

Supplement Material

Atg7 Induces Basal Autophagy and Rescues Autophagic Deficiency in CryAB^{R120G} Cardiomyocytes.

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Running title: Atg7 Induces Autophagy

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SUPPLEMENTARY METHODS

Western Blot Analyses

To lyse RNCs, growth media was aspirated, plates were washed with 1X PBS, which was then aspirated and the cells were lysed with 250uL Cell Lytic M (Sigma) lysis buffer, supplemented with Complete protease inhibitor cocktail (Roche). After 5 minutes of lysis, cells were scraped and transferred to 1.5 mL tubes and sonicated for 6 seconds at 0.06 watts. The lysates were centrifuged at 14,000 x g for 15 minutes to sediment any insoluble material. The protein content of the soluble lysates was measured using the modified Bradford protocol/reagent relative to a BSA standard curve (BioRad). Equal protein concentrations of each lysate were diluted in 3X SDS loading dye (New England Biolabs). Samples were boiled for 5 minutes prior to loading on SDS-PAGE gels. Proteins were separated on SDS-PAGE gels (BioRad) and transferred to PVDF membranes (BioRad). Membranes were blocked for 1 hour in 5% non-fat dried milk and exposed to primary antibodies overnight. Membranes were then washed in 1X TBS-T, 2 times for 5 minutes and incubated for 1 hour with alkaline-phosphatase-conjugated secondary antibodies (Santa Cruz Biotechnology). Membranes were washed in 1X TBS-T 2 times for 5 minutes and exposed with ECF reagent (Amersham) for up to 5 minutes. Bands were detected on a STORM 820 fluorescent scanner (Molecular Dynamics). The following primary antibodies were used for immunoblotting: anti-LC3B (2775, Cell Signaling), anti-Atg7 CT (3615, ProSci), anti-Atg7 (2631, Cell Signaling), anti-FLAG (F7425, Sigma), anti- α B crystallin (SAP-223, Assay Designs), anti-p62 COOH-terminal (GP62-C, ProGen), Anti Atg5-12 (CAC-TMD-PH-AT5, Cosmo Bio Co), anti-Beclin1 D-18, anti-cathepsin D, C-20, anti-ubiquitin P4D1 and anti LAMP1 1D4B (sc-10086, sc-6486, sc-8017, sc-19992, Santa Cruz Biotechnology) and anti-GAPDH (MAB374, Chemicon). Densitometry on scanned membranes was done using ImageQuant version 5.2 (Molecular Dynamics).

EM analyses

RNCs were cultured for 5 days on 10 cm dishes coated with gelatin. Cells were washed with cardioplegic buffer and fixed in the dishes with 1% paraformaldehyde, 2% glutaraldehyde, 0.05M cacodylate buffer for 15 minutes on ice. The cells were then scraped and pelleted by centrifugation. The pellets were post-fixed in 1% OsO₄ in cacodylate buffer, for 2 hours on ice, then dehydrated in series of acetone washes and embedded in EMBED812 in silicone pyramid tip molds. Ultrathin sections were counterstained with uranium and lead salts and examined with a Hitachi7600 transmission electron microscope. Images were acquired with an AMT digital camera.

