UNIVERSITY OF CALIFORNIA SAN DIEGO

# Examining Cre Recombinase Toxicity in Beclin 1-Deficient Cells & Evaluating Mitochondrial Import of Misfolded alphaB-Crystallin

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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The Thesis of Logan B. Holland is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2022

# DEDICATION

"Try to be one of the people on whom nothing is lost!" – Henry James

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ABSTRACT OF THE THESIS

Examining Cre Recombinase Toxicity in Beclin 1-Deficient Cells & Evaluating Mitochondrial Import of Misfolded alphaB-Crystallin

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Master of Science in Biology

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Professor Åsa Gustafsson, Chair
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Beclin 1 is a critical regulator of autophagy which functions as a scaffold protein to initiate autophagosome formation and maturation. Global Beclin 1 knockout mice are embryonically lethal, while heterozygous Beclin 1 mice display a reduction in autophagy and increased susceptibility to myocardial ischemia/reperfusion injury. Cre-mediated recombination is a powerful tool that is commonly used in research to generate tissue-specific and inducible knockout mouse lines. To specifically delete Beclin 1 in the adult heart, our lab has generated cardiac specific, inducible MerCreMer Beclin 1 f/f mice. In establishing this model, Cre toxicity (rapid cardiac dysfunction and increased mortality) was observed in Beclin1 f/f mice (BMCM) but not in MerCreMer cassette-carrying mice (MCM) upon

injection with tamoxifen to induce Cre expression. Here, we assess Cre toxicity in Beclin1-deficiency *in vitro* and *in vivo* to determine whether the Beclin1 deficiency increases the sensitivity to Cre expression. We observed a dose-dependent toxicity of Cre induction in Beclin1-deficient MEFs and hearts. Overexpression of Cre did not cause cell death in Rab7-deficient MEFs or Beclin 1-deficient HeLa cells. Our findings suggest that lack of Beclin 1 leads to increased susceptibility to Cre-mediated toxicity.

The R120G mutation in alpha-b-crystallin (CryAB-R120G) causes a proteotoxic cardiomyopathy that is characterized by the accumulation of misfolded protein aggregates. Recently, mitochondria have been shown to import misfolded proteins as a novel mechanism of protein quality control in *S. cerevisiae*. However, this import mechanism has not been investigated in a mammalian system. Here we disrupt mitochondrial protein import to characterize the potential uptake of CryAB-R120G by mitochondria. We observed that mitochondrial import can be perturbed by knocking down a subunit of the translocase of the mitochondrial outer membrane (Tom20) and that this results in cytosolic accumulation of CryAB-R120G. Our findings suggest that mitochondria play a role in cytosolic proteostasis amid CryAB-R120G-induced aggregation.

# INTRODUCTION

## Autophagy

Autophagy is an intracellular degradation pathway through which cytoplasmic cargo is delivered to the lysosome. Conserved in all eukaryotic organisms, this pathway aids cells in protein and organelle turnover to maintain homeostasis under basal conditions. Autophagy is upregulated in response to various stressors such as nutrient deprivation and hypoxia (Levine & Kroemer, 2008). Under metabolic stress, activation of autophagy induces degradation of cytosolic components to generate free fatty acids and amino acids to be recycled by the cell (Guo et al, 2016). While autophagy aids in the turnover of cellular components, it also selectively eliminates harmful cytosolic material such as intracellular microorganisms and toxic protein aggregates. (Levine & Kroemer, 2008; Dikic & Elazar, 2018).

Initiation of autophagy is promptly followed by the recruitment of autophagy-related proteins (Atg) which coordinate the elongation of the membranes and engulfment of cargo into double-membraned vesicles, or autophagosomes, which are then transported to the lysosome for degradation. Autophagy begins with phagophore formation, which is nucleated by components of the class III PI3K (PI3K) complex. The PI3K complex entails vacuolar protein sorting 34 (Vps34), Beclin 1, Atg14, and Vps15. This signaling pathway, in concert with downstream pathways, initiates the expansion of the phagophore membrane into an autophagosome sequestering cargo (Bento et al, 2016). Following cargo engulfment, the double-membrane autophagosome is sealed and matures. After Atg proteins have been removed from matured autophagosomes, the vesicles will fuse with the lysosome where hydrolases degrade cargo and recyclable nutrients are released back into the cytoplasm (Dikic & Elazar, 2018).

Aberrant autophagy is particularly problematic in post-mitotic cell types where unwanted proteins or dysfunctional organelles are unable to be diluted via cell division (Kilonsky et al, 2021). Defects in autophagy lead to proteotoxicity and cellular dysfunction as undesired cellular components accumulate, often resulting in cell death. As autophagy is a critical quality control pathway required for maintaining homeostasis and responding to stress, its disruption is well-linked to cancer, neurodegenerative disorders, cardiomyopathies, and infectious disorders (Sridhar et al, 2011).

**Beclin 1 and Autophagy**

Beclin 1 was the first identified mammalian autophagy gene, recognized as the ortholog of the yeast autophagy gene Atg6 (Yue et al, 2003). Beclin 1 functions as a scaffold protein through its interaction with lipid kinase Vps34 to form the PI3K complex. The PI3K complex promotes autophagosome formation and maturation (Diao et al, 2022).

It has been established that the inhibition of autophagy via Beclin 1 interference sensitizes cells to metabolic stress, which may lead to necrotic cell death, ensuing inflammation and tumorigenesis. Beclin 1 has been found to be monoallelically deleted in 40-75% of sporadic human breast, ovarian and prostate cancers, and is considered a tumor-suppressor protein (Sridhar et al, 2011). Global Beclin 1 knockout mutant mice die early in embryogenesis while heterozygous Beclin 1 mice develop spontaneous tumors (Yue et al, 2003). Heterozygous Beclin 1 mice display decreased autophagy in the heart and are more susceptible to myocardial ischemia/reperfusion injury (Matsui et al, 2007).

**Cre/loxP System**

Cre recombinase is a 38 kDa enzyme belonging to the λ integrase family whose original function is to convert DNA dimers into plasmid monomers in the circular plasmid of the P1

bacteriophage. It is a site-specific recombinase which recognizes 34-base pair loxP sites (Kilby et al, 2003). Each loxP site consists of two 13-base pair inverted repeats which are recognized and bound by Cre homodimers, along with an 8-base pair spacer which allows for Cre-mediated cleavage and recombination (Garcia & Mills, 2002). Conditional mutagenesis strategies enabled by the Cre/loxP system have enabled researchers with the ability to unveil gene functions in a milieu of cell and tissue types. The Cre/loxP system has allowed for the elucidation of the impact of gene deletions in diseases that affect several organs and develop over time.

