Cystinosis Research Foundation Progress Report

Title of Grant: "Dissect the protein turnover mechanism of cystinosis mutants"
Principal Investigators: Ming Li, Ph.D. and Jacob Kitzman, Ph.D.
Grantee Institution: University of Michigan, Ann Arbor, Michigan
Grant Period: February 1, 2019 – January 31, 2021
Progress Report: August 1, 2020-January 31, 2021

Overview: In this proposed research, we hypothesize that a group of pathogenic Cystinosin mutants are selectively ubiquitinated by an unidentified human lysosome protein quality control system and degraded prematurely in the lysosome. In our preliminary study, we have identified a pathogenic CTNS mutant, ΔITILELP (hereafter referred to as $CTNS_{7\Delta}$), as a fast degrading mutant in humans. We plan to pursue two specific aims to test the central hypothesis. For **Aim 1,** we planned to measure the half-lives of all pathogenic Cystinosin mutants to identify more fast-degrading mutants using cycloheximide (CHX)-based chase assay. For **Aim 2,** we planned to identify the corresponding E3 ubiquitin ligase and downstream degradation machinery. We will use a CRISPR- and flow cytometry-based high throughput screening method to determine the E3 ligase and the downstream machinery.

Progress in the fourth six-month period:

Our lab is still running on a limited capacity in the past six months of the funding period due to COVID-19 pandemic. My lab members are working in shifts to maintain social distancing. So, we focused our efforts on 1) developing a new screening system to isolate fast-degrading CTNS mutants, 2) analyzing the subcellular localization of $CTNS_{7\Delta}$-GFP, 3) generating the HRD1 knockout (KO) and HRD1 GP78 double KO cell lines.

Aim1a: Screening for new CTNS mutants that are quickly degraded by the lysosome protein quality control system

In our previous efforts to identify fast-degrading CTNS mutants, we used a low throughput method by testing all reported disease-causing CTNS mutants one by one.

This way, we only identified one mutant ($CTNS_{7\Delta}$) out of 41 tested mutants. For the last six months, we decided to take a more direct approach. Instead of screening the reported Cystinosin mutants for fast degrading mutants, we used PCR-based random mutagenesis to create a CTNS mutant library (Fig. 1). The library will be fused to a GFP-mCherry tag. If a CTNS mutant is recognized by the lysosome membrane quality control system, it will be constitutively degraded inside the lysosome lumen. Consequently, the GFP to mCherry ratio will be low because GFP is sensitive to the lumenal proteases, but mCherry is resistant. In contrast, if the mutant is stable, the GFP-mCherry tag will be localized to the cytoplasmic side of the lysosome membrane, and the GFP/mCherry ratio will be high. We will then use flow cytometry to sort mutants with a low GFP/mCherry ratio and determine their coding sequences by Illumina sequencing (Fig1A).

So far, we have finished the construction of the phage-based CTNS library for transfecting the HEK293 cells (Fig. 1B). We are in the process of evaluating the library by determining the mutation rate for each Kb DNA in the CTNS coding region, as well as testing the transfection efficiency with HEK293 cells.

Aim1b: Determining the subcellular localization of CTNS7Δ-GFP mutant

In our previous study, we have shown that $CTNS_{7\Delta}$-GFP appears as five distinct bands on a Western Blot. While Band 5 is a breakage product of free GFP falling off $CTNS_{7\Delta}$-GFP, bands 1-4 are localized to the membrane fraction, either at the lysosome membrane or at the ER membrane. Using the lysosome immunoprecipitation (IP) experiment, we have shown that band 2 and band 4 are localized to the lysosome membrane. We suspected that band 1 and band 3 are localized to the ER membrane and rapidly degraded through the ER-associated degradation pathway. In support of this hypothesis, knocking down P97, an essential AAA ATPase involved in the ERAD pathway, stabilized both bands 1 and 3 (Figure 3C). However, the direct evidence to support their ER localization is still lacking. When we checked the $CTNS_{7\Delta}$-GFP under the fluorescence microscope, the mutant protein appeared to localize to both ER and lysosomes.

To provide further evidence of the ER-localization for bands 1 and 3, we are developing other biochemical approaches. So far, we have tried two methods, including immunoprecipitation of the microsome vesicles and differential centrifugation. Unfortunately, both methods have failed. As shown in Figure 2A-B, we tested IP with antibodies against two different baits (Sec61β-3HA and HRD1). Although we were able to pull-down ER microsomes, there was no difference between the control and the antibody reaction, suggesting that the observed pull-down was due to non-specific binding to resins. We then tested differential centrifugation and Oxiprep density gradient to enrich ER microsomes(Figure 2C-D). Under these conditions, however, lysosomes are also enriched together with the microsomes.

