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# Mitochondrial Reprogramming Induced by CaMKII $\delta$ Mediates Hypertrophy Decompensation

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**Rationale:** Sustained activation of G $\alpha$ q transgenic (Gq) signaling during pressure overload causes cardiac hypertrophy that ultimately progresses to dilated cardiomyopathy. The molecular events that drive hypertrophy decompensation are incompletely understood. Ca<sup>2+</sup>/calmodulin-dependent protein kinase II  $\delta$  (CaMKII $\delta$ ) is activated downstream of Gq, and overexpression of Gq and CaMKII $\delta$  recapitulates hypertrophy decompensation.

**Objective:** To determine whether CaMKII $\delta$  contributes to hypertrophy decompensation provoked by Gq.

**Methods and Results:** Compared with Gq mice, compound Gq/CaMKII $\delta$  knockout mice developed a similar degree of cardiac hypertrophy but exhibited significantly improved left ventricular function, less cardiac fibrosis and cardiomyocyte apoptosis, and fewer ventricular arrhythmias. Markers of oxidative stress were elevated in mitochondria from Gq versus wild-type mice and respiratory rates were lower; these changes in mitochondrial function were restored by CaMKII $\delta$  deletion. Gq-mediated increases in mitochondrial oxidative stress, compromised membrane potential, and cell death were recapitulated in neonatal rat ventricular myocytes infected with constitutively active Gq and attenuated by CaMKII inhibition. Deep RNA sequencing revealed altered expression of 41 mitochondrial genes in Gq hearts, with normalization of  $\approx$ 40% of these genes by CaMKII $\delta$  deletion. Uncoupling protein 3 was markedly downregulated in Gq or by Gq expression in neonatal rat ventricular myocytes and reversed by CaMKII $\delta$  deletion or inhibition, as was peroxisome proliferator-activated receptor  $\alpha$ . The protective effects of CaMKII $\delta$  inhibition on reactive oxygen species generation and cell death were abrogated by knock down of uncoupling protein 3. Conversely, restoration of uncoupling protein 3 expression attenuated reactive oxygen species generation and cell death induced by CaMKII $\delta$ . Our in vivo studies further demonstrated that pressure overload induced decreases in peroxisome proliferator-activated receptor  $\alpha$  and uncoupling protein 3, increases in mitochondrial protein oxidation, and hypertrophy decompensation, which were attenuated by CaMKII $\delta$  deletion.

**Conclusions:** Mitochondrial gene reprogramming induced by CaMKII $\delta$  emerges as an important mechanism contributing to mitotoxicity in decompensating hypertrophy. (*Circ Res.* 2015;116:e28-e39. DOI: CIRCRESAHA.116.304682.)

**Key Words:** calcium-calmodulin-dependent protein kinase type 2 ■ G-protein ■ Gq ■ heart failure ■ mitochondrial uncoupling protein 3 ■ oxidative stress

Myocardial hypertrophy is the evolutionarily conserved cardiac reaction to hemodynamic overload or injury. This genetically programmed response improves ventricular ejection performance by restoring a more normal ratio of left ventricular wall thickness to intracavitary pressure, 2 determinants of wall stress.<sup>1</sup> Although initially compensatory, reactive hypertrophy inevitably decompensates and fails. Thus, cardiac hypertrophy is an independent risk factor for both heart failure and death.<sup>2,3</sup>

The mechanistic underpinnings of hypertrophy decompensation are poorly understood. The genetic growth program in adult hearts recapitulates growth of the embryonic heart and includes a reversion to fetal muscle isoforms, alterations in calcium handling proteins, and a lowered threshold for programmed cell death that may ultimately prove detrimental to the adult heart.<sup>4</sup> Because it would be therapeutically beneficial to retain the physical features of cardiac hypertrophy that

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**Nonstandard Abbreviations and Acronyms**

|                                 |   |
|---------------------------------|---|
| <b>Ad-Q209L</b>                 | adenoviral expression of constitutively active G $\alpha$ q |
| <b>CaMKII</b>                   | Ca <sup>2+</sup> /calmodulin-dependent protein kinase II    |
| <b>GqTG</b>                     | G $\alpha$ q transgenic                                     |
| <b>Gq/KO</b>                    | Gq mice in a CaMKII $\delta$ knockout background            |
| <b>KO</b>                       | CaMKII $\delta$ knockout                                    |
| <b>NRVMs</b>                    | neonatal rat ventricular myocytes                           |
| <b>PPAR-<math>\alpha</math></b> | peroxisome proliferator-activated receptor $\alpha$         |
| <b>ROS</b>                      | reactive oxygen species                                     |
| <b>siRNA</b>                    | small interfering RNA                                       |
| <b>TAC</b>                      | transverse aortic constriction                              |
| <b>UCP3</b>                     | uncoupling protein 3  |
| <b>WT</b>                       | wild type   |

lower wall stress if they could be dissociated from deleterious collateral effects, the molecular dissection of hypertrophy signaling pathways has long been a goal. A central pathway transducing reactive hypertrophy signaling has been linked to the heterotrimeric G protein G $\alpha$ q transgenic (Gq), which is stimulated by prohypertrophic hormones such as angiotensin II, endothelin, and  $\alpha$ -adrenergic agonists. Accordingly, mice with cardiomyocyte-specific overexpression of the catalytically active  $\alpha$  subunit of Gq develop marked cardiac hypertrophy that recapitulates hypertrophy development and subsequent decompensation.<sup>5</sup> Conversely, G $\alpha$ q/G $\alpha$ <sub>11</sub> knockout mice are unable to develop hypertrophy in response to pressure overload.<sup>6</sup>

Gq signaling has been linked to increases in intracellular calcium, suggesting the Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) as a potential downstream signaling effector of Gq-mediated hypertrophy and heart failure.<sup>7</sup> Indeed, myocardial overexpression of the predominant cardiac CaMKII isoform (CaMKII $\delta$ ), like overexpression of G $\alpha$ q, is sufficient to induce hypertrophy that transitions to severe cardiac dysfunction.<sup>8</sup> Conversely, inhibition or genetic ablation of CaMKII $\delta$  prevents cardiac dysfunction after transverse aortic constriction (TAC), myocardial infarction, or ischemia/reperfusion.<sup>9–12</sup> The ability of CaMKII to phosphorylate key calcium handling proteins was initially considered to be the major driver underlying its involvement in cardiac disease, but normalization of CaMKII-induced changes in sarcoplasmic reticulum calcium proved insufficient to prevent heart failure induced by CaMKII $\delta$  overexpression.<sup>13</sup>

