

UNIVERSITY OF CALGARY

Investigating the Consequences of Accumulated STARD1 on Glucocorticoid Production

by

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A THESIS

SUBMITTED TO THE CUMMING SCHOOL OF MEDICINE
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE
DEGREE OF BACHELOR OF HEALTH SCIENCES HONOURS

Bachelor of Health Sciences
Cumming School of Medicine
University of Calgary
Calgary, AB

March 2019

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Abstract:

Mitochondria are crucial for many cellular processes including energy production and metabolic pathways, such as steroidogenesis (steroid hormone production). Classic mitochondrial disease is caused by a lack of ATP production, and can present as a multitude of pathologies such as encephalopathy, ataxia, and lactic acidosis. Mitochondrial chaperonopathies are a new class of mitochondrial disease characterized by impaired mitochondrial protein homeostasis (proteostasis) that present with novel phenotypes such as skeletal abnormalities and growth deficiencies. Notably, increased levels of glucocorticoids, a class of steroid hormones, could explain these atypical mitochondrial disease phenotypes. Our goal is to understand the molecular mechanisms through which impaired proteostasis could lead to increased levels of glucocorticoids to better understand and eventually treat patients with mitochondrial chaperonopathies. The rate-limiting step in steroidogenesis, cholesterol import into mitochondria, is regulated by the steroidogenic acute regulatory protein (STARD1). STARD1 is degraded by LONP1 protease, mutations in which impair STARD1 turnover, and can cause a mitochondrial chaperonopathy phenotype. In the current study, we assessed the link between LONP1 impairment leading to accumulation of STARD1 protein, resulting in increased cholesterol import. Subsequently, we posit that higher amounts of cholesterol import ultimately lead to an increased production of glucocorticoids, which may be an underlying cause of the novel phenotypes we see in mitochondrial chaperonopathies. Results indicated increased cholesterol import following accumulation of active STARD1. Findings from this study provide novel insight into the effects of mitochondrial dysfunction on steroid signaling, which are also linked to other common pathologies including diabetes and neurodegenerative diseases.

Acknowledgements:

I would like to sincerely thank my Principal Investigator, Dr. Timothy Shutt, for his continuous support and mentorship. His guidance throughout my undergraduate research and this thesis was crucial to my success, education, and development of both my long-term personal and career goals. Thank you for always being patient and encouraging, and for guiding me in everything from troubleshooting experimental procedures to presenting and writing my work as a story. As well, thank you to my fellow labmates in the Shutt Lab for their endless encouragement and guidance throughout my research. I would like to recognize Tian Zhao for his mentorship and assistance during this thesis. Tian, thank you for always taking the time to assist me, I am extremely grateful for your supervision. A special thank you to Rasha Sabouny for her constant support, kindness, and instruction. Rasha, thank you for taking the time to teach me, you were essential to my learning and development of laboratory skills. Finally, thank you to my family and especially my parents, for your unconditional support and motivation to work hard and persevere throughout all my endeavors.

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List of Abbreviations

1. AIFM1 = Mitochondrial apoptosis-inducing factor 1
2. ATP = Adenosine triphosphate
3. ATPase domain = Adenosine triphosphate domain
4. CDDO = Triterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid or Bardoxolone
5. CODAS = Cerebral, ocular, dental, auricular, skeletal syndrome
6. CYP11A1 = Cholesterol side-chain cleavage enzyme or P450 side-chain cleavage
7. DbcAMP = Dibutyryl cyclic adenosine monophosphate or Bucladesine
8. DNA = Deoxyribonucleic acid
9. DSMO = Dimethyl Sulfoxide
10. ELISA = Enzyme-linked immunosorbent assay
11. HEK-293 = Human embryonic kidney 293 cell line
12. HSPA9 = Mitochondrial 70kDa heat shock protein
13. H295R = Human adrenocarcinoma cell line
14. IMM = Inner mitochondrial membrane
15. kD or kDa = Kilodalton
16. LONP1 = Lon protease homolog
17. MG132 = N-benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal
18. mtDNA = Mitochondrial deoxyribonucleid acid
19. MTS = Mitochondrial targeting sequence
20. mtUPR = Mitochondrial unfolded protein response
21. N-terminal domain = Amino-terminal domain

- 22. OMM = Outer mitochondrial membrane
- 23. OTC = Ornithine transcarbamylase
- 24. Δ-OTC = Mutant ornithine transcarbamylase
- 25. P-domain = Proteolytic domain
- 26. o-Phen = Phenanthroline
- 27. PINK1 = PTEN-induced kinase 1
- 28. PISD = Phosphatidylserine decarboxylase
- 29. pcDNA 3.1 (-) = Mammalian expression vector
- 30. STARD1 = Steroidogenic acute regulatory protein
- 31. TSPO = Translocator protein

Chapter 1: Introduction and Background

1.1 Mitochondria and Mitochondrial Disease

Mitochondria are double membrane-bound organelles crucial for basic cellular processes including apoptosis, energy (ATP) production, and metabolic pathways such as steroid synthesis (steroidogenesis). This research project focuses on the role of mitochondrial proteins in steroidogenesis, and the consequences of protein impairment caused by dysfunctional protein turnover.

Classic mitochondrial disease is caused by a lack of ATP production, typically due to dysfunctional mitochondrial oxidative phosphorylation (1). Recent epidemiological studies suggest at least 1 in 5000 individuals are affected by mitochondrial dysfunction (2, 3). Most mitochondrial protein subunits involved in ATP production are encoded by nuclear genes, however, some essential subunits are encoded by mitochondrial DNA (mtDNA), thus allowing pathogenic variants in both nuclear and mtDNA to impair ATP production (1). Patients with mitochondrial disease can present with a multitude of phenotypes such as encephalopathy, ataxia, and lactic acidosis (1).

1.2 Potential Link Between Mitochondrial Chaperonopathies and Glucocorticoids

A new class of mitochondrial disease, known as “mitochondrial chaperonopathies”, has been characterized by impaired mitochondrial protein homeostasis (proteostasis). Patients present with novel phenotypes including skeletal abnormalities and growth deficiencies (4). Currently, pathogenic variants found in genes involved in maintaining mitochondrial proteostasis including LONP1, HSPA9, AIFM1, and more recently PISD have resulted in similar phenotypes

of mitochondrial chaperonopathies (5). However, the mechanisms by which impaired protein homeostasis leads to these phenotypes is unknown.

Notably, glucocorticoids are a class of steroid hormones regulated by mitochondria whose accumulation can cause the phenotypes seen in mitochondrial chaperonopathies, such as skeletal abnormalities and growth deficiencies (6). For example, Cushing's syndrome is characterized by excess cortisol, which results in skeletal weakness and growth impairment (7). Similar skeletal abnormalities are also seen in osteoporosis, when cortisol (glucocorticoid released in stress response) induces bone loss by altering calcium metabolism leading to bone resorption by osteoclasts (6, 8, 9). Excess glucocorticoid production has also been seen to negatively affect the nervous system and the visual system (10, 11). Growing evidence showing neurodevelopmental issues including autism and cataract formations in the eyes (10, 11). Given that glucocorticoids are synthesized in the mitochondria, and high levels of glucocorticoids can cause the novel phenotypes seen in mitochondrial chaperonopathy patients, we hypothesize that excess glucocorticoid production may be the mechanistic link between impaired mitochondrial proteostasis and patient phenotypes. However, mechanisms by which dysfunctional mitochondria may lead to increased levels of glucocorticoids are unknown.

1.3 STARD1 and Glucocorticoid Production

The mitochondria of specialized cells of the adrenal cortex, gonads, and placenta are involved in steroid synthesis in steroidogenesis (Figure 1) (12). Cholesterol acts as the exclusive precursor for this pathway, which produces the necessary components of cell membranes and cell signaling pathways (13). The rate-limiting step in steroidogenesis, cholesterol import into mitochondria, is regulated by the steroidogenic acute regulatory protein (STARD1), located on

the outer mitochondrial membrane (OMM) (12). STARD1 mediates the cholesterol transfer from the OMM to the inner mitochondrial membrane (IMM), where cholesterol can then be immediately converted to pregnenolone by the enzyme CYP11A1 (14). Pregnenolone can then be converted to various glucocorticoids including cortisol (14).

The exact mechanism by which STARD1 regulated cholesterol import in the IMM is unknown (15). However, current research suggests cholesterol is bound to the translocator protein (TSPO) at the OMM with high affinity, and cholesterol is released from TSPO's hold through interaction with STARD1 (16). It has been proposed that subsequently, STARD1 interacts with voltage dependent anion channel at the OMM to connect the two membranes for cholesterol transfer (17). While there are gaps in our knowledge of STARD1 regulatory mechanics, pathogenic mutations in LONP1, a gene that regulates STARD1 turnover, cause mitochondrial chaperonopathy phenotypes of cerebral, ocular, dental, auricular, and skeletal (CODAS) abnormalities (16, 18).