Immunohistochemistry

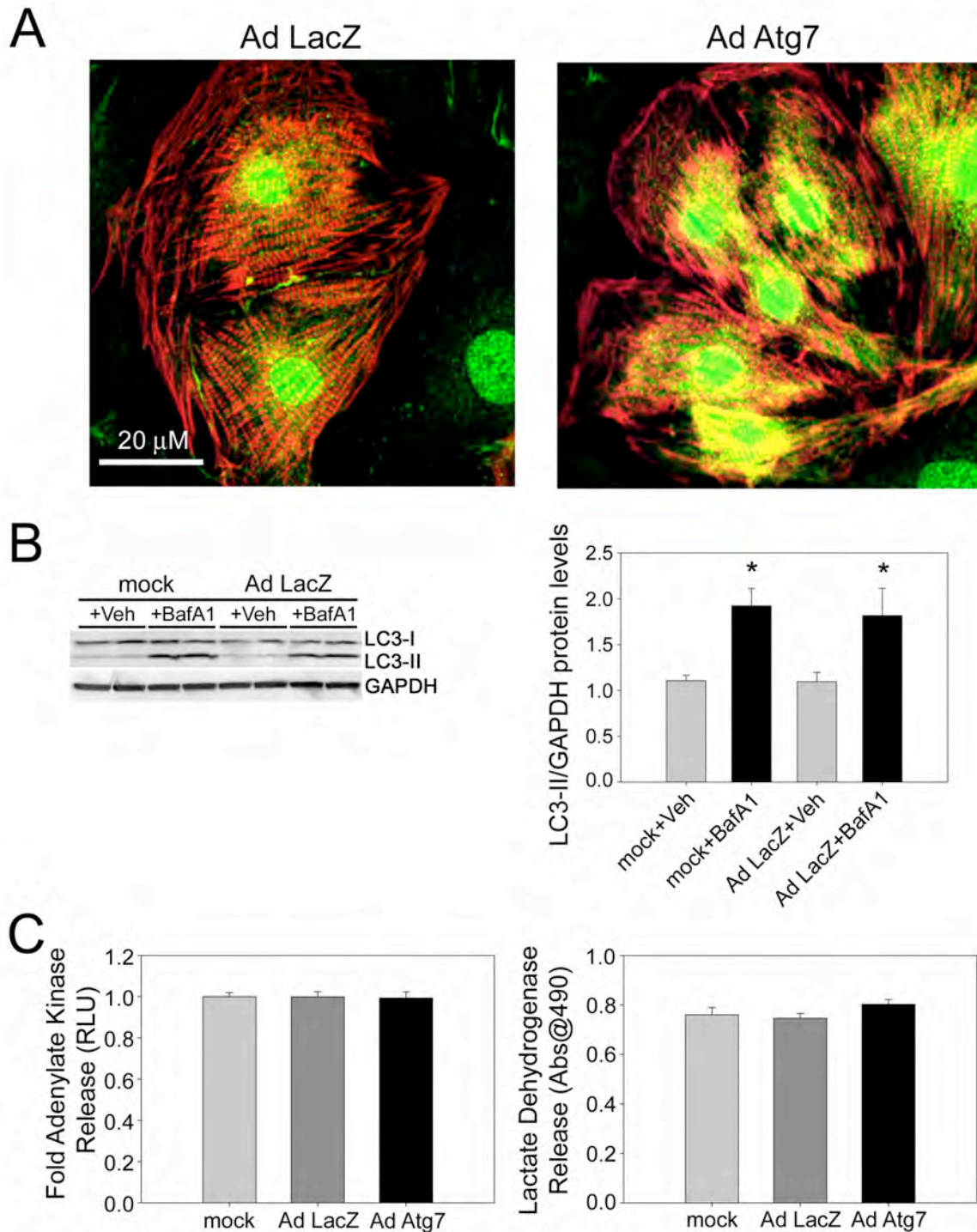
RNCs were grown on 2-well chamber slides and plated at a density of 1×10^5 cells/well (Nalgene). Cells were first washed with 1X PBS, and then fixed for 20 minutes in 4% paraformaldehyde, 0.5% Triton-X 100, in 1X PBS. Fixed cells were washed twice with 1X PBS and then exposed to an antigen retrieval solution of 0.1 mol/L glycine (pH=3.5) for 30 minutes. The retrieval solution-treated cells were then twice washed with 1X PBS. The fixed cells were incubated in blocking solution (1% BSA, 0.1% cold water fish gelatin, 0.1% Tween-20, 0.05% sodium azide in 1X PBS) for 1 hour. Primary antibodies were diluted 1:100 in blocking solution and incubated overnight. The following antibodies were used for immunohistochemistry: anti- α B crystallin (SAP-223, Assay Designs) and anti-A-11/PAO antibody (courtesy of C. Glabe, University of California, Irvine). Cells were washed twice with 1X PBS, followed by incubation with a species-specific secondary antibody –conjugated to Alexa488 (Molecular Probes) for 1 hour. Cardiomyocytes were counterstained using an anti-troponin I antibody (MAB3152, Chemicon) at a 1:1000 dilution for 1 hour, followed by washing and incubation with a goat-anti mouse Alexa568 secondary antibody (Molecular Probes) for one hour. Nuclei were