Gq-induced cardiac decompensation has been associated with mitochondrial dysfunction and increased mitochondrial reactive oxygen species (ROS) generation.<sup>14–16</sup> Adverse effects of CaMKII have also been shown to be associated with altered mitochondrial calcium handling and oxidative phosphorylation.<sup>13,17,18</sup> These observations raise the possibility that CaMKII $\delta$  might serve as the downstream mediator of the maladaptive mitochondrial effects of Gq signaling. Were this the case, combined manipulation of Gq and CaMKII $\delta$  would shed light on the specific molecular mechanisms by which Gq stimulates and CaMKII $\delta$  mediates hypertrophy decompensation. We demonstrate here that CaMKII $\delta$  deletion attenuates the maladaptive cardiac effects of Gq, including mitochondrial dysfunction, by

modulating the expression of nuclear-encoded mitochondrial genes. We suggest that CaMKII $\delta$ -induced repression of uncoupling protein 3 (UCP3) is one of the drivers of mitochondrial dysfunction and decompensation to heart failure.

**Methods**

A more detailed description of the methods has been included as an Online Data Supplement.

**Animal Models**

The generation of G $\alpha$ q-40 transgenic mice and CaMKII $\delta$  knockout mice has been described previously.<sup>5,9</sup> To study the role of CaMKII $\delta$  in G $\alpha$ q-induced heart failure, Gq mice were crossed into a CaMKII $\delta$  knockout background, to yield wild type (WT), CaMKII $\delta$  knockout (KO), Gq, and Gq mice in a CaMKII $\delta$  knockout background (Gq/KO). Studies involving pressure overload used the recently generated cardiac-specific CaMKII $\delta$  knockout mice.<sup>12</sup>

**RNA Sequencing**

Left ventricular RNA was isolated from 4 mice from each group and processed for RNA sequencing studies as previously described.<sup>19</sup>

**Mitochondrial Bioenergetics**

Mouse heart mitochondria were isolated by differential centrifugation, and respiration was measured using a Seahorse XF24 analyzer as previously described.<sup>20</sup>

**Biochemical and Histological Analysis**

Tissue fractionation, RNA isolation, and Western blot analysis were performed as described previously.<sup>9,21</sup> Cardiac mitochondrial protein carbonyl was measured using OxiSelect protein carbonyl ELISA kit (Cell Biolabs, San Diego, CA).

**Neonatal Ventricular Cardiomyocyte Culture and Live Cell Imaging**

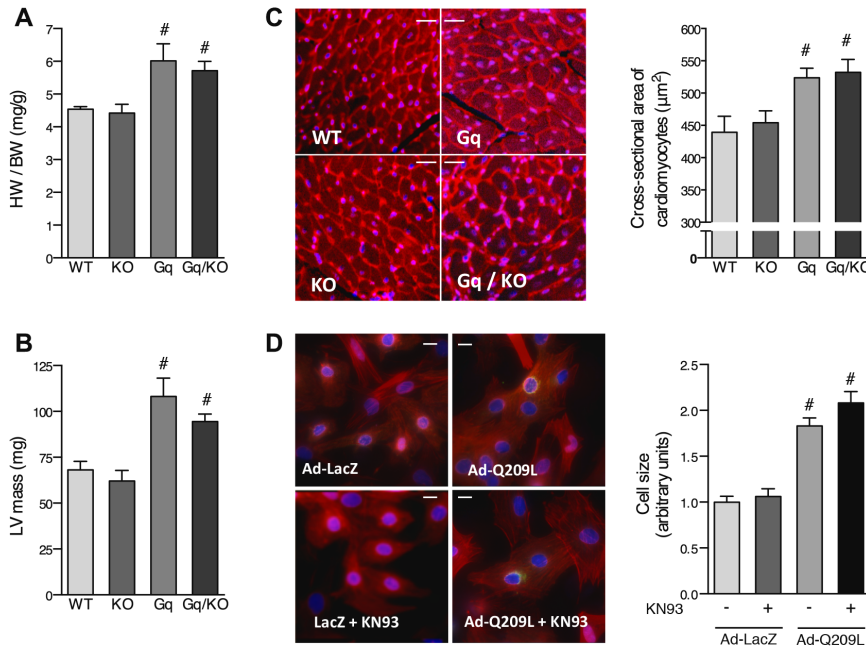
Neonatal rat ventricular myocytes (NRVMs) were isolated, cultured, infected, and transfected as described previously.<sup>21</sup>

**Statistical Analysis**

All data are presented as mean $\pm$ SEM. Comparisons between groups were performed using the Student *t* test, the Mann–Whitney *U* test, Kruskal–Wallis test, or 1-way ANOVA, followed by the Tukey post hoc test, where appropriate. A *P* value <0.05 was considered statistically significant.

**Results****CaMKII $\delta$  Deletion Does Not Affect G $\alpha$ q-Induced Cardiac Hypertrophy**

Cardiac hypertrophy and depressed contractile performance induced by G $\alpha$ q overexpression have been thoroughly described.<sup>5</sup> Consistent with previous reports, gravimetric, echocardiographic, and histological analysis revealed significant increases in left ventricular mass and histological cardiomyocyte cross-sectional area in Gq mice compared with WT mice (Figure 1A–1C). We also observed activation of CaMKII $\delta$  in the Gq compared with WT mouse heart, as indicated by increased CaMKII autophosphorylation and oxidation as well as by increased phosphorylation of phospholamban at threonine 17, a CaMKII-specific site (Online Figure I). Genetic ablation of CaMKII $\delta$  did not diminish Gq-induced cardiac hypertrophy as assessed by comparison of Gq and Gq/KO mice on multiple readouts (Figure 1A–1C). Likewise, hypertrophy of NRVMs induced by adenoviral expression of constitutively active G $\alpha$ q (Ad-Q209L) and