The LONP1 gene encodes a conserved mitochondrial matrix protein, LONP1, which has both chaperone and protease activities (4). Protease activity of LONP1 mediates STARD1 turnover (Figure 2). At the OMM, STARD1 is expressed as an unprocessed 37 kDa protein actively transporting cholesterol (15). During turnover, STARD1 is transported to the mitochondrial matrix where LONP1 cleaves the N-terminal mitochondrial import sequence, inactivating STARD1 to a 30kDa protein (12, 19). LONP1 also has an active role in selective degradation of misfolded and damaged proteins, maintenance of the mitochondrial genome, and regulation of mitochondrial genes (18).

1.4 Mitochondrial Unfolded Protein Response

When mitochondrial protein homeostasis is impaired and there is an accumulation of misfolded proteins, genes including LONP1 are upregulated through a process called the mitochondrial unfolded protein response (mtUPR) (20). In this response, protein translation is halted, chaperone expression is upregulated to assist in correct folding, and LONP1 is targeted to the misfolded proteins for degradation (20). Part of this stress response may be leading to STARD1 accumulation, however, mechanisms by which this occurs is unknown (21). We hypothesize that glucocorticoid production is a branch of the mtUPR, mediated by retrograde signaling from the mitochondria to the nucleus (21). This would occur by part of the response eliciting an increase of glucocorticoids in response to cell stress, and therefore, accumulation of STARD1 at the same time as upregulation of LONP1 protease for targeted degradation of misfolded proteins (22).

Current research has found that different variant combinations in LONP1 have different impacts on LONP1 chaperone activity which correspond to different clinical representations, including CODAS (18). Variants associated with these novel phenotypes have been reported in the N-terminal and ATPase functional domains of LONP1, with variants found in the ATPase domain corresponding to mitochondrial chaperonopathy phenotypes (Figure 3) (18, 23). Notably, pathogenic variants found in LONP1 that impair STARD1 turnover, result in mitochondrial chaperonopathy phenotypes of skeletal abnormalities and growth deficiencies, phenotypes also seen in disorders with excess glucocorticoids (6, 18). As well, patients with other LONP1 mutations that do not affect STARD1 activity do not show these novel phenotypes, suggesting the significance of STARD1 and steroidogenesis pathway. This thesis delves into the molecular mechanisms by which mitochondrial chaperonopathies have these unusual phenotypes.

We will use the PTEN-induced kinase 1 (PINK1) gene as a model for our research with STARD1 (24). Due to similarities, disease mechanisms of pathogenic variants of PINK1 may be used analogously in determining mechanisms for pathogenic variants found in LONP1 (Figure 4). In “healthy” mitochondria, PINK1 is imported from the OMM to the IMM, however when mitochondria are damaged, PINK1 accumulates on the OMM and induces mitochondrial degradation (autophagy) (24). Accumulation of PINK1 on the OMM can also occur due to variants in the PINK1 gene that impair PINK1 import, leading to over-activation of PINK1 and consequently, early-onset Parkinson’s disease (24). This research project follows this parallel between the PINK1 mechanisms and the hypothesized STARD1 mechanisms where an accumulation of PINK1 or STARD1 in our case, on the OMM results in excess function and leads to mitochondrial dysfunction.

Chapter 2: Hypothesis and Methods

2.1 Hypothesis

We hypothesize that pathogenic variants in LONP1 which cause impaired proteostasis result in over-accumulation of STARD1, which increases cholesterol import, and consequently glucocorticoid production. As other diseases characterized by excess glucocorticoid production, show similar phenotypes to mitochondrial chaperonopathies, we suspect an overall enhancement of steroidogenesis to be the cause of these mitochondrial disease phenotypes (6-9). Enhancement of steroidogenesis is believed to be the source for these novel mitochondrial disease phenotypes.

2.2 Cell Lines and Growth Conditions

The human steroid-producing adrenocortical cell line known as H295R from American-Type Culture Cell (ATCC) was used throughout this thesis. H295R cells were initially isolated in 1990 from a patient with adrenocortical carcinoma, showing symptoms of mineralcorticoid, androgen, and glucocorticoid excess (25). These cells were chosen because H295R cells naturally undergo steroidogenesis and express STARD1 and LONP1 (25) . This allowed us to detect STARD1 expression in cells, to look for STARD1 accumulation on the OMM. H295R cells were cultured using a base medium comprised of a 50:50 ratio of Dulbecco's Modified Eagle Medium and Ham's F12 Nutrient Mixture, and additives including an Insulin-Transferrin-Selenium premix (1%) and an Nu-Serum (2.5%) (26). Along with the growth medium, H295R cells were routinely cultured following the ATCC cell culture protocol using Trypsin-0.53 mM EDTA solution (0.25%) for cell dissociation and washed with 1X Phosphate-buffered Saline (PBS) (26). Cells were incubated at a temperature of 37°C and 5% CO₂.

2.3 Immunofluorescence

Cells were seeded onto 12mm glass coverslips at 1×10^6 cells and incubated for 24 hours. Some cells were incubated for 30min with 10mM Dibutyryl-cAMP (dbcAMP) dissolved in H295R media (sc-201567A, Santa Cruz Biotechnology). After incubation, cells were fixed in 4% paraformaldehyde and stained with primary antibodies for STARD1 (sc-166821, Santa Cruz) and TOMM20 (HPA011562, Sigma-Aldrich) at a 1:1000 dilution, and Alexafluor-conjugated secondary antibody.

2.4 Western Blot Analysis

STARD1 expression was analyzed qualitatively using a Western blot protocol. Cells were seeded at 1×10^6 cells and 5×10^5 cells. After growing for 1-2 days, cells were washed with 1X PBS and harvested using 0.25% Trypsin-0.53 mM EDTA solution. Cell lysis was performed using RIPA buffer containing protease inhibitors. Proteins ($50\mu\text{g}$) from total cell lysates were separated on SDS-PAGE gels by gel electrophoresis and transferred onto PVDF membranes for analysis. The blots were probed with a specific anti-STARD1 antibody to detect STARD1 expression at 1:1000 dilution (sc-166821, Santa Cruz). VDAC (ab14734, Abcam) and B-Actin (A5316, Sigma-Aldrich) loading control antibodies were also used at 1:1000 dilution. Horseradish peroxidase conjugated secondary antibody was used at 1:3000 dilution, followed by Clarity ECL substrate (Biorad, 1705061). After all probes and washes with 1X Tris-Buffered Saline Polysorbate 20, blots were imaged using an Amersham Imager AI600.

2.5 Mitochondrial Isolation

A differential centrifugation protocol was used to isolate mitochondria. Cells were treated with 0.25mM dbcAMP for 6hrs, harvested, and re-suspended with mitochondrial isolation buffer at pH 7.2 (280mM sucrose, Magnesium acetate, 10mM HEPES). Cells were then kept on ice and mechanically homogenized using a Potter-Elvehjem homogenizer. Samples were then centrifuged at 800 RCF for 10min to remove the nuclear fraction. A final spin at 10000 RCF for 15min was then performed to pellet the mitochondria from the cytosolic fraction.

2.6 Cell Transfection

Plasmid DNA was designed using InFusion cloning (121416, Takara Bio) with designed primers and restriction enzymes. Cells were seeded at 1×10^6 cells and transfected with plasmid DNA using a lipid based transfection (Lipofectamine 3000 (L3000015), Thermo Fisher Scientific). Untreated cells acted as a negative control, and cells transfected with a plasmid expressing GFP acted as a positive control.

2.7 Pregnenolone Enzyme-linked Immunosorbent Assay

Levels of pregnenolone were measured as a direct equivalent to cholesterol import. This was done using a competitive pregnenolone enzyme-linked immunosorbent assay (ELISA) following protocol from the manufacturer (55R-1B59107, Fitzgerald). Media samples from H295R cells were collected and absorbance results were analyzed to determine pregnenolone levels.

Chapter 3: Aim 1 - Develop tools and techniques to detect STARD1 OMM accumulation

3.1 Detection of Processed Form of STARD1 by Western Blot in H295R Cell Extracts

To investigate consequences of accumulated STARD1 on glucocorticoid production, we first optimized methods to detect STARD1 expression. Using a specific STARD1 antibody and Western blot procedure with H295R cell samples, we could only see expression of the 30kDa processed form of STARD1. However, to better analyze STARD1 expression we wanted to investigate the 37kDa, unprocessed form of STARD1.

To continue to investigate the active unprocessed form of STARD1, we then pursued optimization of our Western blot analysis procedure. This optimization included testing a shorter blocking time to increase protein binding, increasing the primary antibody concentration and incubation time, and increasing the temperature of antibody incubation to increase background and abundance of target antigen. Results showed no expression of the unprocessed STARD1 isoform.