counterstained using TOPRO 1:500 for 45 minutes (Molecular Probes). Following two final washes with 1X PBS, coverslips were mounted using Vectashield Hard Set mounting media (Vector labs) and allowed to dry for 45 minutes. Slides were kept at 4 °C until visualized by confocal microscopy (PCM 2000, Nikon) with images captured by Simple PCv4 software (Compix Inc). To quantify aggregate and PAO content from fluorescent images, images were captured from each experimental group of 4-8 separate chamber slide wells. Each well had 8-12 images from different regions captured per well. The area of aggregate or PAO staining was quantified relative to the area of cardiomyocyte staining using MetaMorph 7 software (Molecular Devices).

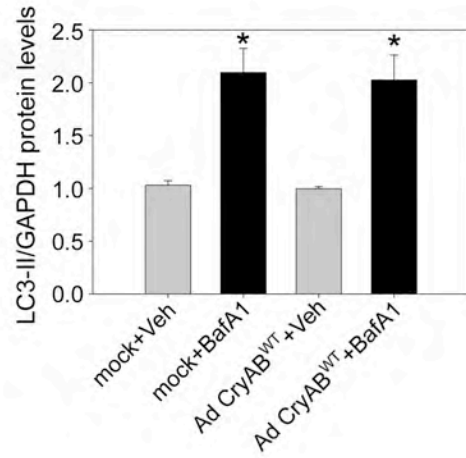
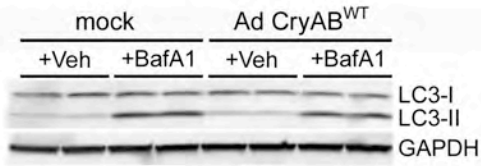
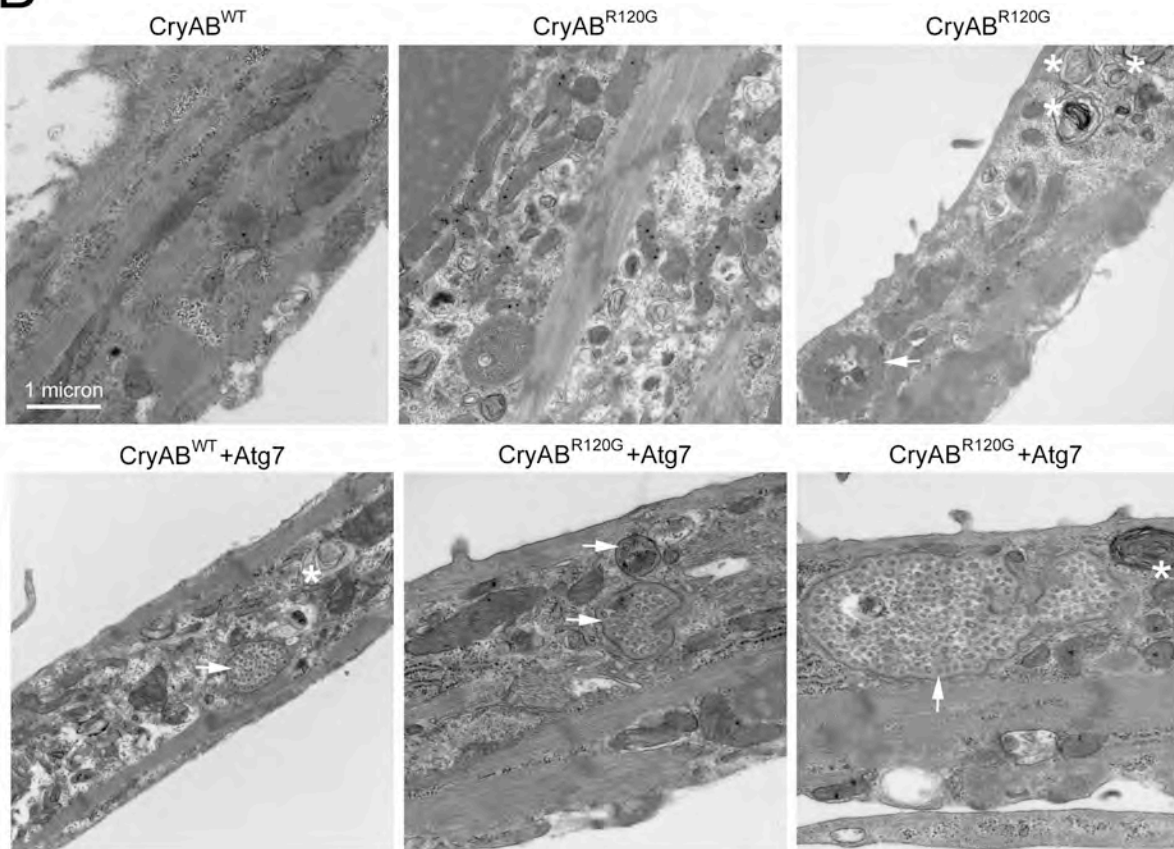
Cytotoxicity assays