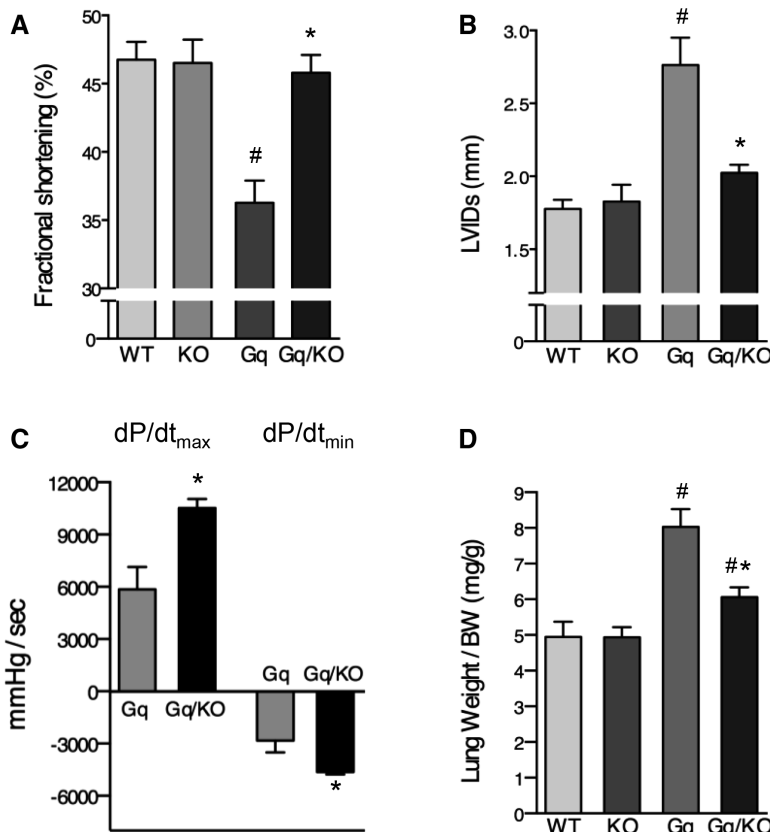


**Figure 1.  $Ca^{2+}$ /calmodulin-dependent protein kinase II  $\delta$  (CaMKII $\delta$ ) is not required for Gq $\alpha$  transgenic (Gq)-induced cardiac hypertrophy.** A and B, Gravimetric and echocardiographic indices of left ventricular (LV) hypertrophy. Shown are heart weight/body weight ratio (HW/BW, A, n=6–9) and LV mass (B, n=7 for wild type [WT], n=5 for CaMKII $\delta$  knockout [KO], n=9 for Gq, and n=8 for Gq mice in a CaMKII $\delta$  knockout background [Gq/KO]). C, LV sections stained with rhodamine-labeled wheat germ agglutinin (red) and 4',6-diamidino-2-phenylindole (DAPI, blue). Scale bar is 20  $\mu$ m. Bar graph depicts the cross-sectional area of cardiomyocytes expressed in  $\mu$ m<sup>2</sup>. D, Neonatal rat ventricular myocytes stained with phalloidin (red), atrial natriuretic factor (green), or DAPI (blue) after infection with an adenovirus expressing constitutively activated Gq $\alpha$  (Ad-Q209L) or  $\beta$ -galactosidase (Ad-LacZ) and cultured in the presence or absence of the CaMKII inhibitor KN93. Scale bar is 10  $\mu$ m. Bar graphs represent average cardiomyocyte circumference 24 hours after infection. All values are expressed as mean $\pm$ SEM. #*P*<0.05 vs WT or Ad-LacZ.

demonstrated by increased cardiomyocyte size and ANF (atrial natriuretic factor) immunostaining (Figure 1D) was unaffected by pharmacological blockade of CaMKII with KN93. Thus, the ability of Gq to elicit genetic and morphological changes characteristic of cardiomyocyte hypertrophy does not depend on CaMKII activation.

### CaMKII $\delta$ Deletion Prevents Functional Decompensation in Gq Mice

Compared with WT littermates, 8-week-old Gq mice had reduced fractional shortening (Figure 2A) and left ventricular systolic dilatation (Figure 2B), decreased load-independent ventricular contractility and relaxation indices (Figure 2C),



**Figure 2.  $Ca^{2+}$ /calmodulin-dependent protein kinase II  $\delta$  (CaMKII $\delta$ ) knockout prevents Gq $\alpha$  transgenic (Gq)-induced cardiac dysfunction.** A and B, Echocardiographic indices of left ventricular (LV) function and LV dilatation. Shown are fractional shortening (A) and LV internal systolic diameter (LVIDs, B; n=7 for wild type [WT], n=5 for CaMKII $\delta$  knockout [KO], n=9 for Gq, and n=8 for Gq mice in a CaMKII $\delta$  knockout background [Gq/KO]). C, Indices of cardiac contractility and relaxation. Shown are maximal rates of LV pressure increase ( $dP/dt_{max}$ ) and decrease ( $dP/dt_{min}$ ), n=4 per group. D, Lung weight/body weight (BW) ratio, an indicator of the severity of functional decompensation (n=6–10 per group). All values are expressed as mean $\pm$ SEM. #*P*<0.05 vs WT; \**P*<0.05 vs Gq.