In mice, the Cre/loxP system requires two components in the mouse genome: the gene of interest flanked by loxP sites, or floxed, and a Cre transgene. The mouse genome can be manipulated to accommodate these two elements. Floxing of the gene of interest is accomplished via gene targeting of pluripotent embryonic stem (ES) cells isolated from the inner cell mass of a developing mouse blastocyst (Heine et al, 2005). Floxed alleles are generated by injecting ES cells with a vector containing a floxed version of the gene of interest. Flanking the sequence of a target gene with loxP sites will knock out a gene in a loss-of-function approach, while a gain-of-function approach entails flanking a stop sequence with loxP sites to separate a promoter from its target gene such that Cre excision will remove this termination sequence and enable transgene expression. Mice homozygous for a floxed allele of interest should be phenotypically normal (Garcia & Mills, 2002). Floxed mice can then be crossed with transgenic mice expressing Cre. Cre expression will yield site-specific recombination between loxP sites and excise the floxed region of the gene of interest.

Tamoxifen, an estrogen antagonist, binds to hormone-binding sites of nuclear hormone receptors (NHR). This binding promotes the translocation of the NHR into the nucleus, where genes containing hormone-specific promoters will be expressed. Fusing Cre with a hormone

receptor directs Cre into the nucleus when a hormone is added. Mutated versions of estrogen receptors (Mer) were first generated by fusing Cre to the mutated ligand-binding domain of the human estrogen receptor (Feil et al, 1996). With the addition of tamoxifen, Mer will translocate to the nucleus where a floxed target gene can be excised. Since the estrogen receptor is mutated, endogenous estrogen levels are unaffected (Garcia & Mills, 2002). Inducing Cre recombinase activity with tamoxifen equips the Cre/loxP system with temporal control.

A milieu of cardiac-specific Cre lines exist for characterizing gene function in the heart. Alpha-myosin heavy chain (α-MHC) Cre is the most widely used Cre line for inducing mutagenesis in cardiomyocytes. α-MHC promoter-driven Cre may incur premature lethality due to premature expression during embryogenesis, when present in a mouse model lacking inducible control (Heine et al, 2005). Other caveats of the Cre/loxP system include partial gene ablation. Tissue specific promoters may induce mosaic expression that generates incomplete gene ablation, disrupting phenotypic analysis (Garcia & Mills, 2002). Cre toxicity may also interfere with the Cre/loxP system. Cre expression in cultured mammalian cells results in reduced proliferation and high levels of Cre expression are known to be toxic (Loonstra et al, 2001; Sharma & Zhu, 2014). It is therefore important to minimize Cre expression levels such that sufficient recombination is achieved and toxicity is avoided.

**Rationale**

Beclin 1 is a key autophagy regulator, as evidenced by extensive literature pointing to the various pathologies that emerge from a deficiency in Beclin 1. Our laboratory has developed a cardiac-specific Beclin 1 knockout mouse model to elucidate the role of Beclin 1 in cardiac homeostasis. We have observed rapid cardiac dysfunction and mortality in this model, however

the extent of Cre toxicity in the BMCM mouse model has not yet been investigated. It is unknown whether the Beclin1 deficiency increases the sensitivity to Cre expression.

In my research, I have employed in vitro and in vivo models to investigate the hypothesis that Beclin 1-deficiency leads to heart failure and death due to increased sensitivity to Cre recombinase expression. I have examined the following questions to test this hypothesis:

1. Is Cre recombinase toxic to Beclin 1-deficient cells?
2. Does Cre recombinase induce cardiac dysfunction in a dose-dependent manner in BMCM mice?

## MATERIALS AND METHODS

### Cell Culture

WT, *Becn1*$^{-/-}$ , *Rab7*$^{-/-}$ MEFs, and WT BECN1$^{-/-}$ HeLa cells were cultured in media comprised of Dulbecco's Modified Eagle Medium (DMEM) with GlutaMax (Gibco), 10% fetal bovine serum (FBS, Gibco), and 1% antibiotic-antimycotic (Gibco). Cells were maintained at 37°C in a 5% $CO_2$ atmosphere. All cell lines routinely tested negative for mycoplasma.

### Adenoviral Infection & Assessment of Cell Death Fluorescent Microscopy

MEFs and HeLa cells were plated on 12 well dishes and allowed to adhere overnight prior to infection. Cells were infected with Cre recombinase expressing adenovirus at 0, 5, 10, 20, 40 MOI in DMEM+ GlutaMax with 2% heat-inactivated serum for 3 hours and then rescued with complete culture media. 48 hours following infection, Cell viability was assessed by staining cells with YO-PRO-1 (1:1000; Thermo Fisher Scientific, Y3603) plus Hoechst 33342 for 15 minutes. Fluorescence images were captured using a Nikon Eclipse Ti2-E equipped with a motorized XYZ-stage fitted with a CFI Plan Fluor 10x objective. Images were acquired with a DS-Qi2 camera (Nikon) illuminated by a Solid-state White Light Excitation Source (Lumencor). Cell death was determined by the number of YO-PRO-1-positive cells divided by the total number of Hoechst 33342-positive cells. Image analysis was performed using Image J.

### Mouse Models

*Becn1*$^{f/f}$ have been described previously (McKnight et al, 2014; Komatsu et al, 2005). The floxed mice were bred with *Myh6*-MerCreMer transgenic mice to generate conditional cardiac myocyte-specific knockout mice (Sohal et al, 2001). All mice were on a C57BL/6J background (Jax lab, stock # 000664) and have been backcrossed for at least 10 generations. Mice were housed under standard conditions and given free access to chow and water with a 12/12-hr dark–

light cycle. Echocardiography was performed using a Vevo-3100 LT Imaging System with an MX400 20–46 MHz imaging transducer (FUJIFILM Visualsonics). M-mode, B-mode, and pulsed wave Doppler views were acquired. Mice were kept on a warming pad and maintained under light anesthesia (0.5%–1% isoflurane, 98%–99.5% $O_2$), while measurements were obtained. The Vevo LAB software was used for analysis and quantification.

**MerCreMer Induction**

Tamoxifen (Sigma-Aldrich) was dissolved in ethanol (100%) and diluted in corn oil (Sigma-Aldrich) to a concentration of 10 mg/mL. Tamoxifen was administered by intraperitoneal (IP) injection at dosages of either 20 mg/kg or 30 mg/kg once per day for five consecutive days.

**Western Blot Analysis**

Cells were harvested and homogenized in ice-cold lysis buffer consisting of 50mM Tris-HCl (pH 7.4), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, and Complete protease inhibitor cocktail (Roche). Lysates were cleared by centrifugation at 20,000 x g for 20 minutes. A Bradford assay was performed on lysates to determine the protein concentration. 4X NuPAGE LDS Sample Buffer (Novex) and 50µM dithiothreitol (DTT) were added to the samples. Proteins were separated on 12% NuPAGE Bis-Tris gels (Life Technologies) and transferred to nitrocellulose membranes. Proteins were detected using antibodies against cleaved caspase-3 (Cell Signaling), PARP (BD Biosciences), Cre recombinase (Cell Signaling), and tubulin (Sigma-Aldrich). Proteins were visualized using a BioRad ChemiDoc XRS+, and bands were quantified using ImageLab.