Dibutyryl-cAMP (dbcAMP) was used to stimulate STARD1 production. To test efficiency of dbcAMP, dbcAMP was added directly to cells at various concentrations (0.25mM, 0.50mM, 1.00mM, 1.25mM, and 1.50mM) and incubated for 16 hours at 37°C. To then compare time points, cells were incubated with dbcAMP at concentration 0.50mM for various times (2hr, 1hr 30min, 1hr, 30min, 15min). DbcAMP in combination with other treatments was used at a concentration of 0.50mM and incubation time of 30min. Western blot results indicated an increase in expression of the processed form of STARD1 with no signal of the unprocessed form (Figure 5A). DbcAMP treatment was also used in mitochondrial isolation and immunofluorescence in an attempt to identify the larger OMM isoform of STARD1.

3.2 STARD1 Localizes at the Mitochondria

Immunofluorescence was used to visualize STARD1 localization within the cell. We found STARD1 co-localized with TOMM20, an OMM protein in H295R cells treated with dbCAMP (Figure 5B). However, we were unable to confirm that STARD1 specifically localized to the OMM due to resolution of images.

Given that STARD1 localizes to the mitochondria, we isolated the mitochondria fraction from H295R cells in the hopes of better detecting the unprocessed form of STARD1. Results were inconclusive, showing no change in STARD1 expression from the original cell preparation and analysis methods.

3.3 Over-expression of STARD1 in HEK-293 cells

Our next approach to better visualize STARD1 was to over-express STARD1 in Human Embryonic Kidney (HEK) cells using a plasmid containing the gene for STARD1 in a pcDNA 3.1 (-) backbone. Transfection incubation times of 24hrs, 48hrs, and 72hrs were tested to determine optimal incubation time. Western blot on HEK cells over-expressing STARD1 showed the processed form of STARD1, with no detectable expression of the larger, unprocessed form of STARD1 (Figure 6A).

It was possible that the unprocessed form was being lost or degraded during the cell lysis procedure. To assess this, four different lysis buffers were tested:

1. RIPA buffer (control)
2. 1.0% Triton-X-100 buffer (150mM NaCl, 1.0% Triton-X-100, 50mM Tris (pH 8.0))
3. Extraction buffer (Abcam)
4. 1% Sodium Dodecyl Sulfate (SDS) buffer (1% SDS, 10mM Tris (pH 8.0), 1mM EDTA)

The control protocol used RIPA buffer to lyse cells. A 1.0% Triton-X-100 buffer was used as a milder buffer containing non-ionic detergent to maintain protein with less disruption compared to RIPA. A third extraction buffer containing a mixture of phosphate inhibitors and sodium dihydrogen phosphate buffer was tested. Using sodium dihydrogen phosphate buffer instead of Tris buffer in the extraction mixture provides a lower pH range to assess the stability of the target protein in regards to pH. A final buffer was also tested containing 1% SDS, an ionic detergent to better solubilize difficult proteins. Under all lysis conditions, only the smaller processed STARD1 isoform was detected, indicating no major effect of lysis buffer on STARD1 detection (Figure 6B). Results for cells lysed with 1% SDS buffer were unable to be analyzed accurately using Western blot due to high viscosity.

3.4 Aim 1 – Discussion

Current research suggests STARD1 in its active form on the OMM regulates cholesterol import, the rate-limiting step in steroidogenesis (12). Our studies began by detecting STARD1 expression in H295R cells using a specific STARD1 antibody. H295R cells untreated showed STARD1 expression of the smaller, processed, and inactive form of STARD1. DbcAMP was used to treat cells to induce steroidogenesis in an attempt to increase expression of STARD1 in both isoforms. Results using dbcAMP show increased STARD1 expression when analyzed with Western blot and mitochondrial localization when analyzed with immunofluorescence (Figure 5). However, this detection of STARD1 via Western blot only included the smaller, processed form of STARD1 at approximately 25kD indicating there dbcAMP induced steroidogenesis, but the processing of STARD1 was also induced. Using differential centrifugation, we isolated mitochondria to target STARD1 analysis and also investigated our Western blot protocol in an

attempt to target detection of STARD1, specifically, the unprocessed form. However, optimization of sample preparation and Western blot analysis did not show any significant change in STARD1 expression compared to untreated H295R cells, indicating our protocols were detecting correct STARD1 expression.

Our next step to optimize detection of STARD1 accumulation was to over-express STARD1 in HEK cells. Although HEK-293 does not naturally produce glucocorticoids, previous studies have shown STARD1 over-expression in HEK cells provides a visual of both unprocessed and processed forms of STARD1 (21). Western blot results showed successful over-expression of STARD1 in HEK cells, however, only the smaller form was detected again. Results also confirmed that our inability to detect the larger STARD1 isoform was not due to our H295R cell lines as we were still unable to detect the larger isoform with the HEK cells. Using the HEK cell model, we then tested different lysis buffers to investigate the import assay and location of unprocessed STARD1 after cell harvesting. However, results of the various lysis buffers on our HEK cell model showed no difference in STARD1 detection compared to the control RIPA buffer protocol, suggesting our protein was not being lost or degraded during cell lysis.

Results from aim 1 conclude STARD1 mitochondrial localization, however, there was no detection of the active STARD1 isoform, important for understanding the relationship between accumulated STARD1 and cholesterol import. Our inability to detect the larger STARD1 isoform may have been a result of the specificity of the STARD1 antibody used (sc-166821, Santa Cruz). It may be possible that the antibody used was specific to the processed form only. Other explanations include limitations to our detection mechanisms, and the timing of STARD1 processing, and we may be harvesting cells after STARD1 has been processed.

Chapter 4: Aim 2 – Genetically and pharmacologically induce STARD1 accumulation

4.1 Genetic Approach by Inducing the Mitochondrial Unfolded Protein Response

Another approach to optimize detection of STARD1 and specifically, STARD1 OMM accumulation, was to impair LONP1 protease activity to inhibit STARD1 turnover and allow us to detect STARD1 isoforms before processing. Our genetic approach transfected H295R cells with a terminally misfolded and highly unstable mutant Ornithine Transcarbamylase (Δ -OTC) (71878, Addgene) (Figure 7). Δ -OTC is a mutant form of OTC, an enzyme which catalyzes one of the main reactions in the urea cycle to produce urea from ammonia (27). By deleting the amino acids 30-114 which contain the binding domain of OTC for the urea reaction, we generate Δ -OTC which triggers the mtUPR. Untreated cells acted as a negative control, and transfection with wild-type ornithine transcarbamylase (OTC) and GFP acted as positive controls. Western blot showed no detection of STARD1 isoforms, while pregnenolone levels were increased with Δ -OTC treatment (Figure 8).

4.2 Pharmacological Approach

The results of pharmacological approaches corresponded with the genetic approaches of STARD1 accumulation. CDDO was used as a specific LONP1 protease inhibitor. Growing H295R cells were incubated with CDDO dissolved in dimethyl sulfoxide (DSMO) at various concentrations (0.25 μ M, 0.10 μ M, and 1.00 μ M) for 16hrs. After incubation, cells were harvested and analyzed using Western blot. Treatment with CDDO, alone resulted in no detection of the larger STARD1 form, and a significantly lower level of expression of the smaller STARD1 form

(Figure 9). Also, cells treated with CDDO at 1 μ M resulted in high cell death, and samples were unable to be analyzed via Western blot.

MG132 drug was also used as a general protease inhibitor. MG132 dissolved in DMSO was added directly to growing H295R cells. Two concentrations of MG132 were tested (2.50 μ M and 5.00 μ M) at a 16hr incubation. Time points for MG132 at a 5.00 μ M concentration in combination with dbcAMP (0.5mM) were also tested. The treated cells from both MG132 experiments were harvested and analyzed using Western blot. Cells treated with MG132, a general protease inhibitor, showed expression of the smaller form of STARD1, but not the larger form (Figure 9). When treated in combination with both MG132 and dbcAMP, longer incubations resulted in decreased expression of the smaller form of STARD1.

A mitochondrial inner membrane uncoupling agent of mitochondrial oxygen phosphorylation known as FCCP was also used to inhibit protein transport from the OMM to the IMM. FCCP drug was dissolved in DMSO was used as a pre-treatment in combination with drugs including dbcAMP (0.5mM), CDDO (0.5 μ M), and MG132 (20mM). The FCCP treatment was added directly to growing H295R cells at a concentration of 1 μ M. Cells were incubated with various combinations of FCCP and dbcAMP/CDDO/MG132 for 30min. A following experiment tested a pre-treatment with varying concentrations of FCCP (1 μ M, 5 μ M, 10 μ M) for 1hr incubation followed by a 30min incubation with dbcAMP (0.5mM). Results for both experiments were analyzed using Western blot and pregnenolone ELISA. DbcAMP, CDDO, and MG132 used in combination with FCCP showed no difference in STARD1 expression compared to an untreated control sample. A following experiment specifically testing the combination of dbcAMP and FCCP also resulted in no difference in STARD1 expression compared to untreated samples.