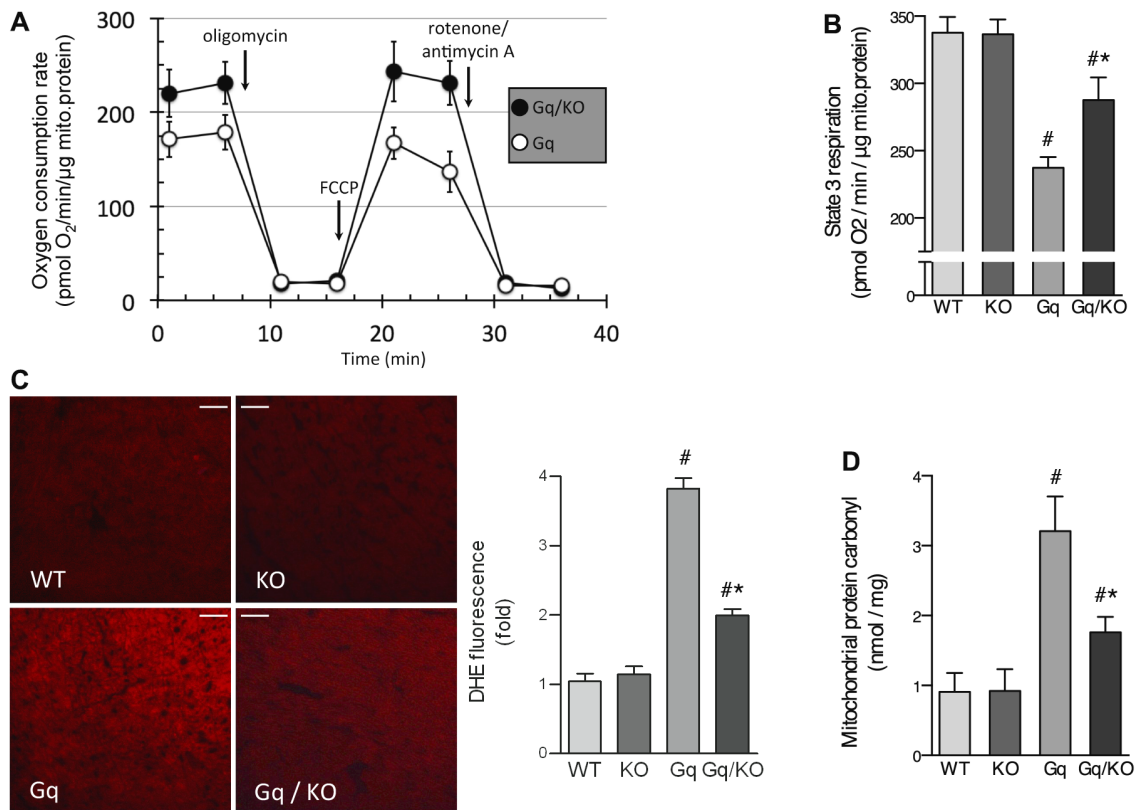
and increased left ventricular filling pressures (Online Figure IIA). Another determinant of functional decompensation, lung weight/body weight ratios, was also significantly increased in Gq mice (Figure 2D). All of these changes were ameliorated by deletion of CaMKII $\delta$  (Figure 2A–2D; Online Figure IIA). Other characteristic heart failure–associated phenotypes of Gq mice, including cardiomyocyte apoptosis, fibrosis, and ventricular arrhythmias, were likewise improved by CaMKII $\delta$  ablation (Online Figure IIB–IIE). Thus, while structural Gq-stimulated cardiac hypertrophy was maintained in the face of CaMKII $\delta$  deficiency (Figure 1), abrogation of CaMKII $\delta$  prevented the associated cardiac decompensation (Figure 2).

### CaMKII $\delta$ Deletion Attenuates Mitochondrial Dysfunction and Mitochondrial Oxidative Stress

The cardiomyopathy of pressure overload is mediated, in part, through disruption of the electron transport chain and increased mitochondrial oxidative stress.<sup>4</sup> To determine whether Gq signaling affects mitochondrial function in a CaMKII-dependent manner, we isolated mitochondria from the 4 mouse lines and measured their oxygen consumption rate drive (Figure 3A). Pyruvate-driven respiration was 40% lower in ventricular

mitochondria isolated from Gq than in those isolated from WT mice (Figure 3B). This impairment was significantly reversed in mitochondria isolated from the Gq/KO mice (Figure 3B). A similar improvement in mitochondrial respiration in Gq/KO mice was observed with the other complex I substrates palmitoylcarnitine and glutamate, whereas respiration on the complex II substrate succinate was not different in Gq/KO versus Gq mice (data not shown). State 4 respiration (oligomycin) and respiratory control ratios were comparable between all 4 mouse lines. Together these data indicate that the effect of CaMKII $\delta$  on complex I contribute to impaired mitochondrial respiration induced downstream of Gq.

To more directly link Gq and CaMKII signaling to mitochondrial function, we examined changes in mitochondrial oxidative stress. ROS were assessed by dihydroethidium fluorescence staining in ventricular sections from hearts of all 4 genotypes. Dihydroethidium fluorescence was markedly increased in Gq mice and was reduced  $\approx$ 50% by concomitant CaMKII $\delta$  deletion (Figure 3C). To confirm data obtained using dihydroethidium fluorescence changes, we evaluated oxidative damage to mitochondrial proteins by quantifying protein carbonyls in isolated mitochondria. In line with



**Figure 3. Ca<sup>2+</sup>/calmodulin-dependent protein kinase II  $\delta$  (CaMKII $\delta$ ) knockout prevents mitochondrial dysfunction and mitochondrial reactive oxygen species (ROS) production in Gq transgenic (Gq) mice.** **A**, Representative experiment to depict the approach for measurement of rates of pyruvate/malate-driven oxygen consumption by isolated cardiac mitochondria from Gq transgenic mice and Gq mice in a CaMKII $\delta$  knockout background (Gq/KO) animals. ADP is present for the first 2 measurements, followed by measurement of state 4 and then uncoupler-stimulated respiration indicating diminished maximal rates of state 3 and uncoupler-stimulated respiration in the GqTg mitochondria. **B**, Bar graph depicting differences in maximal state 3 respiration on pyruvate/malate in all experimental groups ( $n=4$  for wild type [WT] and CaMKII $\delta$  knockout [KO] and  $n=6$  for Gq and Gq/KO). **C**, Cryopreserved myocardial sections were stained with dihydroethidium (DHE), an indicator of total myocardial ROS. Scale bar is 60  $\mu$ m. Bar graph depicts average DHE fluorescence in the different experimental groups ( $n=5-7$  per group). **D**, Carbonylation of mitochondrial proteins in the different experimental groups as measured by ELISA of cardiac mitochondrial homogenates ( $n=6-8$  per group). All values are expressed as mean $\pm$ SEM. # $P<0.05$  vs WT; \* $P<0.05$  vs Gq.

previous observations,<sup>15</sup> mitochondrial protein carbonyls were increased 3-fold in the Gq mouse heart (Figure 3D). Remarkably, this increase was significantly attenuated in Gq/KO mice (Figure 3D).