**Statistical Analysis**

Data are mean +/- S.E.M. Experimental group comparisons were assessed by two-way ANOVA followed by Tukey test using GraphPad Prism 6 software. A p-value of less than 0.05 was considered statistically significant.

CHAPTER 1

**Cell death is promoted by Cre recombinase expression in Beclin 1-deficient MEFs**

Beclin 1 is a critical autophagy regulator involved in autophagosome formation and maturation. Silencing Beclin 1 in mice is known to lead to cell death as cells become more sensitive to metabolic stress without this key regulator of autophagy. BMCM knockout mice incur autophagy dysfunction and rapid cardiac failure following the silencing of Beclin 1. Reports demonstrate that expression of Cre recombinase alone may exert toxicity in mice and should be explored when establishing a mouse model. Here, we investigated the effects of Cre recombinase expression in Beclin 1-deficient MEFs.

To examine the sensitivity of Beclin 1-deficient cells to Cre expression, WT and *Becn1*$^{-/-}$ MEFs were infected with an adenovirus encoding Cre recombinase. Typically, Cre initiates site-specific recombination in the genome between loxP sites by excising a floxed region of a gene of interest. In this cellular context there are no loxP sites for Cre to recognize, so Cre does not initiate recombination and is solely expressed exogenously. Cells were stained with YO-PRO-1 and Hoechst stains to monitor cell death in response to Cre expression. This dye is impermeable to healthy cells but is taken up by dying cells with compromised plasma membranes. We observed a significant increase in cell death in Beclin 1 knockout MEFs infected with 40 MOI Ad-Cre (Fig. 1A & 1B).

Cell death can be coordinated in a caspase-independent or dependent manner. In caspase-independent apoptosis, poly(ADP-ribose) polymerase-1 (PARP-1) facilitates DNA base excision repair through the generation of the product poly(ADP-ribose) (PAR). PAR generation promotes the nuclear translocation of apoptosis-inducing factor, resulting in caspase-independent cell death (Andrabi et al, 2008). We observed an increase in cleaved caspase-3 as well as PARP with

increasing doses of Cre (Fig. 1C & 1D). The increase was not significant due to the variability between experiments. These results indicate that Beclin 1-deficient MEFs are more sensitive to Cre recombinase in a dose-dependent manner.

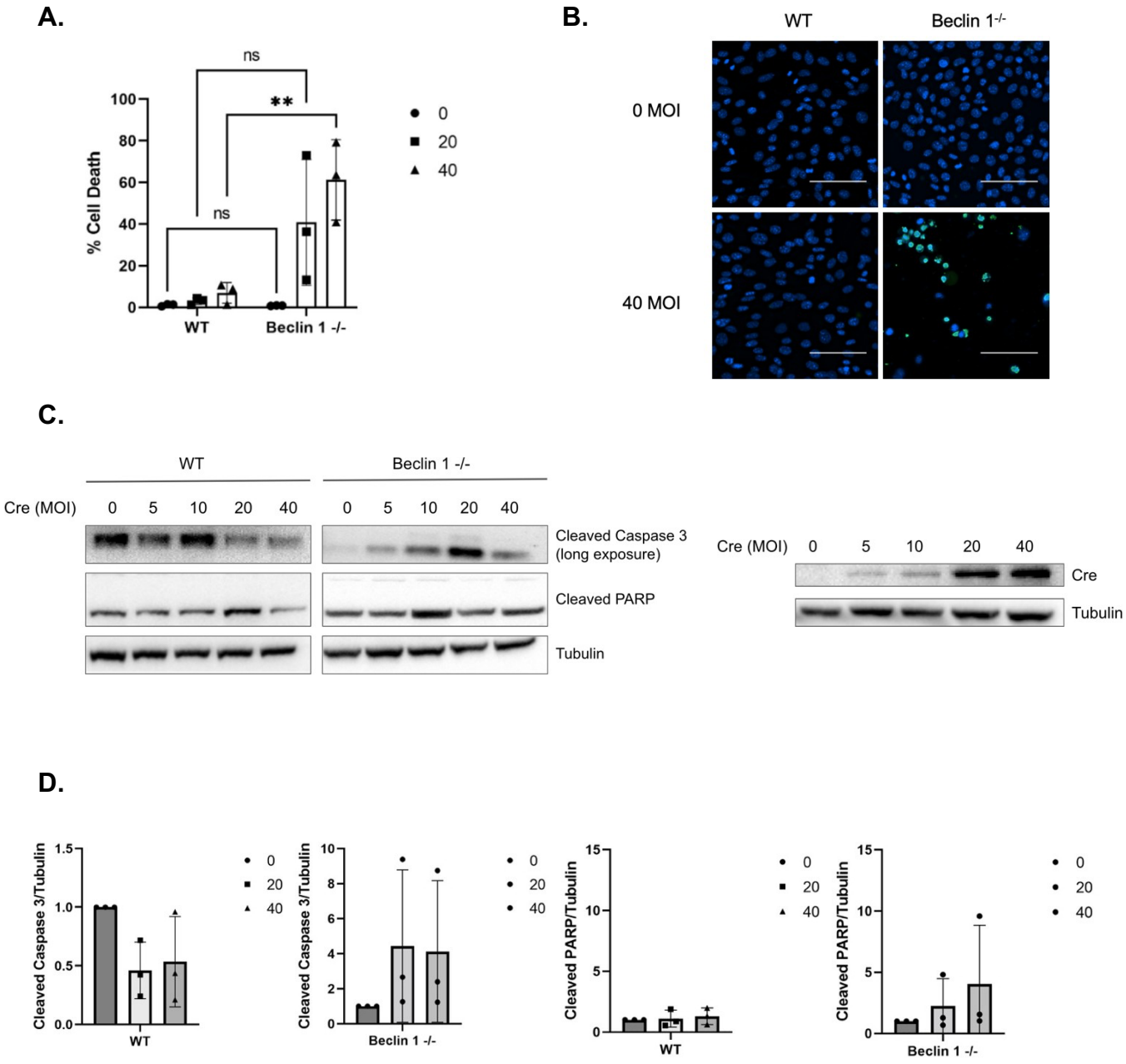



**Figure 1. Overexpression of Cre recombinase induces cell death in Beclin 1-/- MEFs. A)** WT and Beclin 1 -/- MEFs were infected with 0, 5, 10, 20, and 40 MOI adenovirus expressing Cre recombinase for 48 hours after which cell viability was assessed via live cell imaging with 1mM YO-PRO-1 and Hoechst staining. (n=3, **p<0.01, ns= not significant) **B)** Representative images of cell viability assay. Scale bar=100µm **C)** Representative blots of cleaved caspase 3 and cleaved PARP protein levels (left), representative blot of Cre protein levels in Beclin 1-/- MEFs (right). Tubulin was used as a loading control. **D)** Quantifications of cleaved Caspase 3 and cleaved PARP (n=3).