To continue to optimize detection of the larger isoform of STARD1 and changes in expression of the smaller isoform, H295R cells were also treated with phenanthroline (o-phen), a drug shown to prevent formation of smaller STARD1 isoforms, and combinations of o-phen with dbcAMP/FCCP (Figure 10A) (28). o-Phen was dissolved in DMSO and added directly to growing H295R cells. o-Phen was used in combination with FCCP and dbcAMP. Cells were pre-treated by incubation with both FCCP (5 μ M) and o-phen (1.5mM) for 1hr. Cells were then treated with dbcAMP (0.5mM) for 30min post FCCP and o-phen treatment. Samples were harvested and results were analyzed using Western blot. Western blot results showed no difference in STARD1 expression compared to untreated control with the addition of o-phen and combinations of o-phen with dbcAMP (Figure 10B). However, the use of o-phen and FCCP together resulted in no detection of the smaller, processed form of STARD1.

Another drug, hydroxylamine, was then used as a general protease inhibitor and tested on H295R cells to continue the investigation of the isoforms of STARD1. Hydroxylamine was dissolved in double-distilled water (ddH₂O) and added directly to growing H295R cells. Cells were seeded at 1x10⁶ cells and incubated with hydroxylamine for 6hrs at various concentrations (untreated control, 1mM, 3mM, and 5mM). Results only showed expression of the smaller form of STARD1. Lower concentrations of hydroxylamine showed a lower detection of the smaller form of STARD1 (Figure 11).

Overall results of genetic and pharmacological approaches to impair LONP1 function indicated a decreased expression of the smaller form of STARD1, and no detection of the larger, unprocessed STARD1 isoform.

4.3 Aim 2 – Discussion:

Subsequent experiments investigated STARD1 expression after LONP1 impairment, specifically detecting the larger, unprocessed form of STARD1. To artificially impair LONP1 and examine STARD1 turnover, both genetic and pharmacological approaches were used. H295R cells transfected with Δ-OTC were harvested and analyzed using Western blot. Results showed no detection of STARD1 expression compared to untreated controlled cells and wild-type OTC transfected cells (Figure 8).

Compared to untreated controls and cells transfected with wild-type OTC, there appeared to be a disappearance of the smaller, processed form of STARD1 that we had previously seen during protocol optimization. Previous knowledge suggests glucocorticoid production may be a branch of the mtUPR, where there is increased glucocorticoid production during cell stress (29). The disappearance in the smaller form of STARD1 suggests there is an accumulation of the larger, unprocessed, active form of STARD1 present being utilized to import cholesterol for glucocorticoid production via the mtUPR. This in turn allows STARD1 to continue to import cholesterol to produce glucocorticoids in response to stress and stay in its active form rather than be degraded by LONP1. However, our analysis method is unable to detect this larger, active form of STARD1 which is believed to be accumulating. This conclusion is supported by the increased pregnenolone levels measured after treatment with Δ-OTC, suggesting cholesterol import levels are elevated. These results suggest that by inducing the mtUPR, we are increasing active STARD1 production to increase cholesterol import.

We then used a pharmacological approach to impairing STARD1 turnover. CDDO was used to specifically inhibit LONP1 activity. CDDO interacts with LONP1 to form covalent LON-CDDO adducts which irreversibly inhibit LONP1 protease activity (30). Western blot

results matched those of the Δ-OTC results where there was a disappearance of the smaller form of STARD1. This significant decrease in expression of the smaller form of STARD1 suggests impairing LONP1 impairs STARD1 turnover, and there is no processing of the larger form of STARD1. However, detection of this unprocessed STARD1 form was still undetected. Addition of a general protease, MG132, showed expression of the smaller form only, with no significant expression level changes compared to control. MG132 is a potent, reversible proteasome inhibitor that has been shown to impair LONP1 (20). Generally, MG132 works to inhibit proteolytic activity in mammals by blocking proteasome activity that catalyzes the majority of protein degradation (the 26S proteasome complex) (31). However, specific mechanisms by which MG132 inhibits LONP1 are currently unknown (20). When combined with dbcAMP, MG132 and dbcAMP treated cells resulted in decreased expression of the smaller form of STARD1, supporting the interpretation that the unprocessed form of STARD1 is accumulating since degradation is inhibited. These drugs were also used in combination with FCCP, a drug used to inhibit protein transport from the OMM to the IMM. FCCP has been shown to uncouple mitochondrial oxidative phosphorylation, and reduce mitochondrial membrane potential which subsequently impairs import from the OMM to the IMM (32). In the context of this project, FCCP was used to prevent transport of STARD1 from the OMM to be degraded to an inactive, smaller form. Western blot results showed no difference in STARD1 expression compared to the untreated control. However, pregnenolone levels after treatment with FCCP showed increased pregnenolone, and therefore increased cholesterol import. These results indicate that FCCP provides a mechanism to prevent STARD1 transport since increased cholesterol import levels suggest there is accumulated active STARD1 on the OMM. Western blot results contradict this

interpretation because the smaller, inactive form of STARD1 is detected. However, this may be a result of unknown interactions amongst the various drugs used.

Another drug known as phenanthroline (o-phen) was introduced to this approach to specifically inhibit formation of processed STARD1 isoforms (Figure 10A). o-Phen works as a protease inhibitor and protects certain peptides from degradation (28, 33). Previous work identified o-phen as an inhibitor, blocking the formation of the inactive, smaller isoforms of STARD1 (28, 33). Results of o-phen in combination with FCCP show significant decrease in the smaller, processed form of STARD1. Following the pathway of STARD1 isoform formation, the combination of o-phen and FCCP can be interpreted to be working to prevent cleavage to form smaller forms of STARD1. However, the larger, unprocessed form of STARD1 is undetectable similar to previous results using Δ-OTC and CDDO.

Hydroxylamine was used as a final pharmaceutical approach to impair proteostasis and detect STARD1 expression. Hydroxylamine inhibits the production of phosphatidylserine, a phospholipid which has a key role in regulating lipid membrane curvature (34). Since many mitochondrial specific proteases are bound to the IMM, regulated lipid composition is vital for proper mitochondrial function (34). Results did not identify the larger, processed form of STARD1, but showed a significant decrease in the smaller form of STARD1 with lower concentrations of hydroxylamine. These results coincide with the pattern of impaired proteostasis resulting in decreased expression of smaller isoforms of STARD1.

Chapter 5: Aim 3 - Confirm the relationship between STARD1 accumulation with increased mitochondrial cholesterol import

5.1 Pregnenolone Measurement to Assess Cholesterol Import Levels

Pregnenolone was measured using ELISA to directly measure levels of cholesterol import, as imported cholesterol is exclusively and immediately converted to pregnenolone. Increased cholesterol import and glucocorticoid production was expected as a consequence of accumulated active STARD1 (Figure 1). Pregnenolone levels measured from transfected H295R cells with Δ-OTC were higher than untransfected cells after 16hrs of incubation (Figure 8B). Cells transfected for 6hrs with Δ-OTC and cells transfected for both 6hrs and 16hrs with wild-type OTC showed no significant difference compared to untreated control cells.

Pregnenolone levels were also measured after treatment of H295R cells with dbcAMP, CDDO, and MG132 (Figure 9B). A combination of dbcAMP and CDDO resulted in the highest concentration of pregnenolone compared to control and other drug treatments. These results corresponded with pregnenolone levels measured after H295R treatment with the same drugs (dbcAMP, CDDO, and MG132) in combination with FCCP. The addition of FCCP followed a similar trend to non-FCCP drug combinations, with dbcAMP and CDDO together having the greatest pregnenolone level.

In order to generate a more accurate measurement of pregnenolone level, the use of two pregnenolone conversion inhibitors, Abiraterone and Trilostane, were used to prevent downstream conversion of pregnenolone to other compounds. H295R cells were pre-treated with 1μM of both Abiraterone and Trilostane dissolved in DMSO for 90min and then variously treated for 30min with dbcAMP (0.5mM) CDDO (0.5μM), and/or MG132 (5μM). Use of

Abiraterone and Trilostane drugs in combination showed an increase in pregnenolone levels under all treatments when compared to without the pregnenolone conversion inhibitors (Figure 12B). When STARD1 expression was investigated by Western blot, the conversion inhibitors had no effect on STARD1 expression (Figure 12A).

5.2 Aim 3 – Discussion:

Measuring pregnenolone was used as a direct measurement for cholesterol import. Results after treatments to induce STARD1 accumulation showed increased pregnenolone levels. To better reflect the level of cholesterol import, we introduced pregnenolone conversion inhibitors. Abiraterone and Trilostane drugs have been previously shown to inhibit downstream pregnenolone conversion (35). Results indicated using these inhibitors produced a more accurate pregnenolone measurement and therefore, a more accurate measure of cholesterol import. Western blot results indicated that these pregnenolone conversion inhibitors had no effect on STARD1 expression. The use of Abiraterone and Trilostane when measuring pregnenolone as a measure of cholesterol import, allows us to precisely use pregnenolone levels as a direct equivalent to the imported cholesterol.