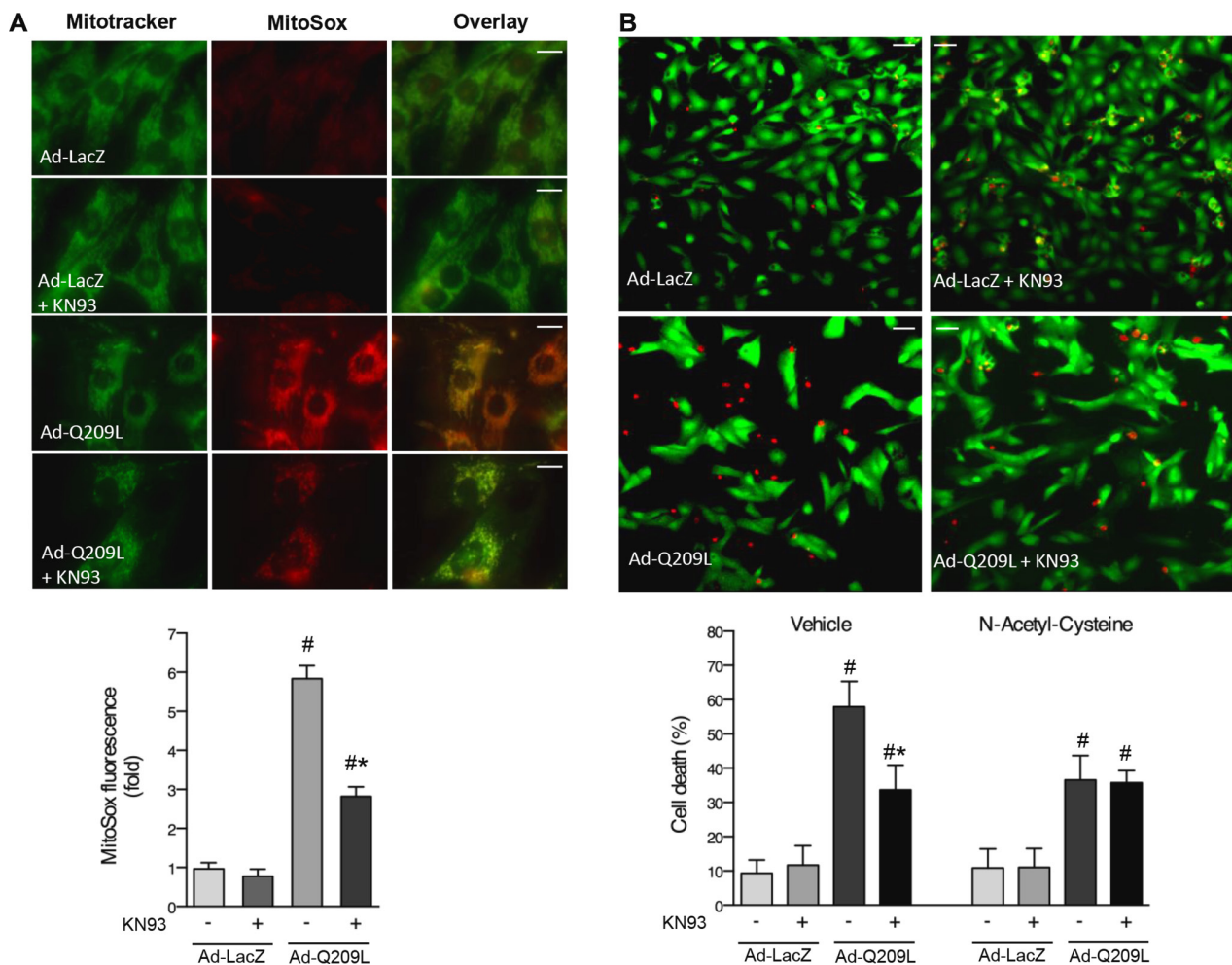
### CaMKII $\delta$ Activation Induces Mitochondrial Oxidative Stress in Cardiomyocytes

To corroborate and add mechanistic insight to these *in vivo* findings, NRVMs were infected with an adenovirus expressing Ad-Q209L, with or without the addition of the CaMKII inhibitor KN93 or of an adenovirus expressing catalytically dead CaMKII $\delta$ . Ad-Q209L infection resulted in a 6-fold increase in mitochondrial ROS accumulation assessed by MitoSox fluorescence. The Gq-mediated increase in MitoSox fluorescence was reduced by >50% in cells treated with KN93 (Figure 4A) or coinfecting with adenovirus expressing catalytically dead CaMKII $\delta$  (Online Figure IIIA). Conversely,

expression of a constitutively activated CaMKII $\delta$  adenovirus (Ad-CaMKII $\delta$ ) significantly increased the MitoSox signal in NRVMs (Online Figure IIIB).

### CaMKII $\delta$ and ROS Generation Mediate G $\alpha$ q-Induced Mitochondria-Mediated Cell Death

Expression of Q209L also led to marked increases in mitochondrial depolarization (assessed using tetramethylrhodamine ethyl ester) and apoptosis (measured by DNA fragmentation), which were prevented by inhibition of CaMKII $\delta$  with KN93 or expression of adenovirus expressing catalytically dead CaMKII $\delta$  (Online Figures IIIC, IIID, and IVA). We used *N*-acetylcysteine as an ROS-scavenger to test the role of ROS in Ad-Q209L-induced cell death. Treatment with *N*-acetylcysteine decreased Q209L-induced cell death by  $\approx$ 60% and did not further reduce cell death in the presence of KN93 (Figure 4B). The observation that *N*-acetylcysteine



**Figure 4. Ca<sup>2+</sup>/calmodulin-dependent protein kinase II  $\delta$  (CaMKII $\delta$ ) induces mitochondrial reactive oxygen species (ROS) generation and cell death in cardiomyocytes downstream of G $\alpha$ q transgenic (Gq). A**, Live cell imaging of neonatal rat ventricular myocytes (NRVMs) stained with the nontoxic mitochondrial marker, mitotracker-green (green), and the mitochondrial-specific ROS indicator MitoSox (red), 12 hours after infection with an adenovirus expressing constitutively activated G $\alpha$ q (Ad-Q209L) or  $\beta$ -galactosidase (Ad-LacZ) and cultured in the presence or absence of the CaMKII inhibitor KN93. Scale bar is 10  $\mu$ m. Bar graph depicts average MitoSox intensities in mitochondria from the experimental groups. **B**, Typical example of NRVMs stained with the live cell indicator calcein (green) and the dead cell indicator propidium iodide (red) after infection with Ad-Q209L or Ad-LacZ and cultured in the presence or absence of KN93. Scale bar is 40  $\mu$ m. Cells were considered viable when positive for calcein and negative for propidium iodide. Bar graph depicts cell death expressed as the % total cells in the different experimental groups, with or without the addition of the ROS scavenger *N*-acetylcysteine or vehicle. #*P*<0.05 vs Ad-LacZ; \*\**P*<0.05 vs Ad-Q209L.

treatment provided no further protection above that afforded by KN93 is consistent with there being a common pathway for the mitochondrial effects of CaMKII and ROS.