**Rab7 deficient cells are not sensitive to Cre expression**

The four main stages of autophagy include: initiation, elongation, maturation, and degradation. Before cargo is degraded, autophagosomes fuse with the lysosome. Rab 7 functions as an important autophagy regulator which assists in autophagosome maturation, transport, and lysosomal biogenesis. Previous reports have shown Rab 7 is required for autophagosomes to fuse with lysosomes, and autophagosomes accumulate in Rab 7-deficient cells in response to amino acid starvation (Wen et al, 2017). Rab 7 is a crucial autophagy regulator, as Rab 7 knockout mice do not survive embryogenesis (Kuchitsu & Fukuda, 2018). Since Rab 7 and Beclin 1 are both key regulators of autophagy, we investigated whether Rab 7-defficiency would lead to increased sensitivity to Cre-induced toxicity.

WT and Rab $7^{-/-}$ MEF viability were assessed by YO-PRO-1 and Hoescht stains as described previously. We observed no significant increases in cell death in Rab $7^{-/-}$ MEFs infected with increasing doses of Ad-Cre (Fig. 2A & 2B). Furthermore, cleaved caspase-3 was not increased in Rab$7^{-/-}$ MEFs compared to WT cells (Fig. 2C & 2D). No significant increase in PARP expression was observed (Fig. 2C & 2D), further suggesting that apoptotic signaling pathways are not initiated in these cells. These results indicate that overexpression of Cre in Rabn$7^{-/-}$ MEFs does not cause cell death.

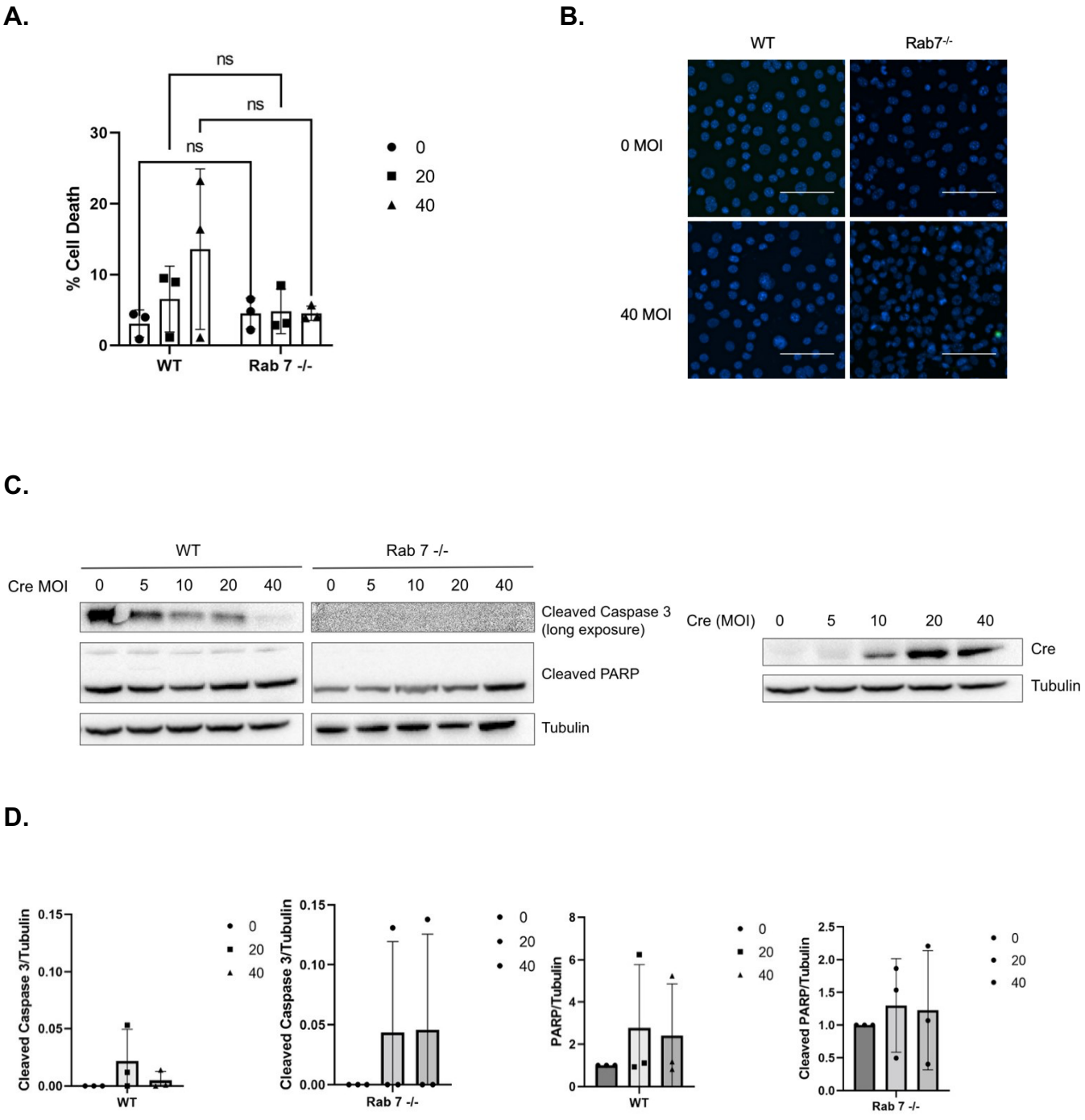



**Figure 2. Rab7$^{-/-}$ MEFs are resistant to Cre recombinase overexpression. A)** WT and Rab7$^{-/-}$ MEFs were infected with 0, 20, and 40 MOI adenovirus expressing Cre recombinase for 48 hours after which cell viability was assessed via live cell imaging with 1mM YO-PRO-1 and Hoechst staining. (n=3, ns= not significant) **B)** Representative images of cell viability assay. Scale bar=100µm **C)** Representative blots of cleaved caspase 3 and cleaved PARP protein levels (left), representative blot of Cre protein levels in WT MEFs (right). Tubulin was used as a loading control. **D)** Quantifications of cleaved caspase 3 and cleaved PARP levels (n=3).

**Cre recombinase overexpression induces cell death in Beclin 1-deficient MEFs**

Next, we investigated Cre sensitivity in *BECN1*$^{-/-}$ HeLa cells to determine whether these cells were also more sensitive to Cre toxicity in a dose-dependent manner. WT and *BECN1*$^{-/-}$ HeLa cells were treated with YO-PRO-1 and Hoechst stains to monitor cell death in response to Cre expression. In contrast to Beclin knockout MEFs, no significant increases in cell death were observed in *BECN1*$^{-/-}$ HeLa cells infected at variant doses (Fig. 3A & 3B). No significant increase in cleaved caspase 3 or PARP expression was observed in *BECN1*$^{-/-}$ HeLa cells (Fig. 3C & 3D), suggesting that *BECN1*$^{-/-}$ HeLa cells are neither initiating cell death in a caspase-dependent or independent manner. These results suggest that *BECN1*$^{-/-}$ HeLa cells are more resistant to Cre recombinase expression.