Chapter 6: Discussion

Overall, we found that STARD1 accumulates when STARD1 turnover and import is inhibited, as well as in response to cell stress. When measured by Western blot we saw the smaller, inactive form of STARD1 after processing by LONP1 protease was significantly decreased after treatments based on increasing cholesterol import. This indicates that the active form of STARD1 has accumulated, resulting in lower detection of the processed form. However, this active form of STARD1 was not detected despite various Western blot optimization steps with the STARD1 antibody. To confirm that levels of the active form of STARD1 had increased, we measured pregnenolone levels as a surrogate for cholesterol import. Pregnenolone levels increased, consistent with increased activity and accumulation of active STARD1 isoform, despite that it was not detected by Western blot. This link between cell stress and LONP1 impairment with STARD1 accumulation is also linked with cholesterol import, supported by results from the pregnenolone ELISA measurements.

A major limitation of this research was our inability to detect the active form of STARD1, whose accumulation following LONP1 impairment, causes the influx of cholesterol for steroidogenesis. Previous studies show over-expression of STARD1 in HEK cells can be detected in two forms, both the larger and smaller isoforms (21). However, in the current study, Western blot results for both H295R and HEK cell lines detected only the smaller form of STARD1. As well, our pharmacological approach to impairing STARD1 turnover as well as using o-phen to specifically inhibit formation of STARD1 isoforms resulted in decreased expression of the processed isoform, but no expression of the larger form. These results taken together, suggest this limitation may be due to specificity and sensitivity of the STARD1 antibody used throughout the project.

Chapter 7: Future Directions

Overarching goals for this project were to studying elucidate the effects of impaired proteostasis, in the pathway of steroidogenesis. By assessing changes in expression of STARD1 after cell stress and impaired proteostasis, this project showed accumulated STARD1 on the OMM elevated cholesterol import. However, as previously mentioned, a major limitation is the detection of the active form of STARD1, responsible for regulating cholesterol import. Future experiments considering this limitation may take other approaches to analyzing STARD1 expression or use a different specific STARD1 antibody.

To continue the investigation of impaired proteostasis on STARD1 accumulation and consequently, cholesterol import and glucocorticoid production, it is important to link the pieces of this mechanism. Our investigation suggests impaired proteostasis results in STARD1 accumulation followed by increased cholesterol import. Future endeavors may take into consideration causes of impaired proteostasis and resulting effects on glucocorticoid production.

Current knowledge suggests mutations in specific domains of LONP1 result in novel phenotypes including skeletal abnormalities, growth defects, and cataracts, classified under mitochondrial chaperonopathies (18). Mutations such as P676S and R721G have been previously shown to result in mitochondrial chaperonopathy expected phenotypes, but mechanisms are unknown (18). Experiments to identify effects of different pathogenic mutations in LONP1 will further develop our knowledge about this new class of disease, and the pathway of impaired proteostasis (18, 36). It is expected that LONP1 mutation location is associated with mutation phenotype, and this experiment will determine the disease mechanism by which mutations targeted to specific domains in LONP1 lead to mitochondrial chaperonopathies.

While we showed that LONP1 impairment increased pregnenolone levels, a glucocorticoid precursor, future studies could also measure glucocorticoid levels. For this reason, it is important to optimize a method to measure glucocorticoid levels. Using an ELISA specific for glucocorticoid measurement similar to the pregnenolone ELISA used in this research, we can measure endogenous glucocorticoid level changes. This ELISA may be used to detect cortisol for example, a glucocorticoid derived from cholesterol import. It is expected that an accumulation of STARD1 due to impaired LONP1 will also result in increased synthesis of glucocorticoids.

Finally, to confirm that STARD1 is responsible for the increased glucocorticoid levels when mitochondrial proteostasis is impaired, future experiments can inhibit STARD1 using silencer siRNAs to reduce its expression and determine whether this reduces the production of glucocorticoids despite impairment of LONP1 (37). By silencing STARD1, we expect cholesterol import to halt. If inhibition of STARD1 activity causes cholesterol and glucocorticoid levels to decrease or remain stable, this will be consistent with a role of STARD1 in glucocorticoid levels.

Chapter 8: Concluding Remarks

A new class of mitochondrial disease, mitochondrial chaperonopathies, present with novel phenotypes including skeletal abnormalities and growth defects (12, 18). Notably, these phenotypes have been previously seen in diseases with increased glucocorticoid production such as osteoporosis (6, 8, 9). This research connects increased glucocorticoid production with impaired proteostasis. By investigating causes and consequences of accumulation of active STARD1 accumulation on the OMM, results from this research support the hypothesized pathway of elevated glucocorticoid production resulting in novel mitochondrial disease phenotypes.

Throughout this investigation, expression of STARD1 in both its active and inactive forms were linked to changes in cholesterol levels. Evidence for impaired proteostasis resulting in accumulated active STARD1 was collected using genetic and pharmacological methods. Western blot analysis showed no detection of the larger, active form of STARD1. However, with treatments to impair proteostasis there was a significantly decreased level of expression of the smaller, processed form of STARD1, suggesting STARD1 remained uncleaved and accumulated in its larger form. This was observed in cells transfected with Δ-OTC, and treated with CDDO, o-phen, and hydroxylamine. Concluding results of decreased expression of the processed STARD1 isoform correlated with increased pregnenolone levels suggesting the active form of STARD1 was accumulating on the OMM, and degradation via proteases was impaired. These results support the hypothesized pathway leading to the novel mitochondrial chaperonopathy phenotypes we predominantly see in diseases with elevated glucocorticoid levels.

Future research is required to further confirm the relationship between impaired proteostasis and glucocorticoid production. Determining an approach to detect the larger form of

STARD1 will provide better insight into the effects of impaired proteostasis, specifically LONP1 protease, on STARD1 expression. Future goals also include confirming that STARD1 accumulation results in increased cholesterol import and consequently, elevated glucocorticoid production. Finally, we can characterize the effects of different pathogenic variants in LONP1 on STARD1 turnover and glucocorticoid production. Results from this investigation will provide more information on the role of specific domains of proteases, important for understanding mitochondrial function and signaling pathways.

This work will be directly applicable to patients with mitochondrial chaperonopathies, and will also provide novel insight into mitochondrial dysfunction and steroid production. Understanding steroid production contributes to our understanding of a multitude of different diseases since steroid have many classes and resulting functions. Such disease include Cushing's syndrome and osteoporosis from hypercortisolism (9). As well, findings from this investigation benefit the knowledge in the biomedical field of other common pathologies including diabetes and neurodegenerative diseases, in which glucocorticoid elevation may play a role through impacting glucose metabolism and immune function (6).

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Appendix

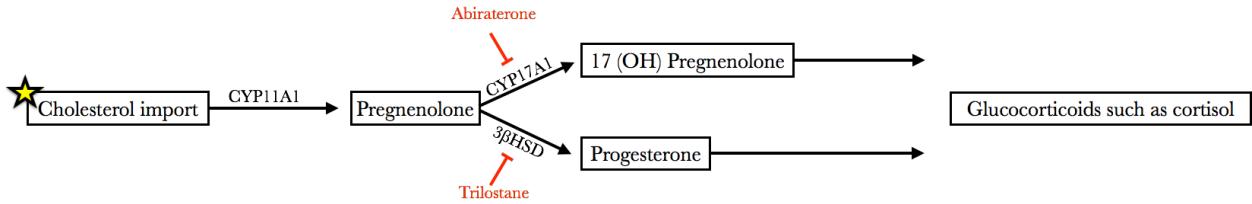


Figure 1: Steroidogenesis pathway. Cholesterol is immediately converted into pregnenolone by the enzyme CYP11A1 after import into the IMM from the OMM by STARD1. Pregnenolone can then continue through steroidogenesis in two pathways to produce various glucocorticoids. Abiraterone and trilostane are drugs that inhibit each downstream steroidogenesis pathway. It is hypothesized that impairing STARD1 turnover will result in STARD1 accumulation and therefore increased cholesterol import. This will then cause over-activation of the steroidogenesis pathway and an increase in glucocorticoids, resulting in mitochondrial chaperonopathy phenotypes.

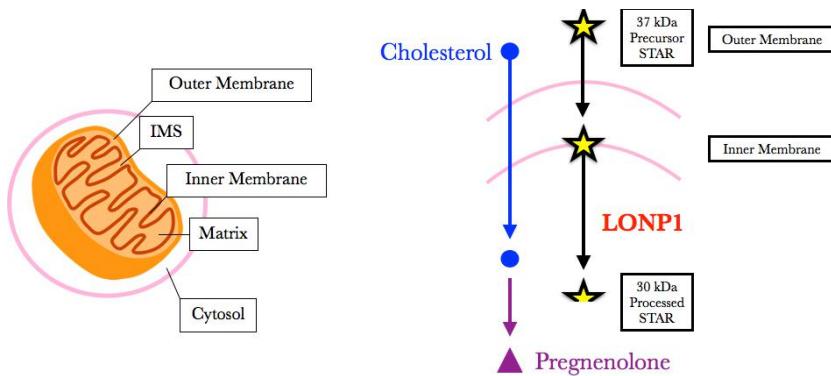


Figure 2: STARD1 processing pathway. STARD1 (yellow star) in its active form on the OMM regulates cholesterol import from the OMM to the IMM. Cholesterol can then be converted into pregnenolone and continue through the steroidogenesis pathway and STARD1 is degraded by LONP1.

