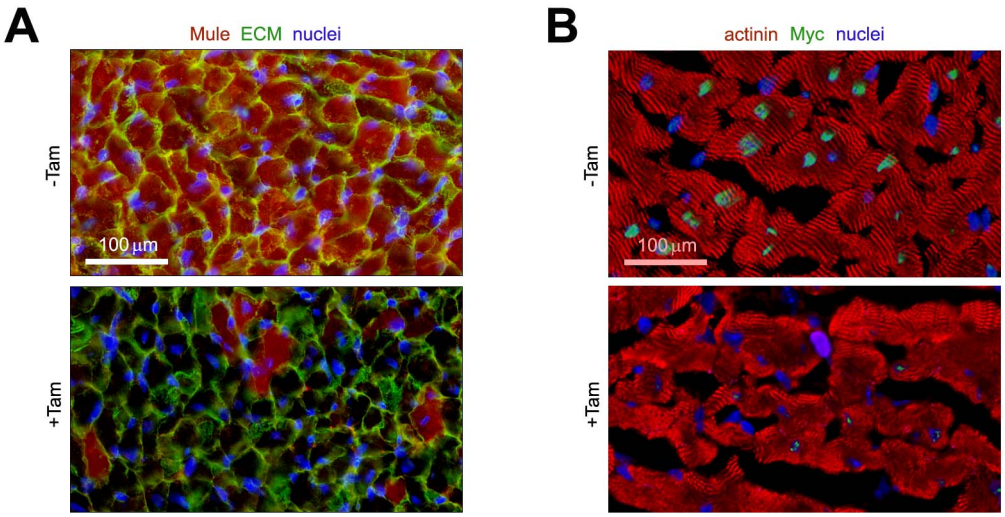
Supplementary Information

The E3 ligase Mule protects the heart against oxidative stress and mitochondrial dysfunction through Myc-dependent inactivation of Pgc-1α and Pink1.

Keith Dadson, Ludger Hauck, Zhenyue Hao, Daniela Grothe, Vivek Rao, Tak W. Mak, and Filio Billia

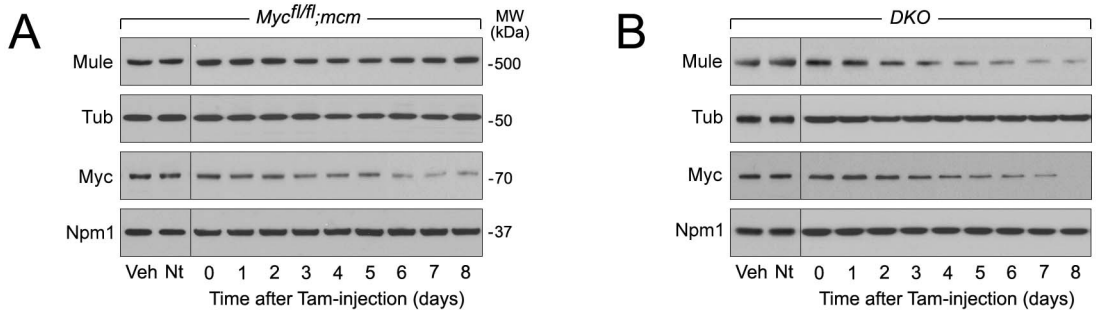


Supplementary Figure S1: Analysis of recombination efficiency.

(A, B) Confocal immunofluorescence microscopical analysis at 7d post Tam shows that four consecutive daily intraperitoneal Tam injections induced genetic ablation of **Mule and Myc** with high recombination efficiency (> 90%). Mice were 12 weeks old at the time of analysis.

(A) Administration of Tam induced marked downregulation of endogenous cytoplasmic Mule expression in left ventricular cardiomyocytes from *Mule*$^{fl/fl(y)}$*;mcm* mice. Specimen were co-stained for immunofluorescence microscopy employing Mule antibodies (red), WGA (green), and Dapi (nuclear DNA; blue).

