Supplemental Materials and Methods

CAP350 silencing and size measurement of HL-1 cells

Accell mouse CAP350 siRNA sequences are: GCGGAAAUUUUAAAGGAAA, CUCUUCGGUUCAAAGGUGA, UGAGCAUGUAGGAAUUUUG, and GGGAGAUGUCUAGAUGAUU (GE Healthcare Dharmacon, Inc., Lafayette, CO). Twenty-four hours after CAP350 siRNA delivery, cells were transduced with AdGFP or AdMuRF1 (MOI 30) for 24 hours, then treated with T3, then harvested 48 hours later. Cells were were lysed in 8M urea buffer or washed three times in DPBS and fixed in 4% formaldehyde for cell size measurement. Fixed cells were mounted with Fluoro-Gel II Mounting media containing DAPI (Electron Microscope Service, Hatfield, PA) and DIC images taken with a Leica confocal microscope. Cell size was measured using Image J software based on standardized pixel adjustment.

TRα mediated Thyroid Response Element (TRE) – Driven Luciferase Activity Assay

After transfection, cells were transduced with GFP or AdMuRF1, followed by treatment with T3 or vehicle as described above. Luciferase activity was detected using the Dual-Light® Luciferase & β-galactosidase Reporter Gene Assay Kit (Applied Biosystems), as described previously (Willis, et al. 2007). Individual wells from 3 independent experiments were evaluated for each group.

Cell Immunoblot Analysis

Protein was transferred to PVDF (EMD Millipore), blocked with 2% Amersham™ ECL™ Prime Blocking Reagent (GE Healthcare Life Sciences, Pittsburgh, PA), and incubated with primary antibodies overnight at 4ºC, followed by washing prior to incubation with horseradish peroxidase (HRP)-linked secondary antibody for one hour at RT. Amersham ECL™ (GE Healthcare Life Sciences) was used to develop blots and chemiluminescence was detected on Amersham™ Hyperfilm ECL™ (GE Healthcare Life Sciences). Densitometry analysis was performed using Quantity One 1-D Analysis Software (Bio-Rad, Hercules, CA).

2.5 Constructs for confocal TRα lysine domain mutants and GST-TRα mapping i.p. studies

Cells were transfected with AdMuRF1 and treated with T3 optimized for the most robust effect on TRα translocation and interaction with MuRF1 and CAP350. Mapping TRα constructs with a GST tag were created as previously reported (Qiu, et al. 2012). Briefly, TRα1-GST-A/B, GST-C, GST-E/F, and full length GST-A/B-C-D-E/F constructs were created by synthesizing the A/B, C, E/F, and full length TRα into the pGEX-KG plasmid (GE Life Sciences, Uppsala, Sweden), expression vector for GST-recombinant protein expression and sequence verified. Validated constructs were used to produce

recombinant proteins corresponding to the nucleotide binding domain (A/B), DNA binding domain (C), and ligand binding domain (E/F), and the full length GST-TRα that were incubated with myc-MuRF1 to determine which domain interacted with MuRF1.

Confocal immunofluorescence analysis

Images were acquired on a FV1000 MPE SIM Laser Scanning Confocal Microscope (Olympus, Center Valley, PA). Co-localization analysis was performed by determining coefficient values for the overlap of two given channels (450, 488, 568, or 647) using Volocity® 3D Images Software Quantitation (PerkinElmer, Waltham, MA), as described previously (Lisby, et al. 2003; Vandenbroucke St Amant, et al. 2012) using the Costes' Pearson's Coefficient correlation statistics function. At least 2 slides from 3 independent wells (where > 15 cardiomyocytes in total were measured) were analyzed. Antibodies used: FLAG-TRα; rabbit (#F7425) or mouse (#F1804) anti-FLAG antibody (F1804) (Sigma-Aldrich, 1:100). myc-MuRF1; rabbit anti-myc or mouse anti-myc (#C3956, C4439, Sigma-Aldrich, 1:100). Endogenous CAP350 (#sc-161481, Santa Cruz Biotechnology, 1:50). Secondary AlexaFluor® anti-goat 488 (#11055), anti-rabbit 568 (#11011), and anti-mouse 568 (#110040) antibodies (Molecular Probes®) and anti-rabbit 647 (#A21443) and anti-mouse 647 (#A21463) (Invitrogen™) were used.

Coimmunoprecipitation

Beads were washed 7 times with the lysis buffer (12.5x bead volume), eluted with 20 µg myc peptide (M2435, Sigma-Aldrich and analyzed by immunoblot. FLAG-TRα was immunoprecipitated using 5% (v/v) EZ View™ Red Anti-FLAG Affinity Gel (Sigma-Aldrich). Lysates were incubated with equilibrated anti-c-myc beads overnight at 4ºC with rotation. Following incubation, beads were recovered by centrifugation at 8,200xg for 30 seconds at 4ºC and washed 7 times with the lysis buffer (12.5x bead volume). After the final wash, immunoprecipitated proteins were eluted using 20 µg FLAG peptide (#F3290, Sigma-Aldrich) at 4ºC for 45 minutes. Eluate was isolated by centrifugation and subsequently analyzed by immunoblot.

Animals, treatment with T3, conscious echocardiography

Blood was allowed to clot at room temperature (RT) in the presence of EDTA, centrifuged at 1,300xg, serum collected and stored at -80°C. In a subset of animals, randomly assigned mice were euthanized and hearts were fixed with 4% paraformaldehyde via perfusion for use in histological studies. All mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of North Carolina and were performed in accordance with federal guidelines.

Echocardiography was performed on conscious mice using a Visual Sonics Vevo 2100 ultrasound biomicroscopy system as previously described (Willis, et al. 2009a; Willis, et al. 2009b).

Tissue immunoblot analysis

Twenty-five micrograms of protein was resolved on 4–12 % NuPAGE gels (Life Technologies, Carlsbad, CA), transferred to Immobilon-polyvinylidene difluoride membrane (Millipore, Billerica, MA), blocked in 5% milk in 1× TBST for 60 min, and incubated with primary antibody overnight at 4°C. After washing, horseradish peroxidase-linked secondary antibody was added to the polyvinylidene difluoride membranes for 1 h at 25°C. Signal was detected using ECL Prime (GE Healthcare Amersham, Buckinghamshire HP7 9NA, United Kingdom), and final immunoblot results were acquired using UVP Imager (UVP, LLC, Upland, CA). Densitometry analysis was performed using UVP Imaging Software (UVP, LLC, Upland, CA).

References

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