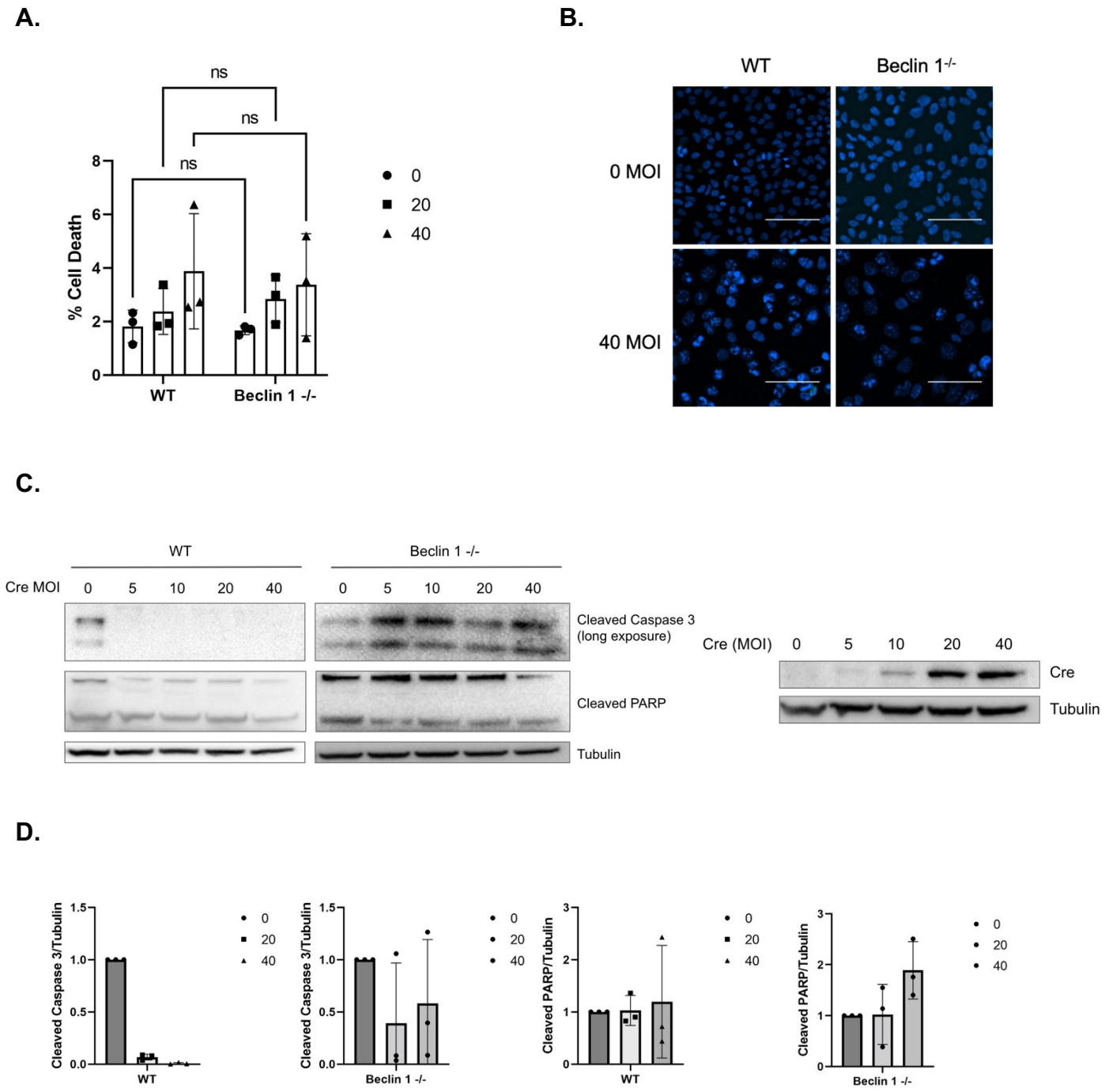



**Figure 3. Cre recombinase expression does not induce cell death in Beclin 1$^{-/-}$ HeLa cells. A)** WT and Beclin 1$^{-/-}$ HeLa cells were infected with 0, 20, and 40 MOI adenovirus expressing Cre recombinase for 48 hours after which cell viability was assessed by live cell imaging using 1mM YO-PRO-1 and Hoechst staining (n=3). **B)** Representative images of cell viability assay. Scale bar=100µm **C)** Representative blots of cleaved caspase 3 and PARP protein levels (left), representative blot of Cre protein levels in WT HeLa cells (right). Tubulin was used as a loading control. **D)** Quantifications of cleaved caspase 3 and PARP levels (n=3).

CHAPTER 2

**High Cre expression induces cardiac dysfunction in BMCM mice**

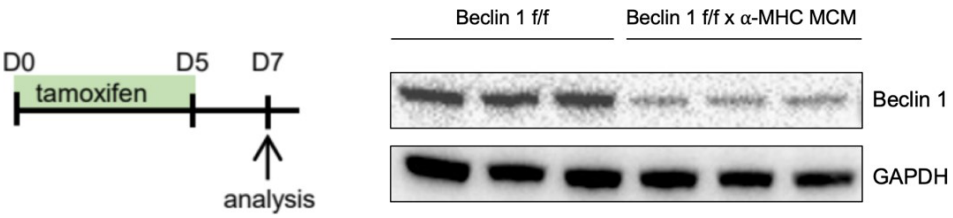
In the Cre/loxP system, the addition of tamoxifen enables mutated estrogen receptors (Mer) to translocate to the nucleus where a floxed target gene will be excised. Previous studies of inducible Cre-mediated mutagenesis in mouse models report tamoxifen administered via intra-peritoneal (IP) injection at doses varying from 40mg/kg to 560mg/kg body weight, given over 1-14 days (Lexow et al, 2013). High levels of Cre recombinase expression are known to be cytotoxic (Buerger et al, 2006). Mice carrying the MerCreMer cassette under the cardiac-specific promoter α-MHC have been shown to display dose-dependent acute cardiac dysfunction after tamoxifen administration (Lexow et al, 2013). In addition, α-MHC-MerCreMer mice express high levels of pro-inflammatory cytokines following tamoxifen treatment (Lexow et al, 2013).

In order to evaluate the role of Beclin 1 in cardiac homeostasis, our lab has generated a tamoxifen-inducible α-MHC-MerCreMer x Beclin 1 f/f mouse model which enables myocyte-specific deletion of Beclin 1 in the heart. Post-tamoxifen administration, we have observed rapid cardiac dysfunction and mortality in this model. However, it was unknown whether the expression of Cre contributed to the phenotype in Beclin 1-deficient hearts.