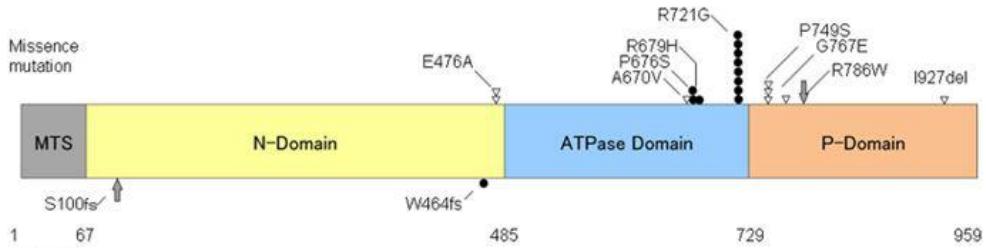


Figure 3. The functional domains of LONP1, as adapted from Inui *et al.* (2007) (18). The sections from left to right are the mitochondrial targeting sequence (MTS), the amino-terminal domain (N-domain), adenosine triphosphatase domain (ATPase domain), and proteolytic domain (P-domain). The black circles indicate mutations found resulting in mitochondrial chaperonopathy phenotypes, specifically found in the ATPase Domain. The white triangles indicate mutations found resulting in atypical mitochondrial chaperonopathy phenotypes. The grey arrows denote mutations found by Inui *et al.* with atypical mitochondrial chaperonopathy phenotypes, suggesting that the location of mutation may play a role in resulting phenotype.

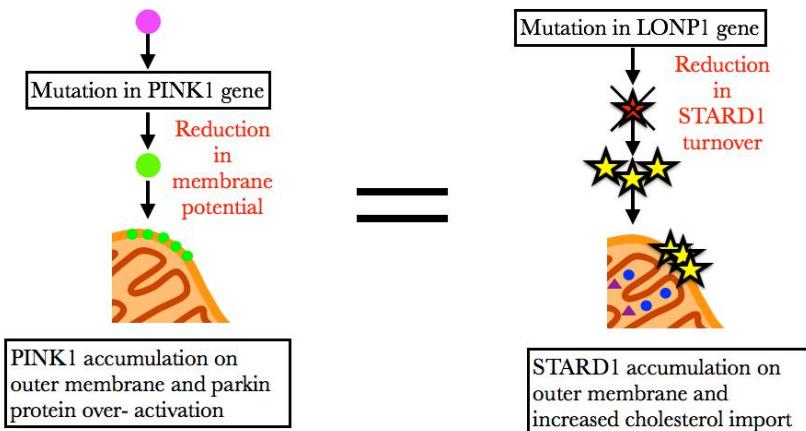


Figure 4: Hypothesized impaired steroidogenesis and STARD1 turnover in parallel to mechanisms of unconventional PINK1 OMM accumulation. PINK1 gene variant (green circle) results in PINK1 OMM accumulation and over-activation of parkin protein. This mechanism can be used as a model for our hypothesized STARD1 accumulation. Impaired STARD1 turnover (red star) results in OMM accumulation of active STARD1 (yellow stars), and consequently, increased cholesterol import.

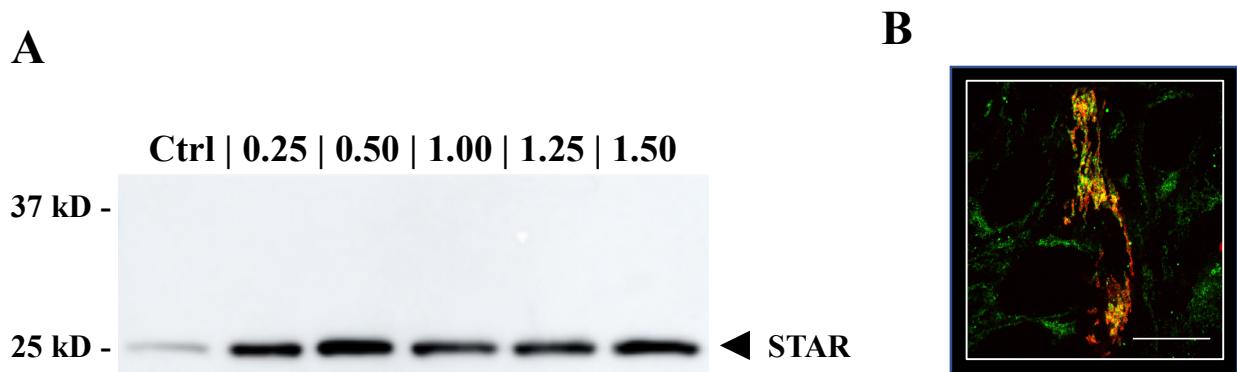


Figure 5: H295R cells treated with dbcAMP and analyzed for STARD1 expression. (A) Western Blot analysis after cells treated with various concentrations of dbcAMP (mM) and incubated for 16hrs. (B) Immunofluorescence results after cells treated with 10mM dbcAMP and incubated for 30min. Samples were imaged using specific STARD1 and TOMM20 antibodies.

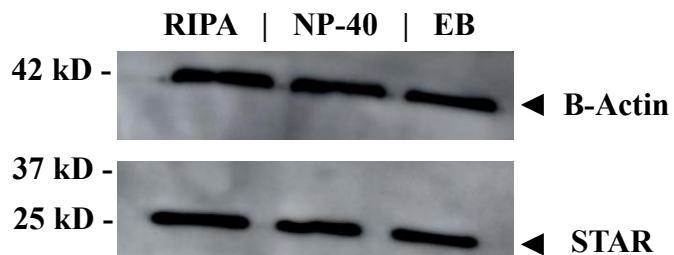
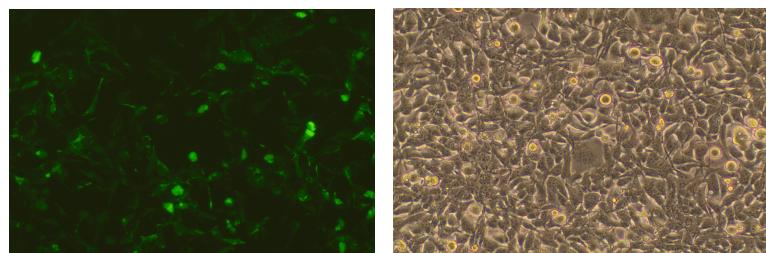
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Figure 6: Western Blot analysis of over-expression of STARD1 in HEK cells. (A) Cells were transfected with plasmid containing the STARD1 gene and incubated for 24hrs, 48hrs, or 72hrs post-transfection. Untransfected HEK cells acted as a negative control and GFP transfected cells acted as a positive control. (B) Cells were lysed with three different lysis buffers to see whether this impacted the detection of unprocessed STARD1. B-Actin was used as a loading control. (C) Cells transfected with GFP as a positive control, imaged after 24hrs incubation.

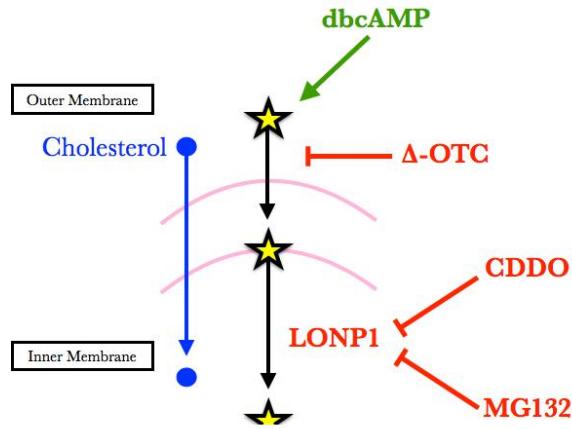


Figure 7: Methodology to investigate the effects of LONP1 impairment on STARD1 (yellow star). The pharmacological approach includes the use of dbcAMP to induce steroidogenesis, and both CDDO, a specific LONP1 inhibitor, and MG132, a general protease inhibitor, to impair STARD1 turnover by inhibiting LONP1 function. The genetic approach introduces Δ -OTC to induce mitochondrial stress, signalling the mtUPR and upregulation of LONP1.

