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DEFINING THE ROLE OF CHAPERONE-MEDIATED AUTOPHAGY IN DEGRADING HARMFUL PROTEINS IN CARDIAC PATHOLOGY

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Background: Protein degradation pathways are essential for maintaining protein homeostasis and thus normal cellular function. Chaperone Mediated Autophagy (CMA) is an important protein degradation pathway that selectively degrades cytosolic and misfolded proteins containing a unique and selective KFERQ-like motif. CMA employs a chaperone complex called, Hsc70 that delivers substrate proteins to a CMA-specific Lysosome-Associated Membrane Protein type 2a (LAMP2a) receptor for lysosomal degradation. Various cardiac proteins were found to contain KFERQ-like motif making them potential substrates of CMA. One such mutant protein, αB crystallin (CryAB^{R120G}) was found to contain a KFERQ-like motif. CryAB^{R120G} is a missense mutation that causes desmin-related cardiomyopathy, which is known to cause proteotoxicity due to the large accumulation of the insoluble CryAB^{R120G} aggregates in the heart. Our goal was to test if increasing CMA function selectively clears the mutant CryAB^{R120G} protein.

Methods: We first screened 12 of the most common cardiac proteins in the heart to see if they contained the KFERQ-motif required for CMA degradation. Uniprot database was utilized to access the cardiac protein sequences and the KFERQ-finder tool was employed to detect the presence of KFERQ-like motifs within the protein sequences. We found that 10 of the 12 common cardiac proteins contained the required KFERQ-motif to be eligible for degradation in the CMA pathway. LAMP2a expression is sufficient and necessary for CMA function. To increase CMA activity, neonatal rat ventricular cardiomyocytes (NRVMs) with the CryAB^{R120G} mutation were infected with a LAMP2a (L2a) adenovirus to overexpress LAMP2a levels and increase CMA function. For loss of CMA, cardiomyocytes were transfected with a LAMP2a (L2a) siRNA to suppress LAMP2a levels. CryAB^{R120G} protein levels were determined by immunoblotting and the protein levels of LAMP2a, CryAB^{R120G} and FLAG-Tag were quantified using Image Studio and Graphpad Prism software. Aggregate content was measured by immunostaining and high-resolution images of the cardiomyocytes were captured using Confocal Imaging.

Results:

Gain of CMA Function

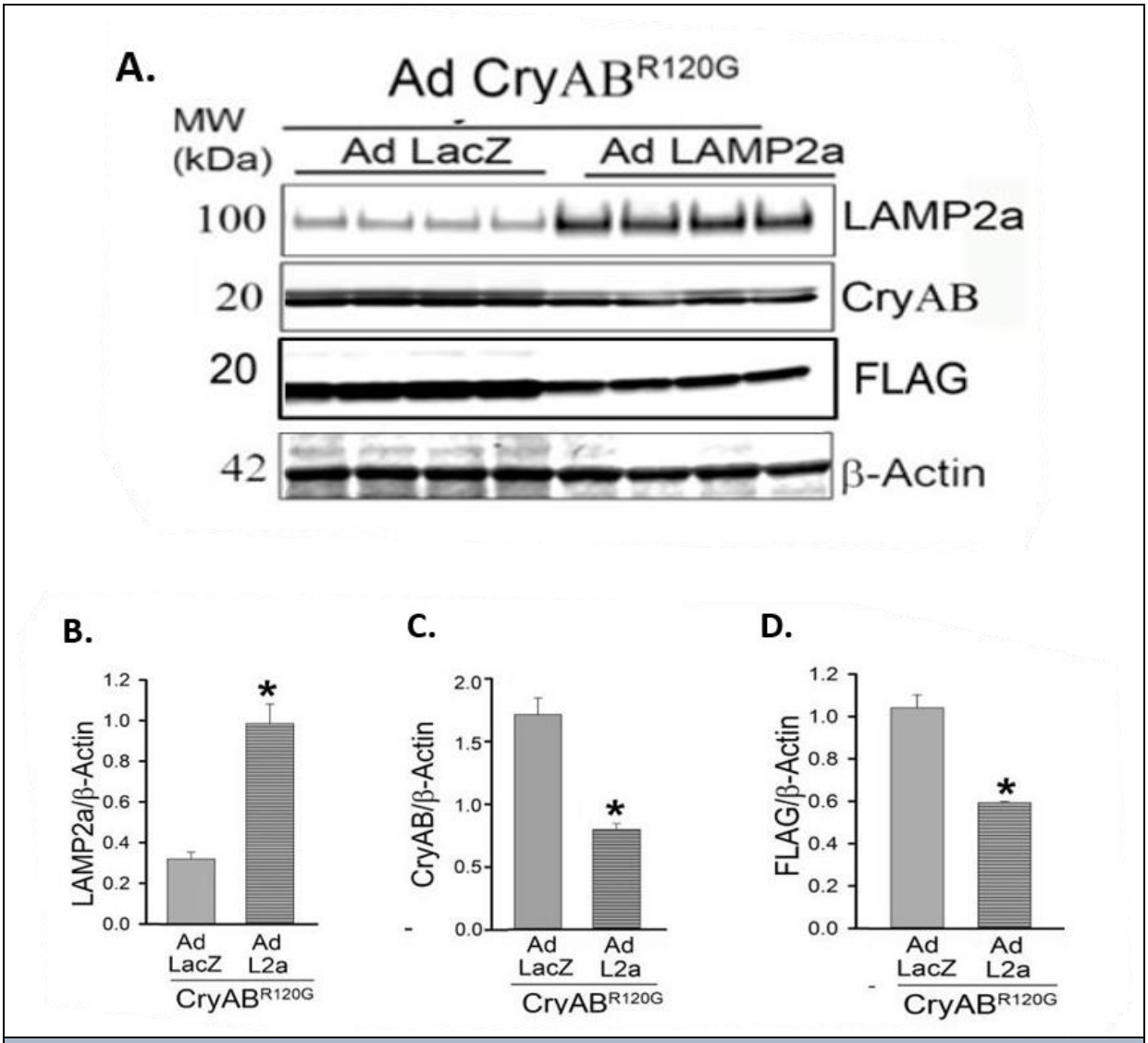

Figure 1. *CryAB$_{R120G}$ mutant protein was degraded by enhancing CMA.* (Figure 1A.) Immunoblot data displaying protein levels in CryAB$_{R120G}$ expressing cardiomyocytes infected with either LacZ or LAMP2A adenoviruses. CryAB$_{R120G}$ was tagged with a FLAG-tag protein to distinguish between the exogenous over-expression of LAMP2a levels and the endogenous expression of basal LAMP2a in the cell. β-Actin was used as a normalizer to correct for variations in loading samples. (Figure 1B-D) Histograms represent LAMP2A, CryAB$_{R120G}$ and FLAG-Tag levels which were normalized to β-Actin levels. Results are expressed as Mean ±SE; N=4/group * significant difference between AdLacZ and Ad LAMP2a; $p<0.05$.

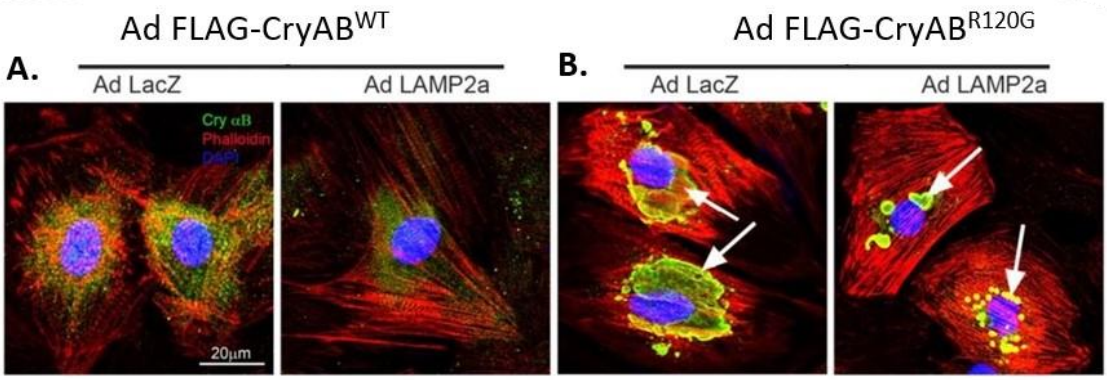

Figure 2. *Immunocytochemistry showed that CryAB$_{R120G}$ protein aggregates were cleared by upregulating CMA.* CryAB$_{WT}$ or CryAB$_{R120G}$ expressing cardiomyocytes were infected with AdLAMP2a or AdLacZ and subsequently stained with CryAB antibody (green). Cardiomyocytes were stained with phalloidin (red) and DAPI was used to stain the nucleus (blue). CryAB$_{WT}$ did not show any aggregate formation (Figure 2A). LAMP2a overexpression markedly reduced the CryAB$_{R120G}$ aggregate content compared to LacZ infected cells (Figure 2B).