To investigate the potential role of Cre toxicity in BMCM mice, mice carrying the α-MHC-MerCreMer cassette as well as mice without the cassette were administered 20 mg/kg or 30 mg/kg tamoxifen via IP injection once daily for five consecutive days. Cardiac function was evaluated by echocardiogram before the tamoxifen injections and seven days after initial tamoxifen administration (Fig. 4A). Beclin 1 protein levels in hearts of mice injected with 20 mg/kg tamoxifen at the D7 time point were assessed via Western blot analysis, and confirmed decreased Beclin 1 protein levels (Fig 4A). Cre induction using 20 mg/kg tamoxifen had no

effect on the heart while the 30 mg/kg dose resulted in a significant decrease in cardiac function as measured by ejection fraction in Cre-positive f/f BMCM mice (Fig. 4B). This finding suggests that Beclin 1 deficiency in BMCM mice leads to increased sensitivity to Cre toxicity.

**A.**

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**B.**

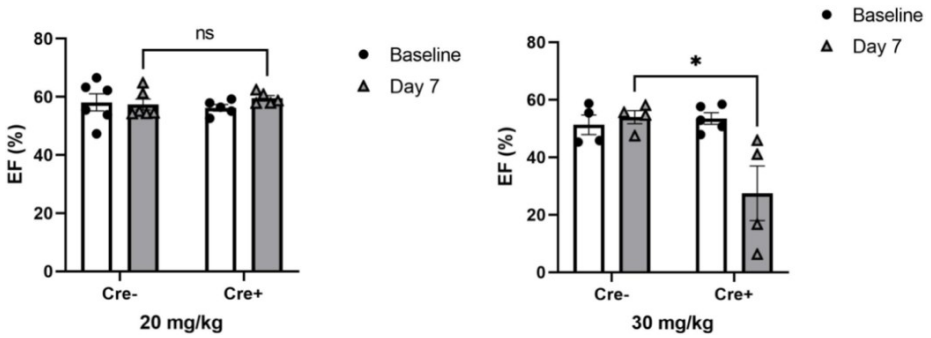


**Figure 4. Increased sensitivity to Cre toxicity in Beclin 1-deficient hearts. A)** Schematic of tamoxifen administration schedule and representative Western blot of Beclin 1 protein levels in hearts at D7 in mice injected with 20 mg/kg tamoxifen. **B)** Echocardiogram measurements of ejection fraction at baseline (before tamoxifen treatment) and day 7 (n=4-6, *p<0.05, ns= not significant).

## SUMMARY

The data shown displays an evaluation of Cre toxicity *in vitro* and *in vivo* to ascertain whether Beclin1 deficiency increases sensitivity to Cre toxicity. Increased cell death is observed with Cre recombinase overexpression in Beclin 1 deficient MEFs, while Rab 7 deficient MEFs and Beclin 1 deficient HeLa cells are not affected. BMCM mice also show a dose-dependent cardiotoxicity upon Cre induction.

## DISUCSSION AND CONCLUSIONS

The results of this investigation point to the role of Cre recombinase in inducing cell death when cells lack Beclin 1. In Beclin 1 deficient MEFs, we observed a significant increase in cell death following Cre expression; however, markers of caspase-dependent pathways do not show a significant upregulation. Additionally, we observed that Rab 7 deficiency does not affect cellular sensitivity to Cre. We also demonstrated that Cre does not initiate cell death in Beclin 1 deficient HeLa cells. *In vivo*, we further explored Beclin deficiency in cardiac-specific Beclin 1 knockout mice and observed a reduction in cardiac function following a tamoxifen treatment of 30mg/kg.

In our *in vitro* investigations we did not evaluate necrotic cell death and propose that necrosis is likely initiated in the Beclin 1 MEFs, rather than the apoptotic pathways we have examined. Beclin 1 HeLa cells were more resistant to Cre expression. The cancerous nature of HeLa cells factors into our observations, and the mutations these cells have acquired assist them in accommodating Cre expression. Additional research identifying the type of cell death induced by Cre overexpression in Beclin 1-deficient cells is needed.

Our research highlights the importance of evaluating Cre toxicity when establishing a new mouse model, as our investigations have shown that sensitivity to Cre toxicity plays a role

in our BMCM mouse model. Further research assessing cardiac morphology *in vivo* following Cre induction is necessitated. Additionally, an evaluation of a broader scope of tamoxifen dosages should be tested to identify a dose that enables sufficient recombination but minimizes toxicity. The cardiotoxicity seen in BMCM mice is suggestive of non-specific recombination induced by Cre, which can be initiated by pseudo-loxP sites in the mouse genome that generate randomized excision. It is possible that the absence of Beclin 1 facilitates non-specific recombination and leads to the significant toxicity we have observed. Further research into potential off-target excision generated by Cre recombinase is necessary to characterize the source of Cre toxicity in this mouse model.

# INTRODUCTION

## Protein Quality Control

Proteostasis is a dynamic process regulated by a network of over 1,000 chaperones and a variety of factors that facilitate protein folding and maintain proteins in their soluble, non-aggregated state (Labbadia & Morimoto, 2015). Present in all extant life, the proteostasis network maintains the total collection of proteins in cells to promote cellular survival. This network encompasses protein synthesis, folding, and conformational maintenance, as well as protein degradation (Klaips et al, 2018). As this network monitors variant stages of protein processing, it is accordingly complex and includes: translational machinery, molecular chaperones, the ubiquitin-proteasome system (UPS), and the autophagy machinery (Labbadia & Morimoto, 2015).

Molecular chaperones in the proteostasis network are highly conserved and take diverse roles such as protein folding, trafficking, and disaggregation in all cellular subcompartments (Sala & Morimoto, 2022). The well-characterized heat shock proteins HSP70 and HSP90 facilitate efficient protein folding and accommodate the re-folding of misfolded proteins. Chaperones can also inhibit proteotoxic aggregation. Proteins that cannot be re-folded by chaperones are targeted for degradation (Labbadia & Morimoto, 2015).

The ubiquitin-proteasome system (UPS) is recognized as the major cytosolic protein quality control system in eukaryotes (Finley, 2009). It functions by targeting proteins for degradation via an enzymatic E1/E2/E3 ubiquitination cascade, after which they are marked for destruction by the proteasome (Chen et al, 2011). Alternatively, large aggregates of misfolded proteins may be delivered to the lysosome through the autophagy pathway (Labbadia & Morimoto, 2015). Autophagy functions by engulfing cytosolic components, such as protein

aggregates, in a double-membraned autophagosome which is trafficked to the lysosome for proteolytic degradation (Glick et al, 2010).