(B) Administration of Tam significantly decreased expression levels of endogenous nuclear Myc in the heart of *Myc*$^{fl/fl}$*;mcm* mice. Left ventricular samples were treated with anti-Myc and anti-α-actinin antibodies, and co-stained with Dapi. Red, α-actinin. Green, Myc. Blue, nuclei.

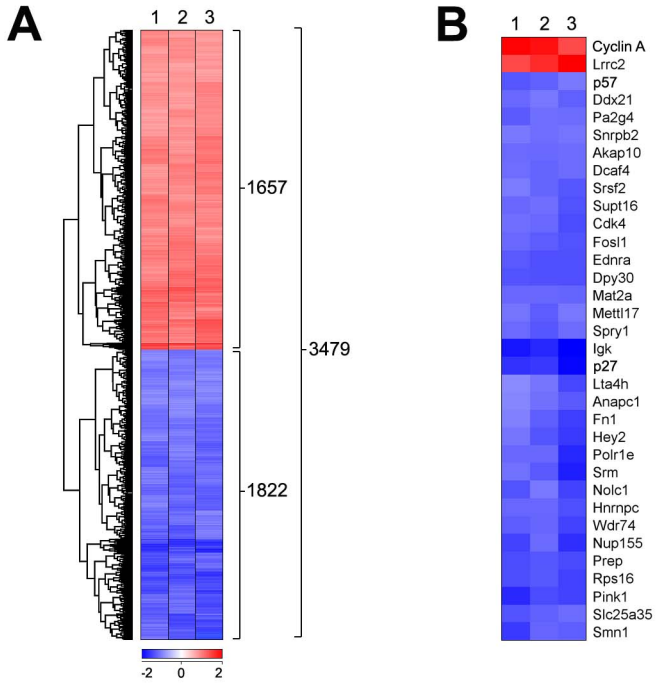


Supplementary Figure S2:

(A) Administration of Tam induced marked downregulation of endogenous nuclear Myc expression in left ventricular (LV) cardiomyocytes from *Myc$^{fl/fl}$;mcm* mice. Immunoblot analysis of Myc in biochemically fractionated LV extracts (60 µg total protein/lane) was done employing specific antibodies as indicated (left).

(B) Administration of Tam significantly decreased expression levels of endogenous cytoplasmic Mule and nuclear Myc in the heart of *Mule$^{fl/fl(y)}$;Myc$^{fl/fl}$;mcm* mice (DKO). LV tissue samples were analyzed by immunoblotting employing antibodies as indicated (left). Western blots were repeated at least once with similar results.

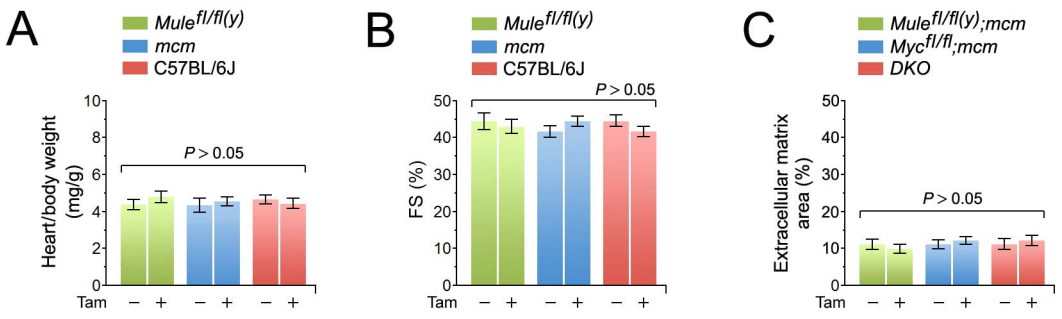
Veh, vehicle injections. Nt, no treatment.



Supplementary Figure S3: Transcriptomic analysis of Mule mutant hearts.