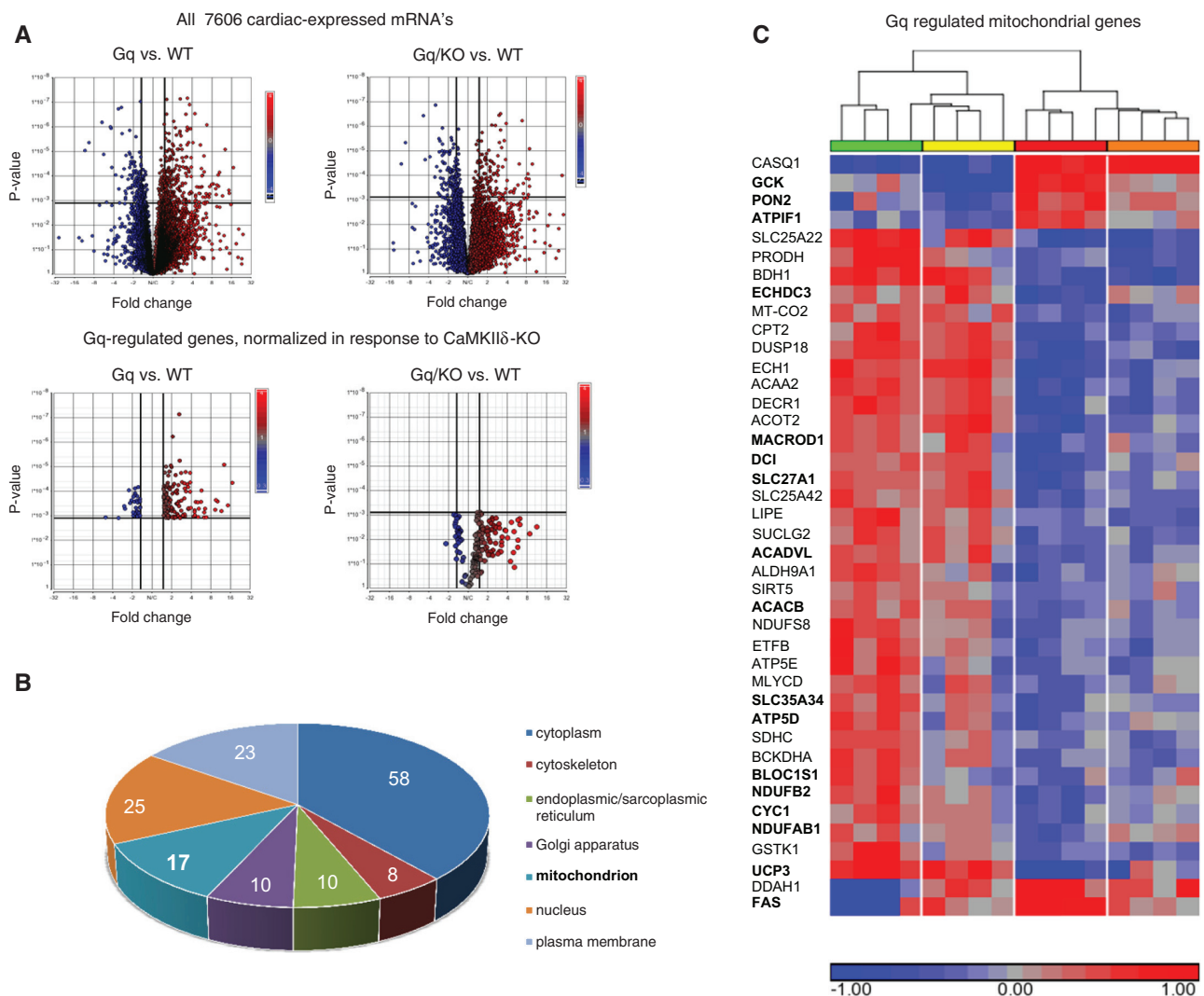
### CaMKII $\delta$ Deletion Prevents Mitochondrial Gene Reprogramming in G $\alpha$ q-Induced Heart Failure

A plethora of transcriptional responses accompany G $\alpha$ q expression and development of decompensation in the mouse heart, including changes in genes that affect mitochondrial function.<sup>19</sup> We applied deep RNA sequencing to discover genes that were differentially regulated in the hearts of G $\alpha$ q mice compared with G $\alpha$ q/KO mice. Of the 335 genes that differed in the hearts of 4-week-old G $\alpha$ q versus WT mice, one third (118 genes) were normalized by CaMKII $\delta$  deletion (Figure 5A). Gene ontology analysis further revealed that 17 genes encoding mitochondrial proteins (41% of the mitochondrial genes

that were differentially regulated in G $\alpha$ q versus WT mice) were normalized in G $\alpha$ q/KO mice (Figure 5B). The 17 mitochondrial genes normalized by CaMKII $\delta$  deletion (bolded in Figure 5C) mediate processes such as mitochondrial substrate transport and oxidation, electron transport chain function, and ATP synthesis. Many of these genes, including cytochrome c (CYC1) and NDU subunits (NDUFAB1 and NDUF58), are components of the electron transport chain and changes in these genes may at least partially explain the alterations in mitochondrial respiration demonstrated in Figure 3.

### G $\alpha$ q and CaMKII $\delta$ Regulate UCP3 and Peroxisome Proliferator-Activated Receptor $\alpha$

One of the mitochondrial gene transcripts found to be markedly downregulated in G $\alpha$ q mice and restored in G $\alpha$ q/KO mice (Figure 5C) was UCP3. The decrease in UCP3 mRNA levels

