactor of key metabolic enzymes, which are involved in various physiological processes. However, an imbalance in copper homeostasis can be very toxic to the body and have been known to cause a number of conditions, including Menkes disease, Wilson's disease, and Alzheimer's disease. This balance is thus maintained by an elaborate system of proteins, enzymes and transporters to ensure that the copper ions are trafficked in an orderly manner.

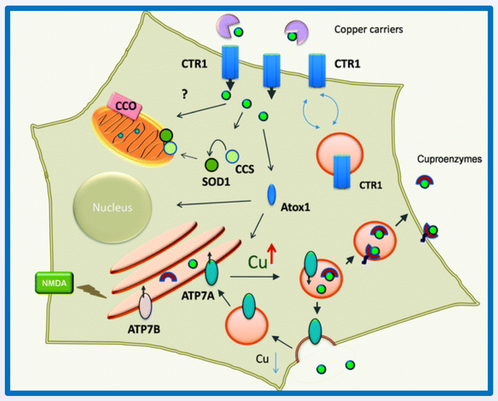

Figure1: Copper trafficking pathway in the cell.
(Figure adapted from Metallomics, 2010, 2, 596–608)

In cells, the Cu-ATPases maintain intracellular copper concentration by transporting copper from the cytosol across cellular membranes. The ATP7A gene provides instructions for making a protein that is important for regulating copper levels in the body. The ATP7B gene provides instructions for making a protein called copper-transporting ATPase 2. Though, these two show high homology, they perform different functions. Recently, ATP7B has been reported as a dimer in the cells, suggesting dimerization as a potential regulatory mechanism. Therefore, this research focuses on testing if the ATP7A can dimerize similar to the ATP7B, and also aim to understand the sub-cellular distribution as well as the interaction dynamics of the dimer complex.

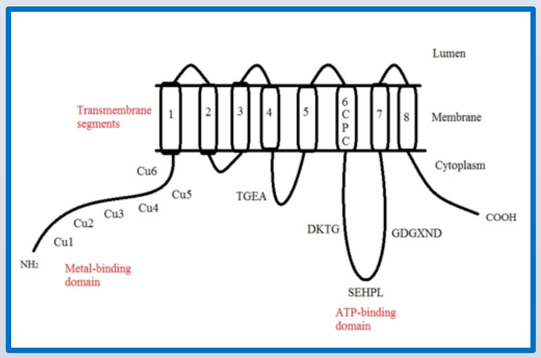

Figure2: Proposed structure of ATP7A.
(Figure adapted from https://en.wikipedia.org/wiki/ATP7A).

METHOD

1. Plasmid Construction: For this purpose, fluorescent protein (FP) tagged ATP7A constructs for mammalian cell expression were generated. Three FP's namely mCherry, mEoS3.2 and PAGFP were used to make three different constructs.

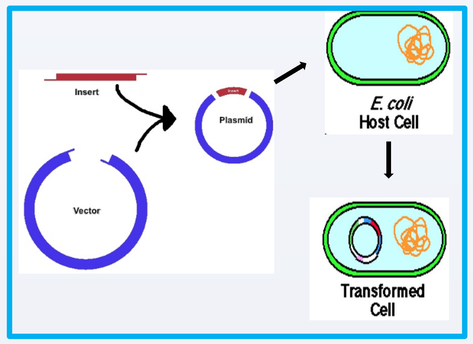

Figure3: Construction of Recombinant DNA
(Figure adapted from https://www.neb.com/tools-and-resources/feature-articles/foundations-of-molecular-cloning-past-present-and-future)

2. Mammalian cell transfection and expression: From the constructs prepared, the DNA was extracted and transfected into COS7, a process of deliberately introducing purified nucleic acid into eukaryotic cells.

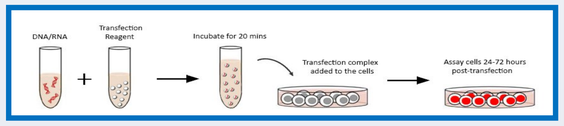

Figure 4: Transfection Protocol at a glance.
(Figure adapted from https://www.abmgood.com/Transfection-Reagent-Protocol-Efficiency.html)

This is followed by imaging to check for functionality assay under different copper conditions. CuCl2 was used to create a copper excessive environment to monitor the ATP7A location within the cell.

3. Functional characterization: Western blotting is performed to ensure the desired protein has been synthesized. It works on the principle that employs electrophoresis to separate samples which are then transferred to a stable membrane and subsequently probed.

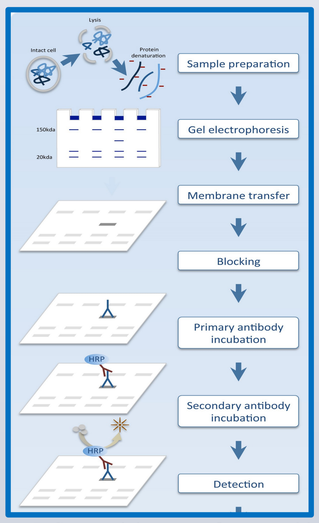

Figure 5: Sequential stages of western blot process.
(Figure adapted from Bass, JJ. *et al.* (2017) Scand J Med Sci Sports;27(1):4-25

RESULTS

1. Plasmid Construction: The figure below shows three bands are obtained for all the 8 colonies. The first two represent the mCherry-hATP7A construct and the rest represent the PAGFP-hATP7A construct. The bands shown are around ~5000bp, ~3000bp and ~2000bp. These are the expected values indicating that all the selected colonies contain the desired construct.