(A) Genome-wide mRNA microarray profiling reveals that conditional genetic ablation of Mule induces profound alterations in the cardiac transcriptome indicating a most unexpected high degree of complexity associated with Mule network regulation in the heart. Heat map of unsupervised hierarchical cluster analysis at a high confidence threshold identifies 3479 transcripts (rows) out of 33561 entities that were enriched in ventricular samples of *Mule*$^{fl/fl(y)}$*;mcm* mice (columns) post-Tam relative to vehicle-injected controls. Mice were analyzed at 4 weeks post-Tam. Values ($\log_2$ expression) are shown by color and intensity of shading. Blue, repressed. Red, induced. $n = 3$ biological replicates. $P < 0.05$. Fold change >1.3 and < −1.3.

(B) Heat map examining the impact of genomic modifications in Mule mutant hearts (columns) on previously validated Myc target genes (rows). The majority of these factors was downregulated in *Mule*$^{fl/fl(y)}$*;mcm* mice post-Tam demonstrating that Myc activation is a consequence of inhibiting Mule, and that Myc is involved in the development of the mutant cardiac phenotype.



Supplementary Figure S4:

(A) Heart-weight corrected for body weight. *n*=4-6. Mice were 12 weeks old at the time of analysis.

(B) Fractional shortening (FS) determined by M-mode echocardiography. *n*=4.

(C) Quantification of extracellular matrix area by immunofluorescence microscopy employing routine wheat germ agglutinin (WGA-) stained cardiac sections. *n*=4

(A-C) Data are means ± s.e.m.

Supplementary Table S1: Primer sequences used in ChIP assays.

Gene Symbol	Forward	Reverse
ATP5a	ACTCGAGGACCCAGGTTTG	CCACGTGAGTAGCAGAACCA
Bckdhb	CTTTAAATCAGTCATTCAACTTGC	CAGACGGAAGCCATACCAAG
Cox4i1	TCTTGGTCTTCCGGTTGC	CACACCTCCTGCGACTAGA
Cox10	CACAGAAATGCTAACCTCATTCC	GGAGCTTATAAAGATCGGATGG
Esrrb	CCGGGAGCTTATGTCAACTG	AGAGAGCCGGTGAGTACAGG
Got1	ACAAAACGCCCAAGTACAGG	GTGGGGAAAGCAGAGAACAG
Gstm1	GGACAGTGTTCTTGGGAGGA	ACCAGAGCAGCAGACCCTAA
Hk2	GGAACACACGTCCCAACTCT	AGTCGGGGCCAAATAGAGAA
Ndufa4	GCCAGTGAAAGTCAGGAAGC	GTCACCCAGGACCTCCTACC
Ndufa8	TGAGCCATCTCTCCAGCTCT	GCCATTCCAGGAAGTCTAGC
Pgc-1a	TGTGGCGGTTTTGTTGACTA	TCCCCAGTCACATGACAAAG
Pink1	CAAGCAGTTTAAGTCATAGGGATTT	TGGGACAACAACAAACTTCG
Ppara	CCTAGGGGGCGGAGTTTC	CAGGGGACAACCAGAGGAC
Pparg	CAGCACCAACCACCTGTAAC	GTGCTGTGCGTCGGTGAG

Supplementary Table S2: Primer sequences used in RT-qPCR assays.