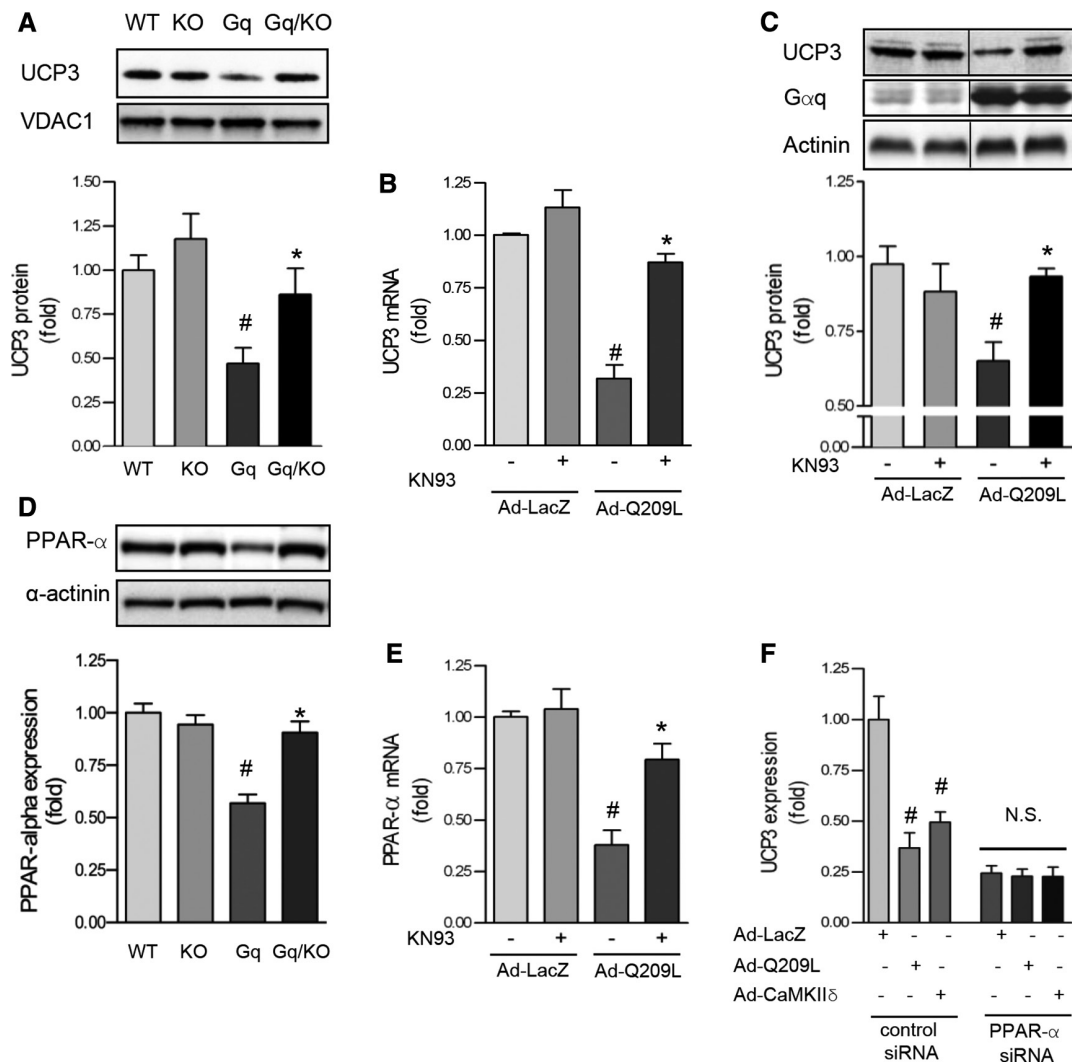


**Figure 5. Ca<sup>2+</sup>/calmodulin-dependent protein kinase II  $\delta$  (CaMKII $\delta$ ) is involved in mitochondrial reprogramming downstream of G $\alpha$ q transgenic (Gq).** **A**, RNA sequencing of Gq and Gq mice in a CaMKII $\delta$  knockout background (Gq/KO) hearts vs wild-type (WT) hearts. Regulated mRNAs at a fold change of  $\pm 50\%$ , false discovery rate (FDR) of 0.02, are shown in the upper panels (thick vertical lines denote fold-change cutoff; thick horizontal lines denote P value cutoff for FDR 0.02). Lower panels show only mRNAs that are regulated in Gq hearts and normalized in CaMKII $\delta$  KO hearts. **B**, Gene ontology cellular component category analysis of Gq-regulated, CaMKII $\delta$  KO-normalized genes. **C**, Unbiased hierarchical clustering of Gq-regulated mitochondrial genes demonstrates nearly perfect clustering between the groups. Those mRNAs that are normalized by CaMKII $\delta$  KO are shown in bold. Blue indicates low expression; and red, high expression.

observed through RNA sequencing was confirmed by changes in UCP3 protein expression in mitochondria isolated from Gq and Gq/KO mice (Figure 6A). To further explore the mechanism and significance of Gq signaling to UCP3, we examined the regulation of UCP3 in NRVMs infected with Ad-Q209L. UCP3 mRNA and protein expression were significantly decreased in cells expressing Q209L, and this was prevented by KN93 (Figure 6B and 6C), confirming a role for CaMKII in the regulation of UCP3 expression.

Peroxisome proliferator-activated receptor  $\alpha$  (PPAR- $\alpha$ ) has been suggested to regulate the expression of UCP3.<sup>22-24</sup> We analyzed the 17 mitochondrial genes normalized by CaMKII $\delta$  deletion for transcription factor binding site enrichment and conservation in their 5-kb proximal promoters as

previously described.<sup>25</sup> This analysis showed significant enrichment ( $P<0.0001$ ) for PPAR- $\alpha$  binding sites. Moreover, analysis of the RNA sequencing data described above demonstrated that PPAR- $\alpha$  mRNA levels were reduced by one third in Gq mice compared with WT mice ( $P=0.00032$ ) but not in Gq/KO versus WT mice. Western blot analysis revealed concomitant changes in PPAR- $\alpha$  protein expression; PPAR- $\alpha$  was decreased in the Gq, and this was normalized by CaMKII $\delta$  deletion (Figure 6D). Expression of Q209L in NRVMs also effectively decreased PPAR- $\alpha$  mRNA through CaMKII signaling (Figure 6E), consistent with a direct effect of Gq activation on PPAR- $\alpha$  expression. Furthermore, the expression of PPAR- $\alpha$ -dependent genes, such as carnitine palmitoyltransferase 1B (CPT-1b) and pyruvate dehydrogenase