Adenylate kinase release into cell culture media was measured using the ToxiLight Bioassay kit (Lonza). The assay luminescence was measured on a MonoLight 3010 luminometer (BD Biosciences). Lactate dehydrogenase release was measured by a Cytotoxicity Detection kit (Roche). Color development was measure using a μ Quant microplate spectrophotometer with an absorbance of 490nm (BioTek Instruments).

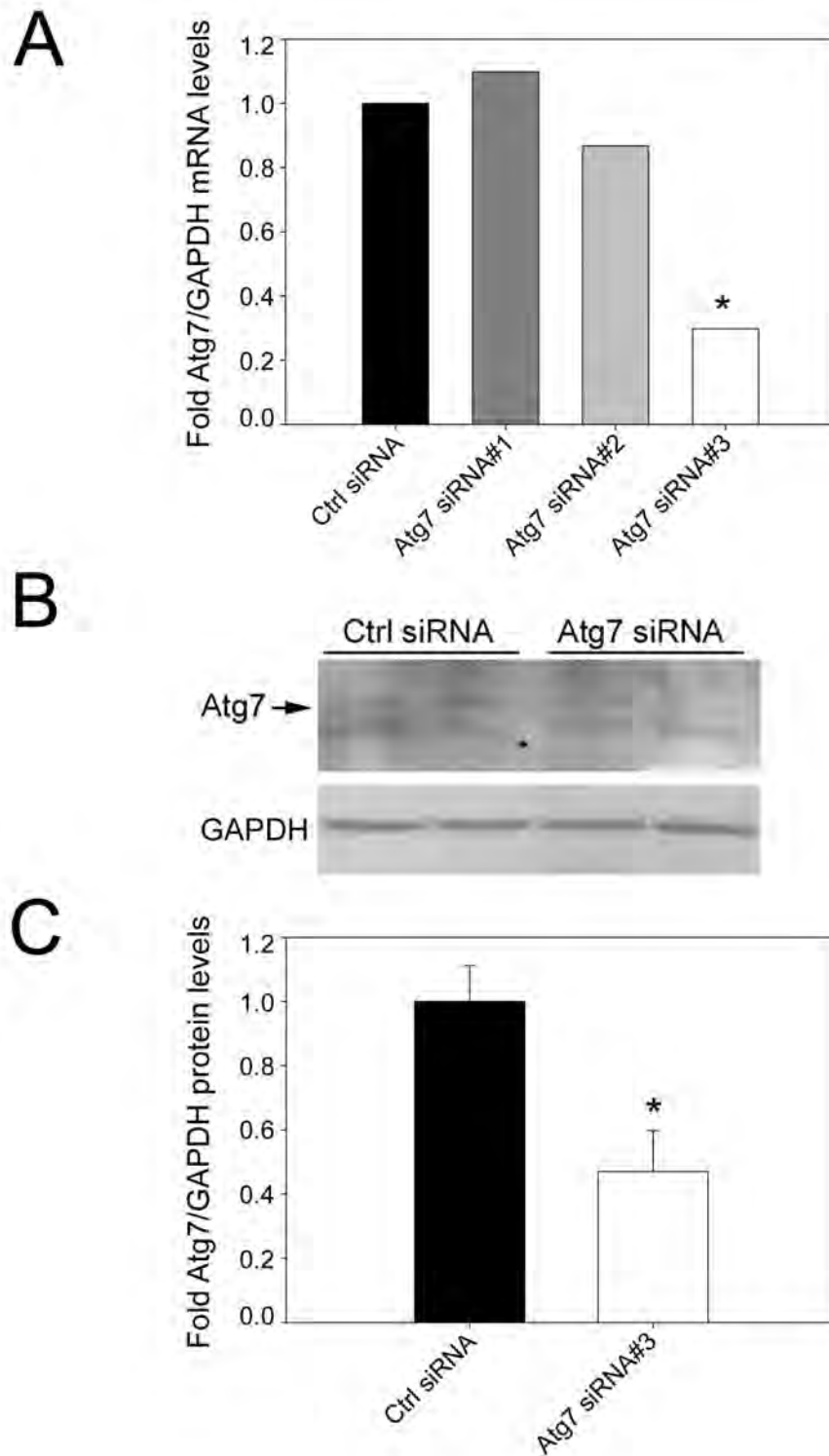
SUPPLEMENTARY FIGURES



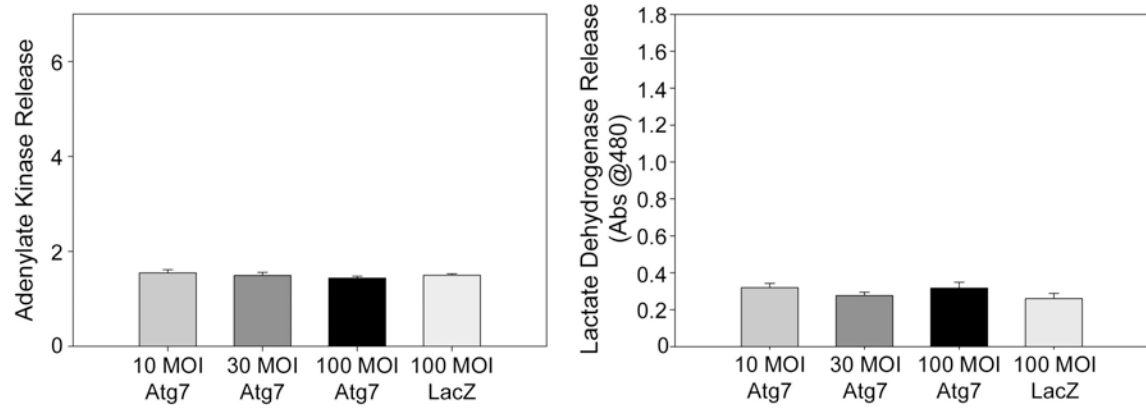
Supplementary Figure 1. A, Atg7 staining (**green**) maintains nuclear and cytoplasmic localization with overexpression. Cardiomyocytes counterstained with TnI antibody (**red**). **B**, Representative blot showing autophagic flux does not differ between AdLacZ and mock-infected cells. * $P < 0.05$ significant difference between Veh (vehicle) and BafA1 treated groups. **C**, Adenylate kinase and lactate dehydrogenase release are unchanged by Atg7 expression at five days post-infection ($n=4/\text{treatment}$).

A**B**

Supplementary Figure 2. A, Representative blot showing that autophagic flux does not differ between mock-infected and AdCryAB^{WT}-infected cells. * $P < 0.05$; significant difference between Veh and BafA1 treated groups. **B**, Atg7 increases the number of autophagic structures visible by EM analysis when co-expressed with CryAB^{WT} and CryAB^{R120G}. >50 fields were randomly scored from both groups and representative fields are shown. Amphisomes denoted by white arrows. Multilamellar bodies denoted by white asterisks.



Supplementary Figure 3. A, Real-time PCR analysis shows that one of three siRNAs targeted to Atg7 effectively reduces Atg7 mRNA levels. * $P < 0.05$ significant difference between Ctrl siRNA and Atg7 siRNA#3. **B,** Representative immunoblot shows Atg7 protein levels are reduced following transfection with Atg7 siRNA. **C,** Plot shows reduced densitometry of in Atg7 protein levels by Atg7 siRNA transfection (n=4/group). * $P < 0.05$ significant difference between Ctrl siRNA and Atg7 siRNA.



Supplementary Figure 4. A, Adenylate kinase and lactate dehydrogenase release are unchanged with increasing Atg7 expression at five days post-infection (n=4/treatment).