Gene Symbol	Forward	Reverse
Acta1	CCCAAAGCTAACCGGGAGAAG	CCAGAATCCAACACGATGCC
Acta2	GTCCCAGACATCAGGGAGTAA	TCGGATACTTCAGCGTCAGGA
β-actin	GAAATCGTGCGTGACATCAAAG	TGTAGTTTCATGGATGCCACAG
Actn1	GACCATTATGATTCCCAGCAGAC	CGGAAGTCCTCTTCGATGTTCTC
Actn4	ATGGTGGACTACCACGCAG	CAGCCTTCCGAAGATGAGAGT
ANP	GCTTCCAGGCCATATTGGAG	GGGGGCATGACCTCATCTT
ATP5a1	TCTCCATGCCTCTAACACTCG	CCAGGTCAACAGACGTGTCAG
ATP5i2	TGCCGAGCTGGATAATGATGC	ACCATGCTAATCCCCGAGATG
B2m	TTCTGGTGCTTGTCTCACTGA	CAGTATGTTCGGCTTCCCATTC
Bckdhb	AGCTATTGCGGAAATCCAGTTT	ACAGTTGAAAAGATCACCTGAGC
Bckdk	ACATCAGCCACCGATACACAC	GAGGCGAACTGAGGGCTTC
BNP	GAGGTCACTCCTATCCTCTGG	GCCATTTCCTCCGACTTTTCTC
Cat	AGCGACCAGATGAAGCAGTG	TCCGCTCTCTGTCAAAGTGTG
Cox4i1	ATTGGCAAGAGAGCCATTTCTAC	CACGCCGATCAGCGTAAGT
Cox10	AGAAGAGCTATACAGGGATTGCC	CTGTGTGACATACATGCGCTT
Cytb	TTCTGAGGTGCCACAGTTATT	GAAGGAAAGGTATTAGGGCTAAA
Got1	GCGCCTCCATCAGTCTTTG	ATTCATCTGTGCGGTACGCTC
Gpi1	TCAAGCTGCGCGAACTTTTTG	GGTTCTTGGAGTAGTCCACCAG
Gpt2	AACCATTCACTGAGGTAATCCGA	GGGCTGTTTAGTAGGTTTGGGTA
Gsta3	AAGAATGGAGCCTATCCGGTG	CCATCACTTCGTAACCTTGCC
Gstm1	ATACTGGGATACTGGAACGTCC	AGTCAGGGTTGTAACAGAGCAT
Gstp1	ATGCCACCATACACCATTGTC	GGGAGCTGCCCATACAGAC
Gstp2	CATTACAGGGCAGCAGGAGT	CATATGGCTCCCTCTTGTCC
Hk1	AGGGCGCATTACTCCAGAG	CCCTGTGGGTGTCTTGTGTG
Hk2	TGATCGCCTGCTTATTCACGG	AACCGCCTAGAAATCTCCAGA

Supplementary Table S2: Primer sequences used in RT-qPCR assays (continued).

Gene Symbol	Forward	Reverse
α-MHC	GCCCAGTACCTCCGAAAGTC	GCCTTAACATACTCCTCCTTGTC
α-MHC	GCCCAGTACCTCCGAAAGTC	GCCTTAACATACTCCTCCTTGTC
β-MHC	ACTGTCAACACTAAGAGGGTCA	TTGGATGATTTGATCTTCCAGGG
Ndufa4	TCCCAGCTTGATTCCTCTCTT	GGGTTGTTCTTTCTGTCCCAG
Ndufa8	GGAGCTGCCAACTCTGGAAG	CCAGCGGCACAGCATAAAC
Ndufv1	TTTCTCGGCGGGTTGGTTC	GGTTGGTAAAGATCCGGTCTTC
Pgc-1α	TATGGAGTGACATAGAGTGTGCT	CCACTTCAATCCACCCAGAAAG
Pgc-1β	TCCTGTAAAAGCCCGGAGTAT	GCTCTGGTAGGGGCAGTGA
Pink1	TTCTTCCGCCAGTCGGTAG	CTGCTTCTCCTCGATCAGCC
Pparα	AGAGCCCCATCTGTCCTCTC	ACTGGTAGTCTGCAAAACCAAA
Pparγ	TCGCTGATGCACTGCCTATG	GAGAGGTCCACAGAGCTGATT
Tgm2	GACAATGTGGAGGAGGGATCT	CTCTAGGCTGAGACGGTACAG
Tnnc1	GCGGTAGAACAGTTGACAGAG	CCAGCTCCTTGGTGCTGAT
Tnnt1	CCTGTGGTGCCTCCTTTGATT	TGCGGTCTTTTAGTGCAATGAG

Supplementary Table S3: Antibodies employed for ChIP, IF, IP and WB.