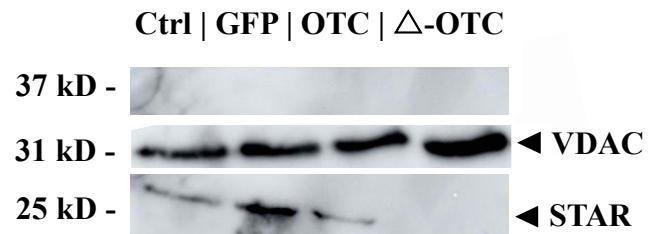
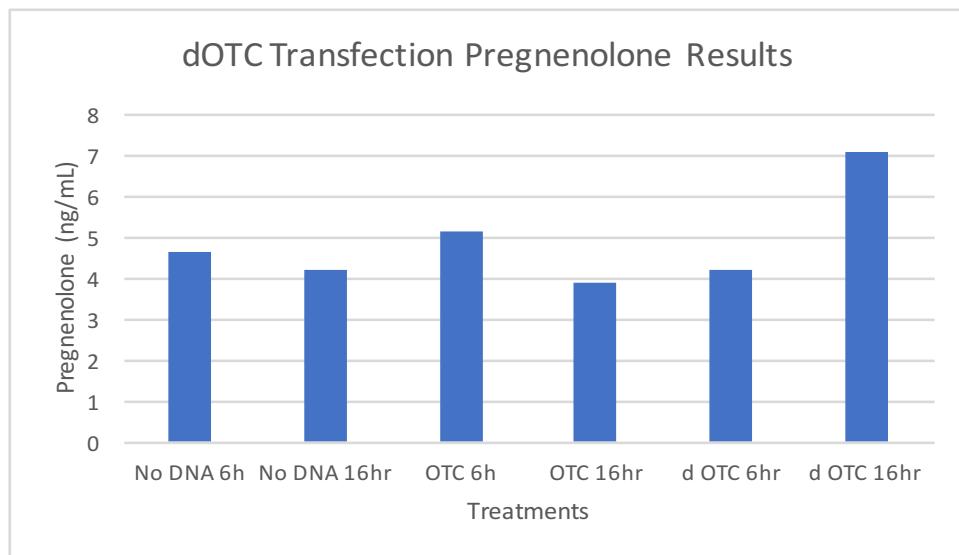
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Figure 8: Expression of mutant ornithine transcarbamylase in H295R cells. (A) Western Blot analysis of STARD1 and loading control VDAC expression in H295R cells after transfection with wild-type OTC or Δ-OTC (dOTC). Untreated H295R cells acted as a negative control and both GFP transfected cells and wild-type OTC transfected cells acted as positive controls. (B) Pregnenolone levels after transfection with incubation times of 6hrs and 16hrs. Pregnenolone was measured by ELISA with one replicate.

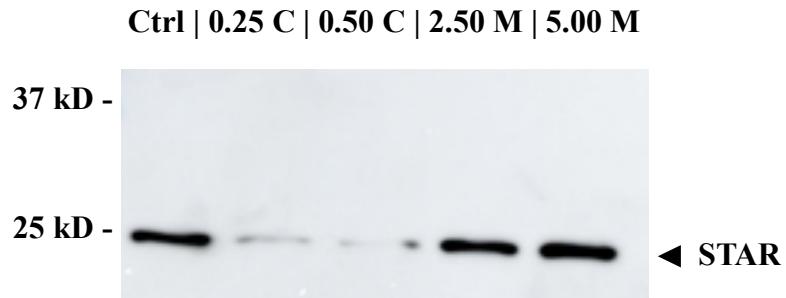
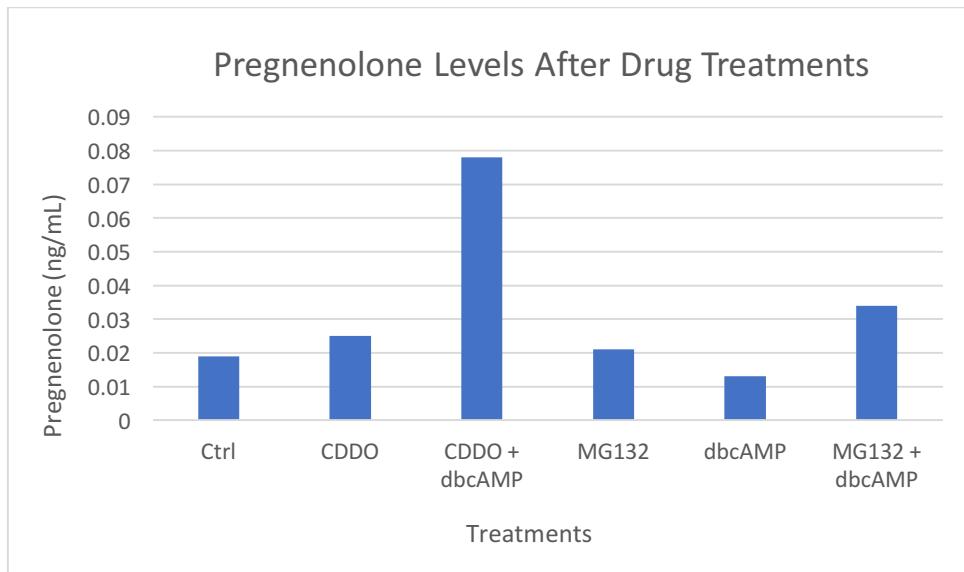
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Figure 9: Drug treatments on H295R cells. Cells were treated with various doses of protease inhibitors CDDO (C) and MG132 (M), and a steroidogenesis inducer, dbcAMP. (A) Cells were treated for 16hrs with (from left to right), untreated, 0.25 μ M CDDO, 0.50 μ M CDDO, 2.50 μ M MG132, and 5.00 μ M MG132. (B) Pregnenolone levels were measured using ELISA after treatment with drugs for 30min.

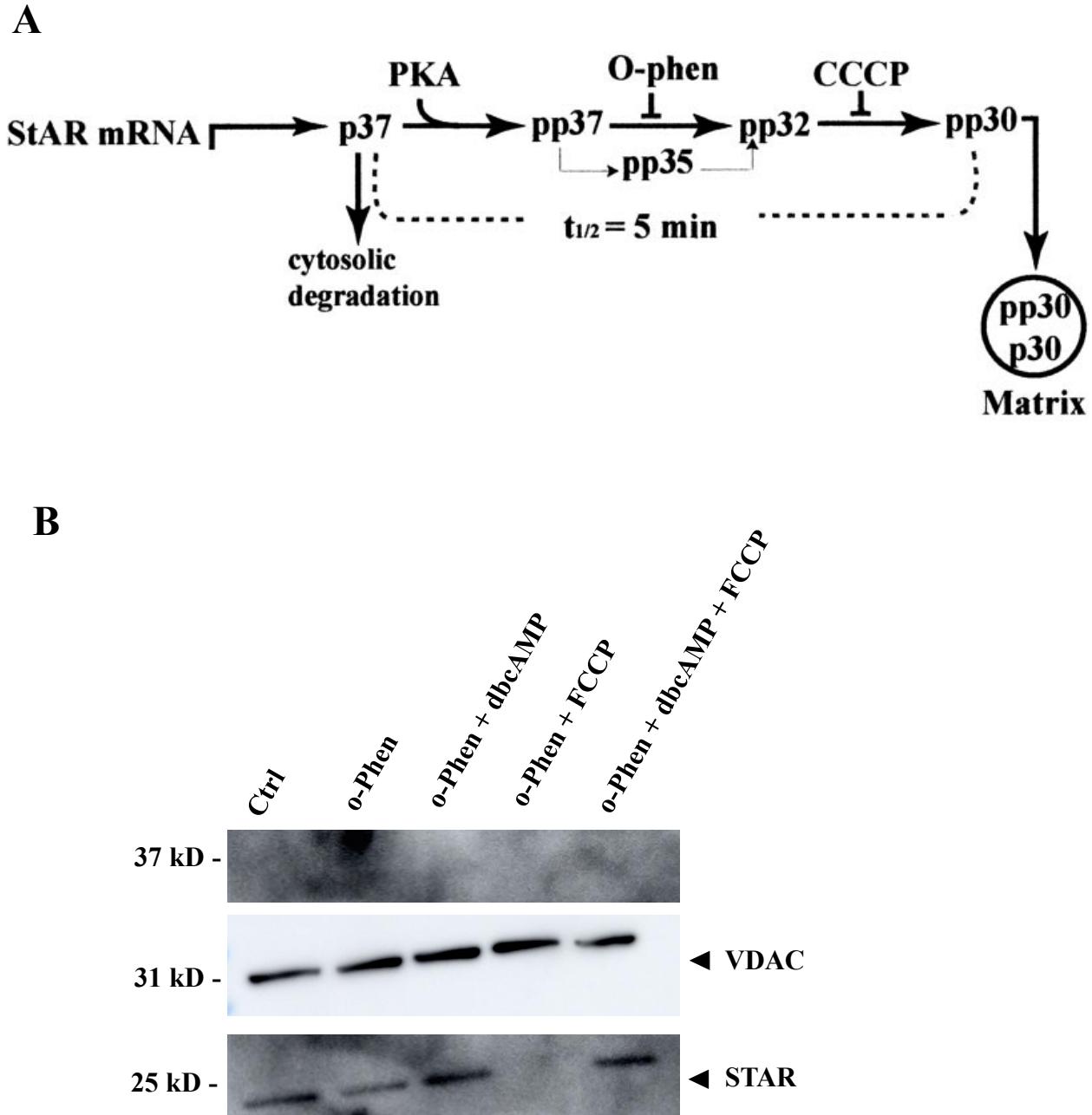


Figure 10: Phenanthroline (*o*-Phen) drug treatment on H295R cells. (A) STARD1 processing pathway to form smaller STARD1 isoforms, as adapted from Artemenko *et al.* (2001) (28). *o*-Phen and FCCP are shown to act as processing inhibitors. (B) Western Blot results after a pre-treatment for 1hr with *o*-Phen (1.5mM) and FCCP (5mM), followed by a 30min treatment with dbcAMP (0.5mM).