We are now testing if we could use FACS sorting to separate the ER microsomes from the lysosome fraction. We plan to label Sec61β with a very bright fluorophore PE/Dazzle and sort the ER microsomes by flow cytometry. Following the FACS sorting, we will use Western Blotting to check if Bands 1 and 3 are enriched in the microsome fraction.

Aim2: In search of the E3 ubiquitin ligase that ubiquitinates CTNS$_{7\Delta}$-GFP

As stated above, we hypothesized that bands 1 and 3 of CTNS7Δ-GFP are degraded through the ERAD pathway. However, knocking out either GP78 or HRD1, the two major E3 ubiquitin ligases that function in the ERAD pathway, did not slow down the degradation of the CTNS7Δ-GFP (Figure 3A-B). We also knocked down HRD1 in the GP78 knockout background and observed minimal CTNS7Δ-GFP degradation. These results strongly suggest that a novel ER-localized E3 ligase might be involved in the degradation of CTNS7Δ-GFP.

We plan to perform a whole-genome CRISPR screening to identify this new E3 ligase. To do this, we need to generate a HEK293 strain with the double knockout of HRD1 and GP78. This double knockout strain was successfully generated two weeks ago(Figure 3D, DKO-5, and DKO-6). We will perform the CRISPR screening soon.

Closing:

We wish to thank the CRF for the generous support. This project did not work as we initially hypothesized. We proposed that CTNS7Δ-GFP was degraded by an unidentified quality control system on the lysosome membrane. After extensive investigation, we now believe that CTNS7Δ-GFP is degraded at the ER by the ERAD pathway. Our results suggest that a novel ER E3 ligase might be involved in the turnover of CTNS7Δ-GFP. We will use the CRISPR screening to identify this E3 ligase.

The other unexpected result was: out of 41 disease-causing CTNS mutants, we only identified one fast degrading mutant (CTNS7Δ-GFP). We are now using a more direct approach to create the fast degrading CTNS mutants. Finding these mutants will enable us to study the mechanism of the lysosome protein quality control, a completely unexplored question in the field.