Gain of CMA by LAMP2a overexpression increased LAMP2a levels by almost 70% *(*Figure 1B*)* and reduced the soluble mutant CryAB$_{R120G}$ protein by 60% (Figure 1C) and Flag/ β-Actin levels by 40% (Figure 1D*)* in NRVMs, suggesting that CMA clears proteotoxic proteins. Immunocytochemistry showed no aggregation and accumulation of CryAB with or without LAMP2a overexpression (Figure 2A). On the other hand, cardiomyocytes containing CryAB$_{R120G}$ proteins displayed much higher aggregation levels (displayed in green) indicating proteotoxicity (Figure 2B). When LAMP2a was over-expressed (to induce CMA function) with the LAMP2a adenovirus, there was a 40% decrease in CryAB$_{R120G}$ aggregate volume, further confirming that CMA plays an important role in degrading the CryAB$_{R120G}$ proteotoxic proteins.

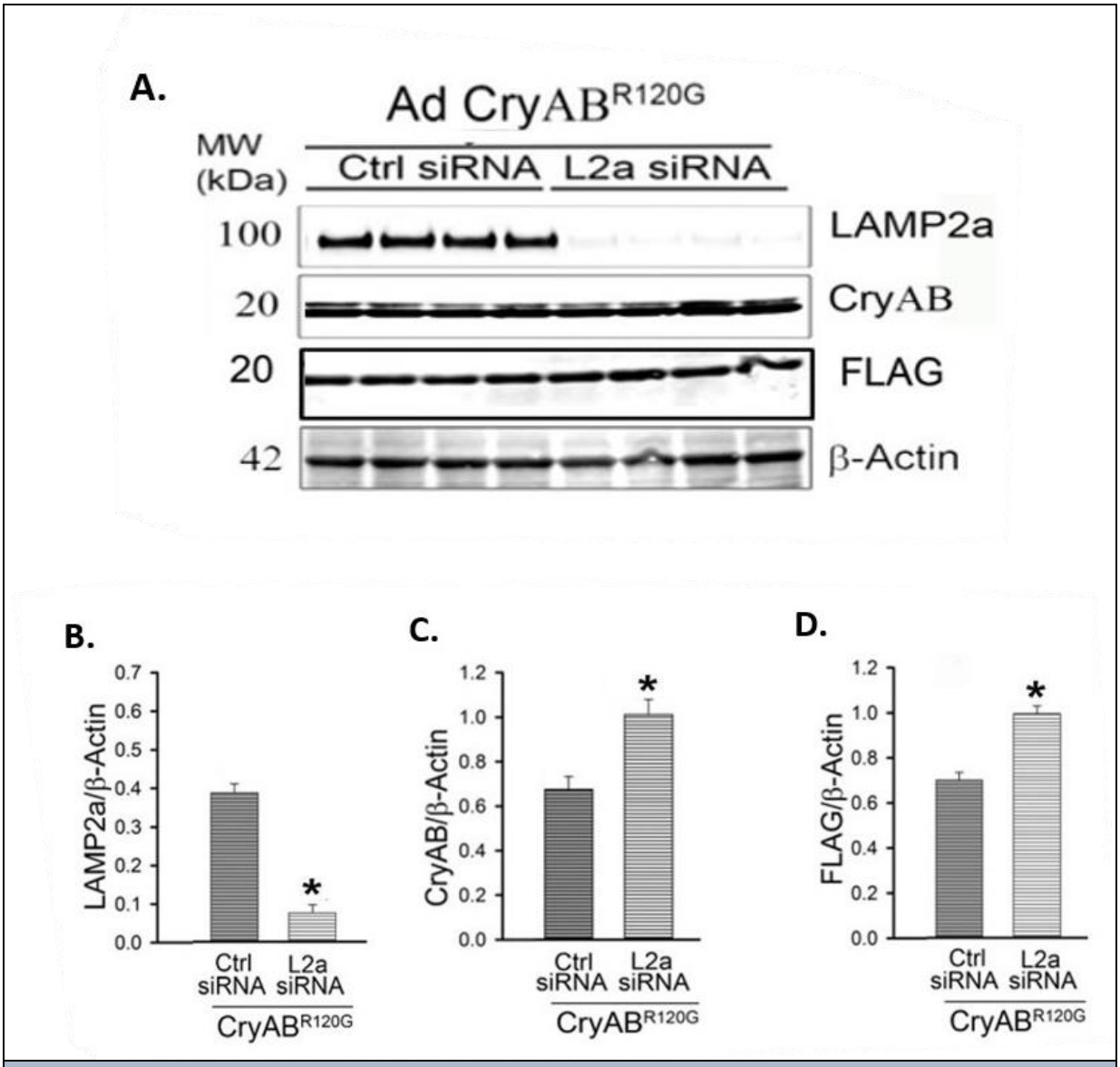

Figure 3. *CMA suppression caused accumulation of CryAB$_{R120G}$ mutant protein in NRVMs.* (Figure 3A). Immunoblot data displaying protein levels in *CryAB$_{R120G}$* expressing cardiomyocytes transfected with Ctrl siRNA or LAMP2a siRNA. β-Actin was used as a normalizer to correct for variations in loading samples. (Figure 3B-D) Histograms represent LAMP2A, *CryAB$_{R120G}$* and FLAG-Tag levels which were normalized to β-Actin levels. Results are expressed as Mean ±SE; N=4/group; * significant difference between AdLacZ and Ad LAMP2a; $p<0.05$.