Loss of proteostasis is a hallmark of cellular dysfunction. Misfolded and damaged proteins can accumulate and form aggregates that exert proteotoxic effects commonly observed with age and disease (Labbadia & Morimoto, 2015). An accumulation of damaged and misfolded proteins can overwhelm the proteostasis network and initiate widespread protein aggregation that leads to cell death (Chen et al, 2011). This mechanism is widely observed in neurodegenerative diseases like Alzheimer's and Huntington's disease in which a loss of proteostasis is associated with proteotoxic aggregate formation.

**Mitochondrial Protein Quality Control**

Mitochondria contain their own resident chaperones and proteases that facilitate protein folding and degradation. Matrix proteases maintain normal protein turnover inside mitochondria (Quiles & Gustafsson, 2020). The most abundant protease present in cardiac mitochondria is LonP1. This enzyme degrades misfolded and damaged matrix and intermembrane proteins (Bota & Davies, 2002).

Mitochondria have recently been shown to participate in cytosolic proteostasis. The importance of this function was identified in yeast, in which mitochondria import misfolded proteins for degradation in the matrix (Ruan et al, 2017). Mitochondria import proteins sequentially through the protein import machinery (PIM), consisting of channels in the outer and inner mitochondrial membranes. Proteins are first imported through the translocase of the outer mitochondrial membrane (TOM). Tom is a complex of Tom20, Tom40, Tom22, Tom70, and the functionally unessential Tom5, Tom6, and Tom7 (Wiedemann & Pfanner, 2017). Tom20 is of particular interest in our investigation as this subunit serves as the initial receptor that recognizes

proteins that have been destined for import. Alongside Tom70, Tom20 exposes receptor domains to facilitate the cleavage of mitochondrial targeting signals (Lenkiewicz, 2021). Thus, Tom20 serves as an ideal target for disrupting mitochondrial protein import at its early stages.

Following import through the outer membrane, proteins are guided to the inner membrane of the mitochondria where they are linearized as they are imported through the translocase of the inner mitochondrial membrane (Tim), before arriving in the mitochondrial matrix. Proteins arriving in the matrix will either be targeted for folding or degradation by resident proteases (Anand et al, 2013).

**Misfolded alphaB-Crystallin (CryAB)**

Alpha B-crystallin (CryAB), or HspB5, is a heat shock protein that is known to function as a molecular chaperone by binding irreversibly to denatured proteins to inhibit the formation of aggregates (Horwitz, 1992). First discovered as a lens protein, CryAB is highly expressed in the lens, heart, skeletal muscle, brain, and lowly expressed in several other tissues (Boelens, 2014). In addition to its chaperone role, CryAB is also involved in the structural organization of intermediate filament proteins, such as desmin (Horwitz, 1992). The introduction of the missense mutation R120G into CryAB (CryAB-R120G) results in a defect in the structural organization of the protein at the quaternary level and a resultant loss of CryAB's chaperone-like functions (Bova et al, 1999 & Vicart et al, 1998). CryAB-R120G expression has been demonstrated to impair the UPS as well as autophagy. Aberrant accumulation of CryAB-R120G has also been shown to directly result in a desmin-related cardiomyopathy (Wang et al, 2001).

CryAB is abundant in the heart, comprising 0.1-2% of soluble protein content in cardiac cells and 3-5% of total cardiac mass (Islam et al, 2020; Boelens, 2014). The R120G mutant has been demonstrated to be detrimental in the heart, as cardiac-specific CryAB-R120G

overexpression culminates in heart failure after five to seven months in transgenic mouse models (Maloyan et al, 2005). CryAB-R120G aggregates disrupt sarcomere organization in the heart (Islam et al, 2020). Isolated aggregates contain both the mutant CryAB protein as well as desmin, among other proteins crucial to the structural integrity of the sarcomere (Wang et al, 2001). Cardiomyocytes respond to proteotoxic CryAB-R120G aggregation by upregulating autophagy and the UPS. Stimulating these pathways has been shown to dampen cardiomyopathy development in transgenic CryAB-R120G mouse models (Pan et al, 2017).

**Rationale**

It is currently unknown whether mitochondria are active participants in protein quality control in the cardiac environment. In my research, I aim to examine the import of CryAB-R120G by mitochondria as a potential regulated, adaptive response to protect against CryAB-R120G-induced aggregation.

I have used in vitro models in my research to examine the hypothesis that Tom20 knockdown will disrupt mitochondrial protein import and increase the accumulation of CryAB-R120G in the cytosol. To test this hypothesis, I have investigated the following question:

1. Does Tom20 participate in the import of CryAB-R120G?

# MATERIALS AND METHODS

## Cell Culture

Mouse embryonic fibroblasts (MEFs) were cultured in media comprised of Dulbecco's Modified Eagle Medium (DMEM) with GlutaMax (Gibco), 10% fetal bovine serum (FBS, Gibco), and 1% antibiotic-antimycotic (Gibco). Cells were maintained at 37°C in a 5% $CO_2$ atmosphere.

## siRNA Knockdown and Adenoviral Infection

MEFs were transfected with either 50 nM negative control siRNA (Sigma Aldrich) or 50 nM Tom20 siRNA (Sigma siRNA ID: SASI_Mm02_0032_7580/TOMM20) using RNAiMax Lipofectamine reagent (Thermo Fisher) according to the manufacturer's protocol. 48 hours following transfection, cells were infected with a CryAB-R120G adenovirus at 30 MOI in DMEM+ GlutaMax with 2% heat-inactivated serum for 3 hours and then rescued with complete culture media. Cells were collected 48 hours post intake for isolation of mitochondria and subsequent Western blot analysis. Silencing of Tom20 was confirmed by Western blot for each experiment.

## Mitochondrial Isolation

Cells were collected by centrifugation at 600 x g for 5 minutes at 4°C and resuspended in ice-cold isolation buffer (220 mM mannitol, 70 mM sucrose, 1 mM EDTA, and 10 mM HEPES, pH 7.4, 10 mM N-Ethylmaleimide, and complete protease inhibitor cocktail (Roche)). Cells were incubated on ice for 45 minutes and gently resuspended every 10 minutes with cut pipette tips. The cells were homogenized using a plastic Eppendorf tube mortar and pestle for 10 minutes on ice to gently release mitochondria. The suspension was centrifuged at 600 x g for 5 minutes at 4°C to pellet cellular debris. The supernatant was decanted and kept on ice while the cell pellet was resuspended in ice-cold isolation buffer, homogenized for 10 minutes on ice, and

centrifuged at 600 x g at 4°C. The supernatant was combined with the previous and centrifuged at 600 x g for 5 minutes at 4°C, after which the supernatant was centrifuged under the same conditions. The supernatant was centrifuged at 14,000 x g for 15 minutes at 4°C to pellet mitochondria. The mitochondrial pellet was gently washed and resuspended in isolation buffer. A Bradford assay was performed to determine the protein concentration.