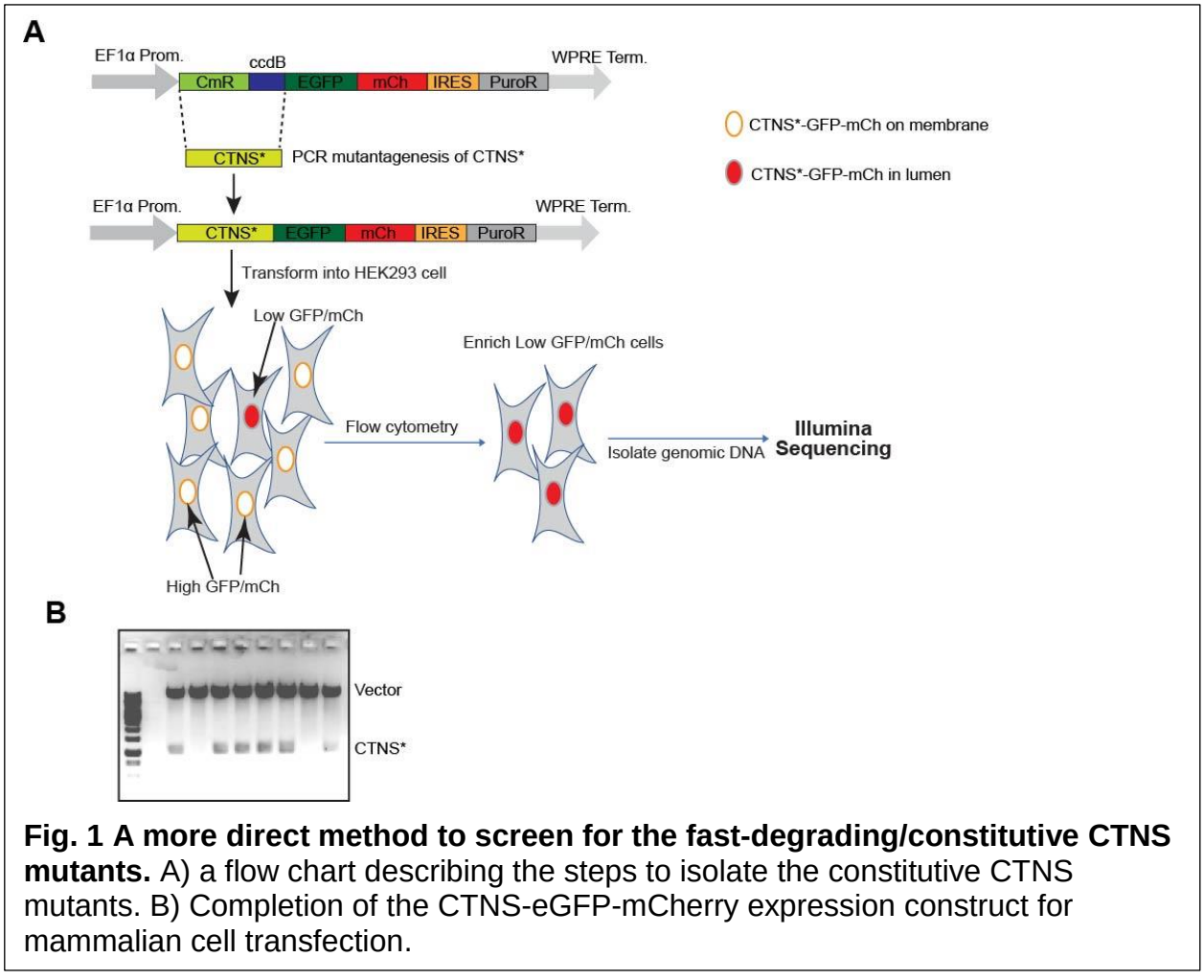

Fig. 1 A more direct method to screen for the fast-degrading/constitutive CTNS mutants. A) a flow chart describing the steps to isolate the constitutive CTNS mutants. B) Completion of the CTNS-eGFP-mCherry expression construct for mammalian cell transfection.

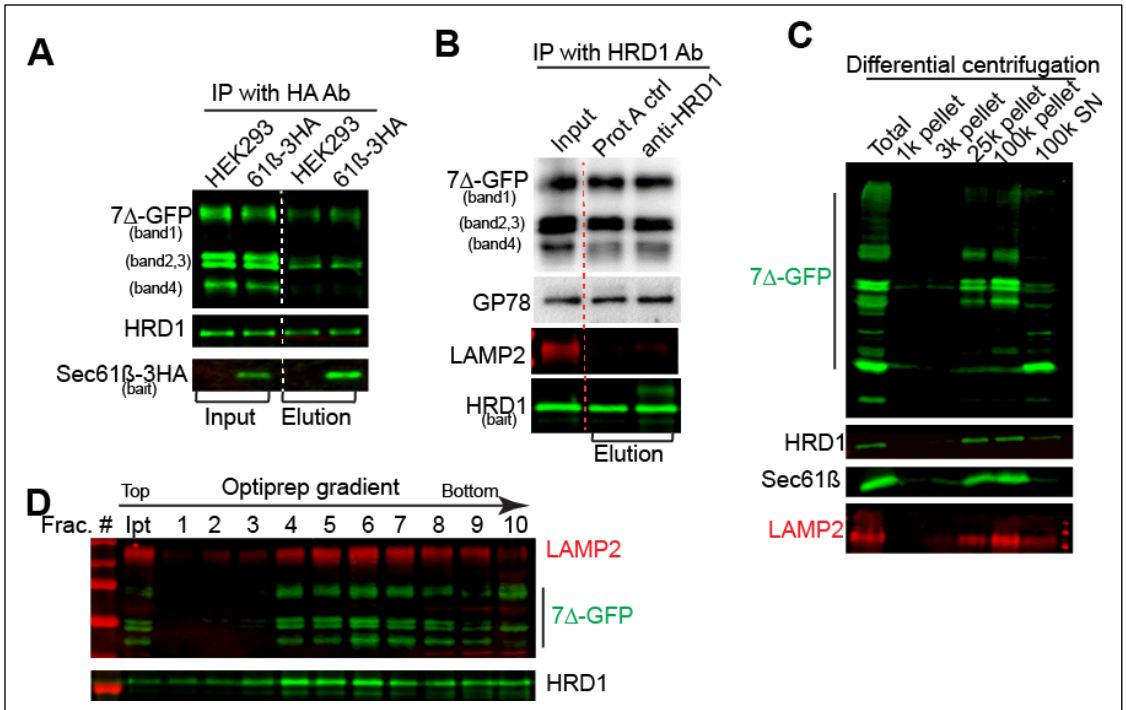

Fig. 2 Developing a method to purify ER microsomes (in progress).

A-B) ER microsomes non-specifically bind to agarose resin during immunoprecipitation experiments. Two different baits(Sec61β and HRD1) have been tested. **C)** ER microsomes are enriched in the same fractions as lysosomes(labeled by LAMP2) during a differential centrifugation. **D)** ER microsomes are enriched in the same fractions as lysosomes (labeled by LAMP2) during a Optiprep gradient centrifugation.