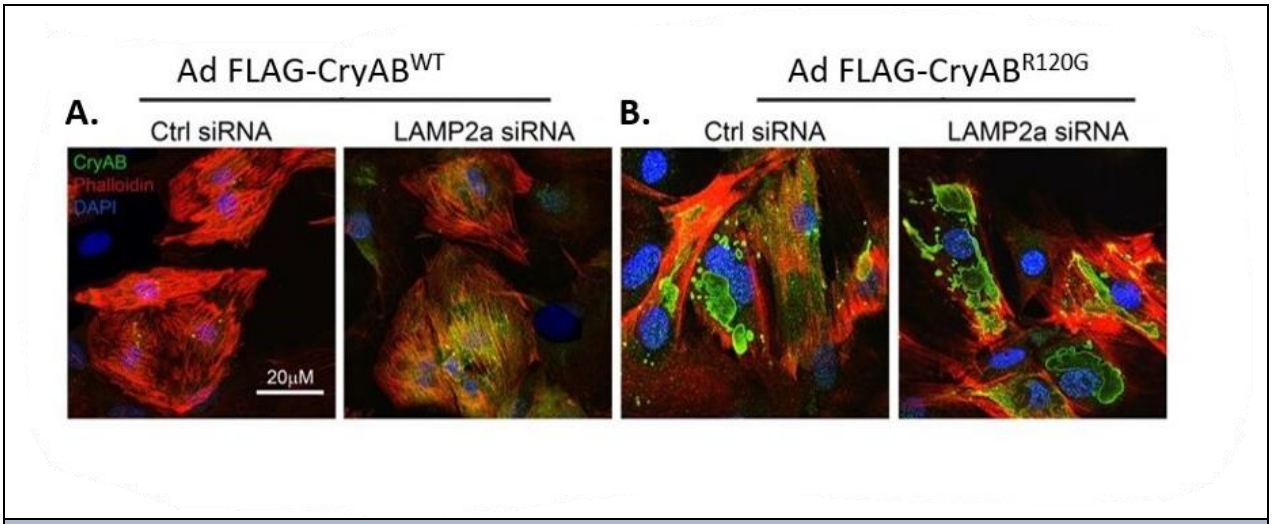

Figure 4. *Immunocytochemistry showed that suppression of CMA caused CryAB$_{R120G}$ protein aggregates to accumulate*. CryAB$_{WT}$ or CryAB$_{R120G}$ expressing cardiomyocytes were transfected with LAMP2a or Ctrl siRNA and subsequently stained with CryAB antibody (green). Cardiomyocytes were stained with phalloidin (red) and DAPI was used to stain the nucleus (blue). CryAB$_{WT}$ did not show any aggregate formation (Figure 4A). LAMP2a knockdown markedly increased the CryAB$_{R120G}$ aggregate content compared to Ctrl siRNA infected cells (Figure 4B).

On the other hand, loss of CMA due to LAMP2a suppression decreased LAMP2a levels by roughly 80% *(Figure 3B*), increased CryAB$_{R120G}$ levels by 40% (Figure 3C), and Flag/ β-Actin levels by 40% *(*Figure 3D*)*. Immunocytochemistry images showed that aggregate volume increases by 40% (more green aggregates) when CMA was suppressed by LAMP2a siRNA compared to the Ctrl siRNA treated cells (Figure 4). These data show that CMA is cardioprotective in NRVMs with proteotoxic stress.

Conclusion: The CMA pathway plays an important role in degrading proteotoxic proteins that contain the KFERQ-motif within the heart. Our studies indicate that targeting the CMA pathway has potential to be used as a therapeutic treatment. For example, it can be used as a therapeutic strategy for treating other pathologies beyond CryAB$_{R120G}$ pathology. For example, our preliminary data have shown that many harmful viral proteins synthesized by SARs-CoV2 virus contain the KFERQ-motif required for CMA degradation. Thus, activating the CMA pathway may be a beneficial approach to eliminate the disease-causing proteins and prevent the corresponding pathology.

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