**Western Blot Analysis**

MEFs were lysed in ice-cold lysis buffer consisting of 50mM Tris-HCl (pH 7.4), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, and Complete protease inhibitor cocktail (Roche). Lysates were cleared by centrifugation at 20,000 x g for 20 minutes. The protein concentrations were determined by Bradford assay. 4X NuPAGE LDS Sample Buffer (Novex) and 50uM dithiothreitol (DTT) were added to the samples. Proteins were separated on 12% NuPAGE Bis-Tris gels (Life Technologies) and transferred to nitrocellulose membranes. Proteins were detected using antibodies against HA (Cell Signaling), Tom20 (Santa Cruz), and tubulin (Sigma-Aldrich). Proteins were visualized using a BioRad ChemiDoc XRS+, and bands were quantified using ImageLab.

**Statistical Analysis**

Data are mean +/- S.E.M. experimental group comparisons assessed by student's t-test using GraphPad Prism 6 software. A p-value of less than 0.05 was considered statistically significant.

# CHAPTER 1

## Tom20 Deficient Cells Display Cytosolic Accumulation of CryAB-R120G

Tom20 acts as a crucial subunit of the TOM complex, serving to initiate the import of mitochondrially-targeted proteins. Tom20 exposes receptor domains to facilitate the cleavage of5 mitochondrial targeting signals (Lenkiewicz, 2021). Thus, Tom20 serves as an ideal target for disrupting mitochondrial protein import at its early stages. Here, we investigated the effects of Tom20 deficiency on CryAB-R120G levels in MEFs.

We first established sufficient Tom20 knockdown. MEFs were transfected with 50nM control or Tom20 siRNA for 48 hours before cells were harvested for mitochondrial isolation. Cytosolic and mitochondrial fractions were analyzed via Western blot, which revealed sufficient knockdown of Tom20.

To examine the role of Tom20 on CryAB-R120G import into mitochondria, MEFs were transfected with 50nM control or Tom20 siRNA for 48 hours prior to infection with 300 MOI adenovirus expressing HA-CryAB-R120G for 48 hours. Mitochondrial and cytosolic fractions were then isolated and the cytosolic fractions were evaluated via Western blot. A significant increase in CryAB-R120G protein levels was observed in the cytosol in cells with Tom20 knockdown (Fig 1A & 1B). These results suggest that CryAB-R120G accumulates in the cytosol due to mitochondrial import disruption.

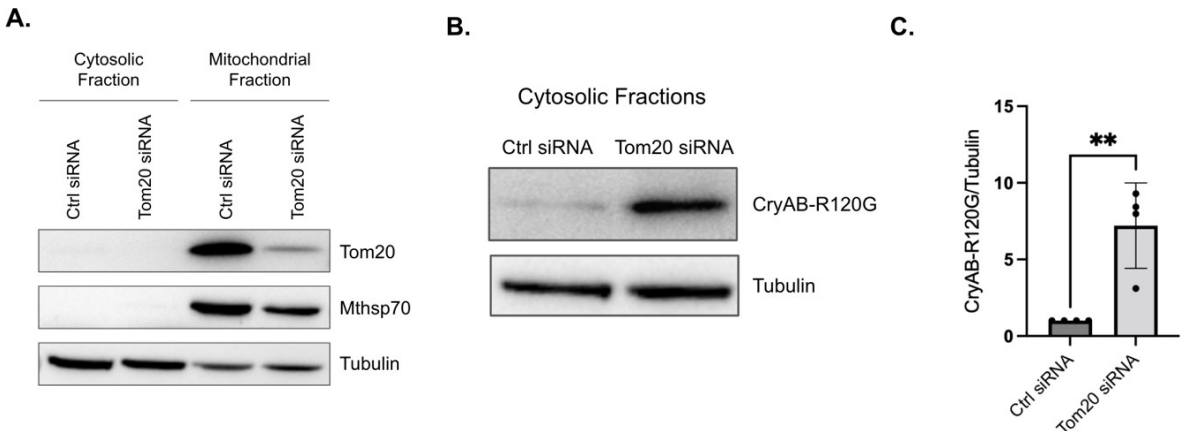


**Figure 5. Tom20 knockdown in cells leads to increased accumulation of CryAB-R120G in the cytosol. A)** MEFs were transfected with 50nM control or Tom20 siRNA and harvested for mitochondrial isolation. Representative blot of Tom20 levels in mitochondrial fractions. Mthsp70 and tubulin were used as loading controls. **B)** MEFs were transfected with 50nM control or Tom20 siRNA and infected with 300 MOI adenovirus expressing HA-CryAB-R120G for 48 hours. Cells were then harvested for isolation of cytosolic and mitochondrial fractions and subsequent Western blot analysis. Representative blot of CryAB-R120G levels using anti-HA in cytosolic fractions. Tubulin was used as a loading control. **C)** Quantification of HA levels. (n=4, **p<0.01).

## SUMMARY

The data shown displays an evaluation of the role of Tom20 in the import of CryAB-R120G. Silencing of Tom20 in MEFs demonstrates that this subunit of the TOM complex is critical in protein import.

## DISCUSSION AND CONCLUSIONS

Our results offer insight into the importance of the TOM complex in importing misfolded proteins. We demonstrated that Tom20, a subunit of the translocase of the outer mitochondrial membrane, critically participates in mitochondrial protein import of CryAB-R120G. In MEFs, we silenced Tom20 with siRNA for 48 hours and then infected cells with an adenovirus overexpressing CryAB-R120G for 48 hours before performing a mitochondrial isolation. We observed a significant increase in CryAB-R120G protein levels in cytosolic fractions. Our research therefore suggests that Tom20 is a critical subunit of the TOM complex for the import of CryAB-R120G.

Silencing Tom20 effectively led to a blockage of mitochondrial import and subsequent accumulation of aggregates in the cytosol. Our knockdown prevented Tom20 from exposing protein domains containing a mitochondrial targeting sequence (MTS), which could not be cleaved and therefore MTS-containing proteins such as CryAB-R120G could not be fully imported (Lenkiewicz, 2021).

As our research has pointed to the importance of mitochondrial import as a potential cytosolic quality control mechanism, the proteolytic processing step of this pathway merits further research. A study in yeast has shown that mitochondria can import misfolded proteins and degrade them with resident mitochondrial proteases (Ruan et al, 2017). The fate of CryAB-

R120G following its import into mitochondria is currently unknown. It is unknown whether CryAB-R120G is actively imported and proteolytically processed by mitochondria in a regulated manner. Our lab is investigating this as well as whether blocking the import of CryAB-R120G further exacerbates CryAB-R120G-induced proteotoxicity.

Additional research is necessary to clarify mitochondrial import's role as a potential cytosolic quality control mechanism. Inhibition of the proteasome and autophagy could provide insights into the interplay between mitochondrial import and established cellular quality control mechanisms for clearing CryAB-R120G aggregates. We speculate that inhibition of the proteasome would lead to enhanced uptake of CryAB-R120G. Overall, further research into mitochondrial import's role as a cytosolic quality control mechanism is needed.

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