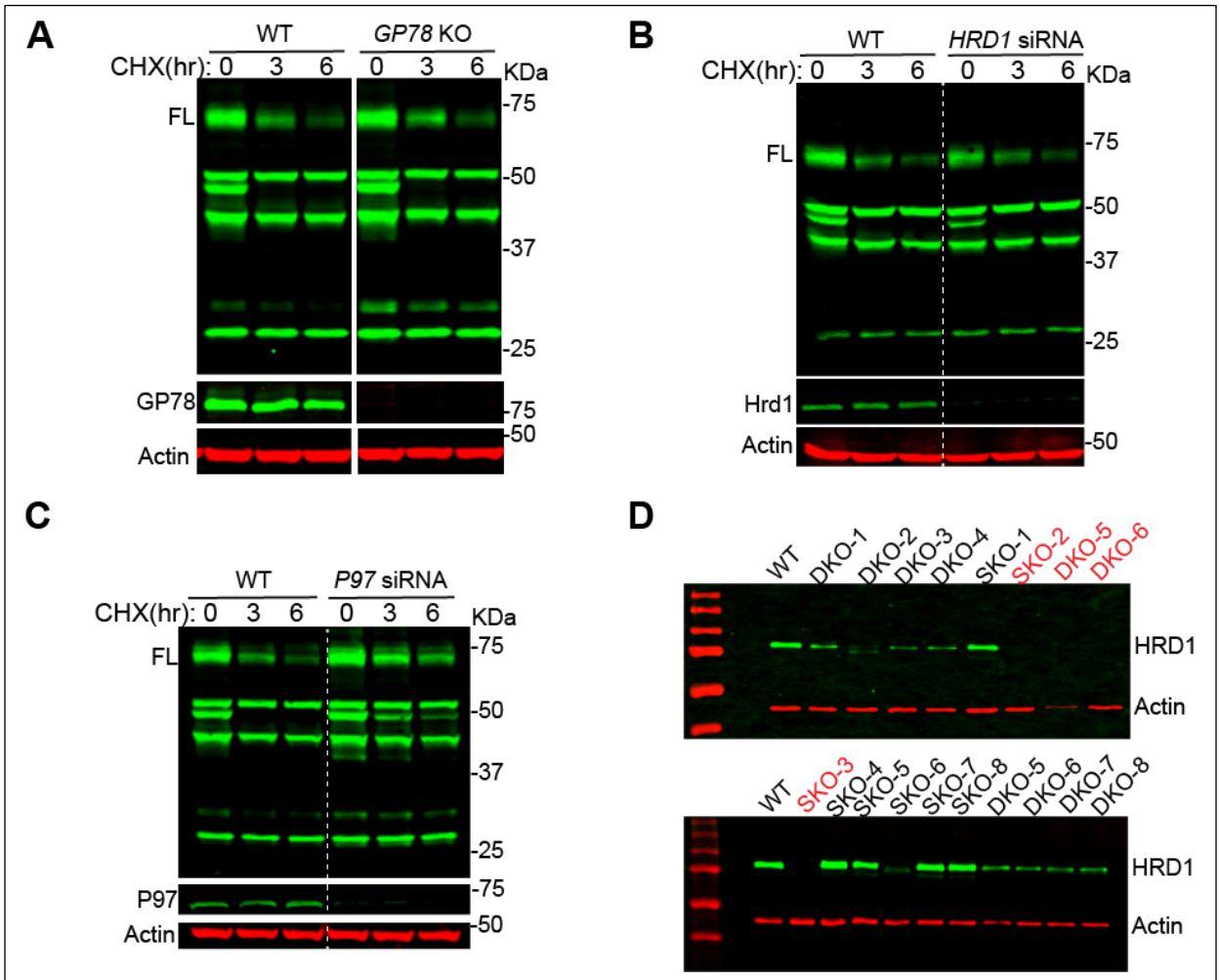

Fig. 3 Bands 1 and 3 of CTNS 7Δ-GFP are degraded by the ERAD pathway via a new E3 ligase. A) GP78 alone is not important for the degradation of CTNS 7Δ-GFP. **B)** HRD1 alone is not important for the degradation of CTNS 7Δ-GFP. **C)** P97 is important for the degradation of CTNS 7Δ-GFP. **D)** Generation of HRD1 single knockout and HRD1 GP78 double knockout lines in HEK293 cells. SKO: single knockout of HRD1. DKO: double knockout of HRD1 and GP78.