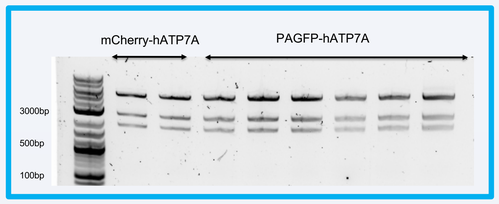

Figure 6: Digestion of the prepared construct using ApaI and XbaI

2. Mammalian cell transfection and expression This figure shows the mCherry-hATP7A (top A) mEos3.2-hATP7A (B) being expressed within the COS7 cells. The functionality assay of mCherry is observed using CuCl2 where the protein is dispersed into the surrounding regions as compared to that under the normal condition.

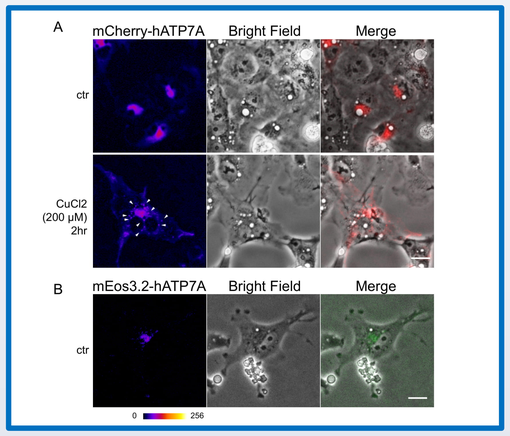

Figure 7: Microscope images of constructs under bright field and red/ green channel

3. Functional characterization: This figure shows the blotting images of mCherry and PAGFP constructs. The first panel shows a band around ~190kDa which corresponds to mCherry-hATP7A. The second panel shows a stronger signal for PAGFP-hATP7A at the same length.

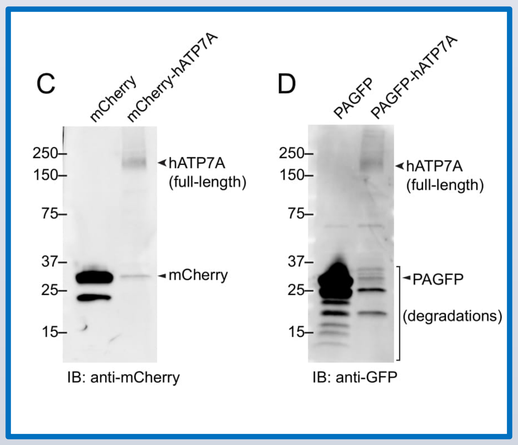

Figure 8: Western blots for mCherry-hATP7A and PAGFP-hATP7A

DISCUSSION & CONCLUSION

The Molecular Cloning results show the correct bands for all eight colonies totaling upto ~9000bp which is the size of the total construct. This serves as a confirmation to show that the desired construct has been obtained.

The Imaging results show under regular growth condition mCherry-tagged hATP7A mainly distributed at perinuclear region, suggesting the fusion proteins were properly synthesized through secretory pathway. After 2hr treatment with 200 µM CuCl2, several mCherry positive puncta (white arrows) scattering away from ER-Golgi condensed region can be observed, suggesting the vesicle trafficking of the fusion proteins in response to Cu stress is still functional. The mEos3.2 tagged hATP7A also showed similar perinuclear distribution under normal condition.

The integrity of mCherry- and PAGFP-tagged hATP7A were analyzed by immunoblotting. Despite the relatively low expression level and some degradation, the intact full-length proteins still can be detected with expected molecular weight at ~ 190 kDa.

Now that all the constructs are ready with confirmed sequence, functionality assay and western blotting, they are ready for imaging using a Super Resolution Microscopy to analyze the dimerization and Interaction dynamics of ATP7A.

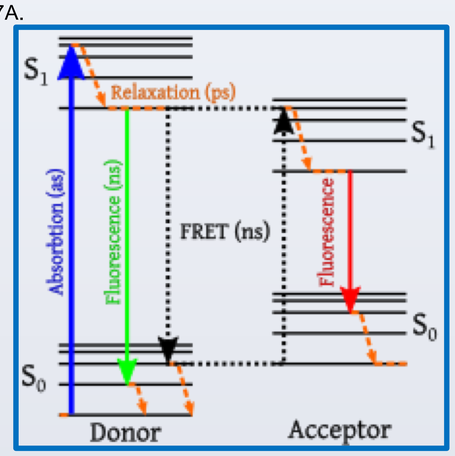

Figure 7: Schematic Representation of FRET
(Figure adapted from https://en.wikipedia.org/wiki/F%C3%B6rster_resonance_energy_transfer)

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