Figure 11: Hydroxylamine drug treatment on H295R cells. Western Blot results after a treatment with hydroxylamine for 6hrs at various concentrations (from left to right), untreated control, 1.00mM, 3.00mM, and 5.00mM.

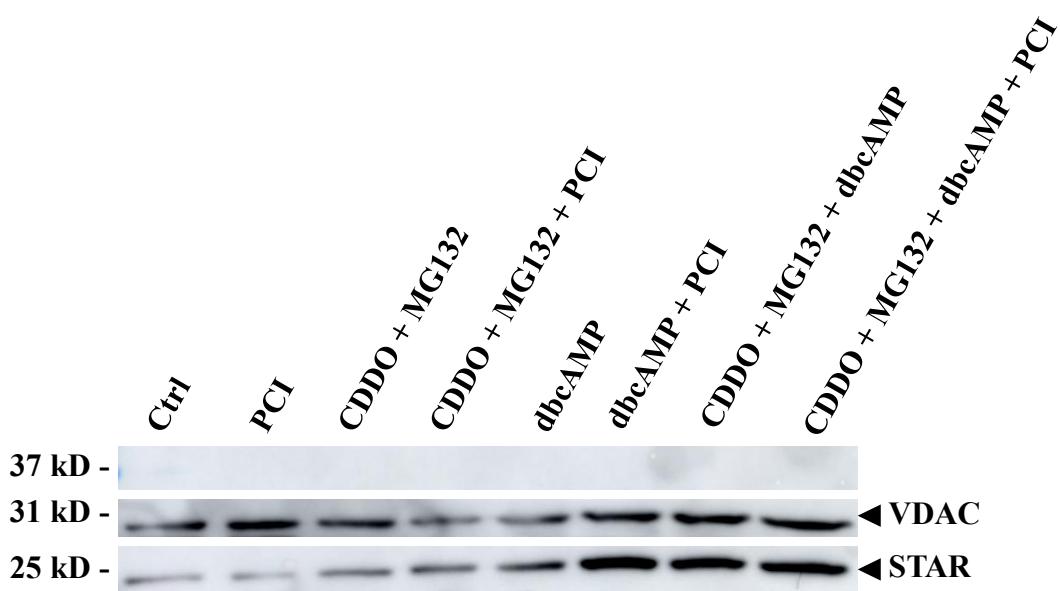
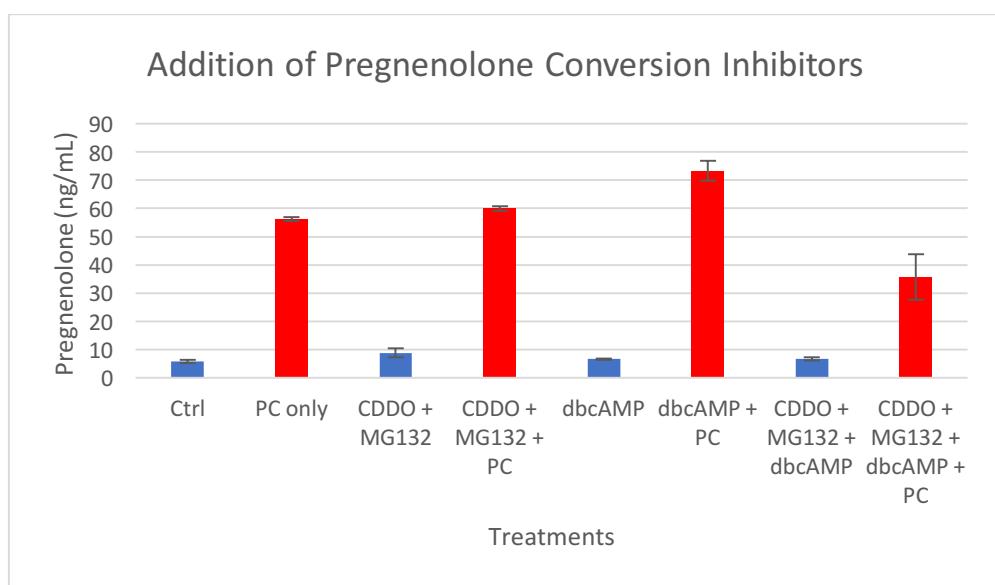
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Figure 12: Combination of pregnenolone conversion inhibitors (PCI) with other drug treatments including protease inhibitors (CDDO and MG132), and steroidogenesis inducer (dbcAMP). H295R cells were pre-treated for 90min with Abiraterone and Trilostane, the PCI drugs. Cells were then treated for 30min with CDDO (0.50M), MG132 (5.0M), and dbcAMP (0.50mM). (A) Western Blot analysis of cells after drug treatments. (B) Pregnenolone assay analysis of cell media after drug treatments. Error bars are created from a set of duplicate samples.

Table of Materials:

Aim	Product	Catalog Number and Source
Aim 1. Develop tools and techniques to detect STARD1 OMM accumulation.	B-Actin Antibody	(A5316, Sigma-Aldrich)
	Dibutyryl-cAMP	(sc-201567A, Santa Cruz)
	H295R cells	(CRL-2128, ATCC)
	HEK-293 cells	(CRL-1573, ATCC)
	pcDNA 3.1 (-) Backbone	(V79520, Addgene)
	Primers for STARD1 over-expression in HEK-293 cells	IDT *
	Restriction enzyme ECORI	(R3101T, NEB)
	Restriction enzyme BAMHI	(R3136, NEB)
	STARD1 Antibody	(sc-166821, Santa Cruz)
	STAR Gene in pDONR221	(HSCD00043889, DNASU)
	TOMM20 Antibody	(HPA011562, Sigma-Aldrich)
	VDAC Antibody	(ab14734, Abcam)
Aim 2. Genetically and pharmacologically induce STARD1 accumulation.	CDDO	(A11324, AdooQ)
	FCCP	(BML-CM120-0010, Enzo)
	Hydroxylamine	(467804, Sigma-Aldrich)
	MG132	(M8699, Sigma-Aldrich)
	OTC	(71877, Addgene)
	Δ-OTC	(71878, Addgene)
	Phenanthroline	(131377, Sigma-Aldrich)
Aim 3. Confirm the relationship between STARD1 accumulation with increased mitochondrial cholesterol import.	Abiraterone	(A10020, AdooQ)
	Pregnenolone ELISA	(59107, Fitzgerald)
	Trilostane	(A10949, AdooQ)

* Cloning primers designed for STARD1 over-expression in HEK-293 cells:

Forward (3' – cGAATTCAAAAGCAGGCTCCACCATG – 5')

Reverse (3' – GTGGATCCGGGTCTAACACCTGGCTTC – 5')

Table 1: Table of materials used in this thesis.



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Conjoint Health Research Ethics Board
Research Services Office
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chreb@ucalgary.ca

CERTIFICATION OF INSTITUTIONAL ETHICS APPROVAL

Ethics approval for the following research has been renewed by the Conjoint Health Research Ethics Board (CHREB) at the University of Calgary. The CHREB is constituted and operates in compliance with the *Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans* (TCPS 2); Health Canada Food and Drug Regulations Division 5; Part C; ICH Guidance E6: Good Clinical Practice and the provisions and regulations of the Health Information Act, RSA 2000 c H-5.

Ethics ID: REB16-1776_REN2

Principal Investigator: Tim Shutt

Co-Investigator(s): There are no items to display

Student Co-Investigator(s): Rasha Sabouny

Study Title: Investigations into the regulation of mtDNA and mitochondrial dynamics

Sponsor: ACHRI
NSERC

Effective: Sunday, September 16, 2018

Expires: Monday, September 16, 2019

Restrictions:

This Certification is subject to the following conditions:

1. Approval is granted only for the research and purposes described in the application.
2. Any modification to the approved research must be submitted to the CHREB for approval.
3. An annual application for renewal of ethics certification must be submitted and approved by the above expiry date.
4. A closure request must be sent to the CHREB when the research is complete or terminated.

Approved By:

Stacey A. Page, PhD, Chair , CHREB

Date:

Wednesday, August 22, 2018

Note: This correspondence includes an electronic signature (validation and approval via an online system).