Gene name	Catalog No.	Vendor	Application	Dilution Factor
α-actinin, sarcomeric	A7811	Sigma	IF	50
Atp5a	ab14748	Abcam	WB	1000
Atp5b	ab14730	Abcam	WB	1000
BrdU	MCA2060	AbD Serotec	IF	50
Cat	14097	Cell Signaling	WB	1000
Complex I	ab109798	Abcam	IP	5 μg
Complex IV	ab109863	Abcam	IP	5 μg
Cox4i1	ab16056	Abcam	WB	1000
Cox10	ab84053	Abcam	WB	1000
CytC	ab13575	Abcam	WB	1000
Gpdh1	ab87230	Abcam	WB	1000
Gst1	ab53940	Abcam	WB	1000
Gstm1	ab178684	Abcam	WB	1000
Pink1	3929-100	BioVision	WB	1000
Prdx1	8499	Cell Signaling	WB	1000
Sod2	13194	Cell Signaling	WB	1000
Mef2a	ab32866	Abcam	IF	50
Mule	Ab70161	Abcam	IF WB	50/1000
Myc	sc-764	Santa Cruz	IF IP WB ChIP	50 - 7.5μg 1000 - 7.5 μg
Ndufa4	ab129752	Abcam	WB	1000
Ndufa8	BS3336	BioWorld	WB	1000
normal rabbit IgG	2729	Cell Signaling	CHIP	7.5 μg
Npm1	B0556	Sigma	WB	1000
Opa1	ab42364	Abcam	WB	1000
Pgc-1α	3934-100	BioVision	WB	1000
Pparα	PAB11321	Abnova	WB	1000
Pparγ	PAB11321	Abnova	WB	1000
Sdhb	ab14714	Abcam	WB	1000
ubiquitin	13-1600	Thermo Fisher	WB	7.5 μg
tubulin	2146	Cell Signaling	WB	1000

ChIP, chromatin immunoprecipitation. IF, immunofluorescence microscopy. IP, immunoprecipitation. WB, Western blot.

Supplementary Table S4: Patient characteristics of human LV samples as in Fig. 1A.

Etiology of HF syndrome	Age (years)	Sex
Familial DCM	47	Female
Familial DCM	50	Female
Idiopathic DCM	51	Female
Idiopathic DCM	47	Male
Idiopathic DCM	24	Female
Idiopathic DCM	55	Male
Idiopathic DCM	48	Male
Idiopathic DCM	59	Male
Idiopathic DCM	60	Female
Idiopathic DCM	71	Female
Idiopathic DCM	64	Male
Idiopathic DCM	61	Male
Idiopathic DCM	44	Female
Idiopathic DCM	24	Female
Idiopathic DCM	26	Male
Idiopathic DCM	58	Male
Idiopathic DCM	56	Female
Idiopathic DCM	46	Male
Congenital CM	16	Female
Congenital CM	31	Male
Congenital CM	57	Female
Ischemic CM	46	Male
Ischemic CM	45	Male
Ischemic CM	61	Male
Ischemic CM	69	Male
Ischemic CM	58	Male
Ischemic CM	53	Male
Ischemic CM	51	Male
Ischemic CM	67	Male

Informed consent was obtained from patients with end-stage HF who were to have a left ventricular assist device inserted as either a bridge to a heart transplant or as destination therapy to end of life. Samples were obtained from the LV apex and frozen in liquid nitrogen at the time of procurement.

Supplementary Table S4: Patient characteristics of human LV samples as in Fig. 1A (continued).

Etiology of HF syndrome	Age (years)	Sex
Ischemic CM	44	Male
Ischemic CM	59	Male
Ischemic CM	62	Male
Ischemic CM	66	Male
Ischemic CM	58	Male
Myocarditis	57	Male
Myocarditis	50	Male
Myocarditis	41	Male
Chemotherapy-induced CM	55	Male
Chemotherapy-induced CM	51	Female
Chemotherapy-induced CM	36	Male
Chemotherapy-induced CM	62	Female
Control	24	Male
Control	21	Male
Control	27	Male
Control	26	Male
Control	21	Male
Control	24	Male
Control	27	Male
Control	24	Male

Informed consent was obtained from patients with end-stage HF who were to have a left ventricular assist device inserted as either a bridge to a heart transplant or as destination therapy to end of life. Samples were obtained from the LV apex and frozen in liquid nitrogen at the time of procurement.