**Figure 6.  $Ca^{2+}$ /calmodulin-dependent protein kinase II  $\delta$  (CaMKII $\delta$ ) downregulates peroxisome proliferator-activated receptor  $\alpha$  (PPAR- $\alpha$ ) and uncoupling protein 3 (UCP3) expression.** **A**, UCP3 protein expression normalized for voltage-dependent anion-selective channel protein 1 (VDAC1) in lysates of mitochondria from mouse ventricle. **B**, UCP3 mRNA expression normalized for the internal control 36B4 in neonatal rat ventricular myocytes (NRVMs) infected with constitutively activated G $\alpha$ q (Ad-Q209L) or  $\beta$ -galactosidase (Ad-LacZ) and cultured in the presence or absence of the CaMKII inhibitor KN93. **C**, UCP3 protein expression normalized for actinin in whole cell lysates from NRVMs treated as in **B**. The lanes were run on the same gel but were noncontiguous. **D**, PPAR- $\alpha$  protein expression in whole cell lysates from mouse ventricle. **E**, PPAR- $\alpha$  mRNA expression normalized for 36B4 from NRVMs treated as in **B**. **F**, UCP3 mRNA expression normalized for 36B4 in NRVMs which were first transfected with control small interfering RNA (siRNA) or PPAR- $\alpha$  siRNA and subsequently infected with Ad-LacZ, Ad-Q209L, or constitutively activated CaMKII $\delta$ c (CaMKII $\delta$ ). All values are expressed as mean $\pm$ SEM. # $P<0.05$  vs wild type (WT) or Ad-LacZ; \* $P<0.05$  vs Gq or Q209L. Gq indicates G $\alpha$ q transgenic; Gq/KO, Gq mice in a CaMKII $\delta$  knockout background; and KO, CaMKII $\delta$  knockout.



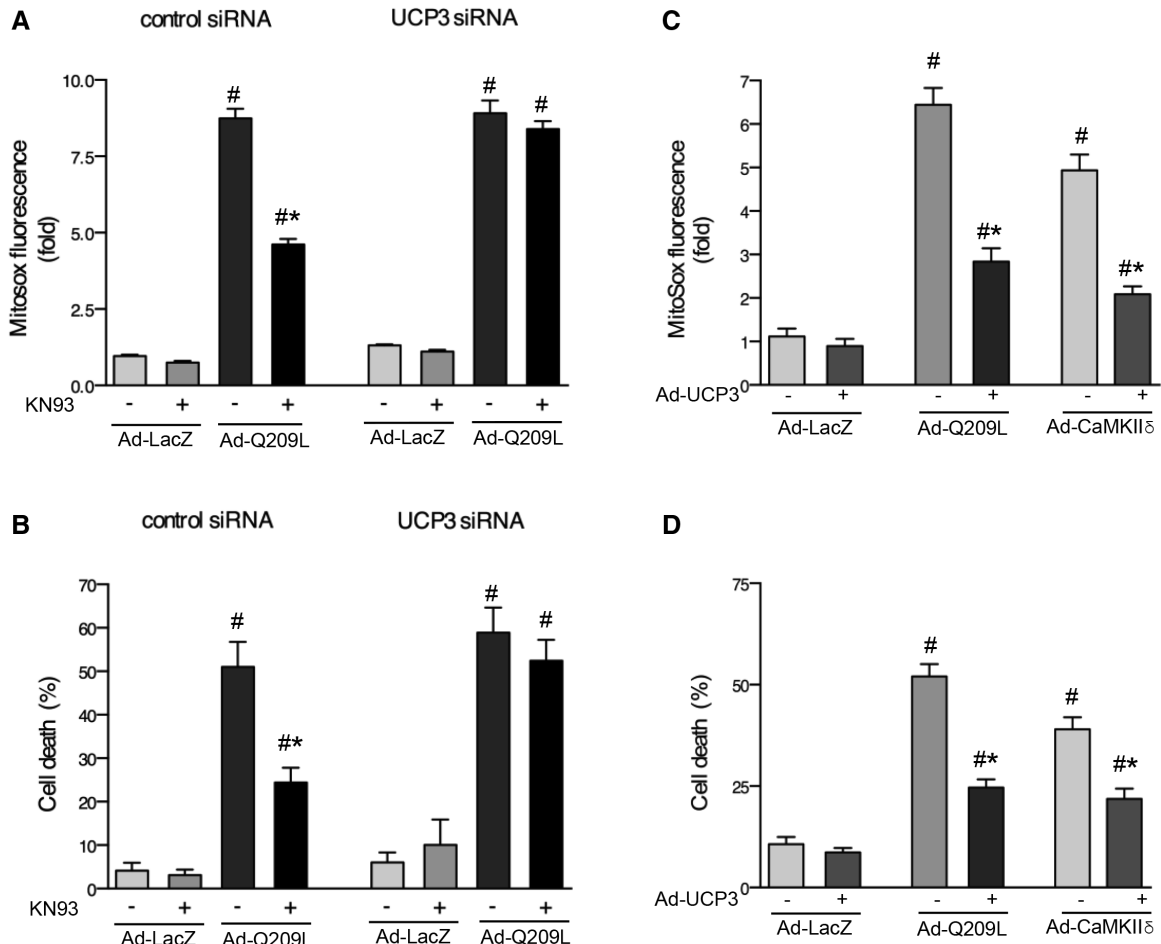
kinase 4 (PKD4), was diminished through a KN93-sensitive pathway in cells expressing Q209L and also by expression of CaMKII $\delta$ c (Online Figure V).

To demonstrate a functional relationship between PPAR- $\alpha$  and UCP3 expression, we downregulated PPAR- $\alpha$  with small interfering RNA (siRNA). Greater than 85% downregulation of PPAR- $\alpha$  protein expression was achieved by siRNA treatment (data not shown). Knockdown of PPAR- $\alpha$  significantly decreased basal levels of UCP3 expression (Figure 6F), directly demonstrating that UCP3 expression is regulated by PPAR- $\alpha$ . Additionally, UCP3 levels were not further diminished by Ad-Q209L or Ad-CaMKII $\delta$  when PPAR- $\alpha$  was downregulated (Figure 6F). Together these findings suggest that CaMKII downregulates UCP3 expression through inhibition of PPAR- $\alpha$  and that this occurs downstream of Gq signaling.

### UCP3 Contributes to ROS Production and Cell Death

We carried out loss- and gain-of-function experiments using UCP3 siRNA and adenovirus to demonstrate a causal

relationship between decreased UCP3 expression and G $\alpha$ q/CaMKII-mediated mitochondrial ROS generation and cell death. Treatment with UCP3 siRNA reduced UCP3 expression in NRVMs by 80% (Online Figure VIIA). The ability of KN93 to inhibit Q209L-induced mitochondrial ROS accumulation (Figure 7A) and cell death (Figure 7B) were both abrogated when UCP3 was depleted. The protective effect of adenovirus expressing catalytically dead CaMKII $\delta$  on cell death induced by Q209L was also blocked (Online Figure IVB). To further establish the importance of UCP3 downregulation in G $\alpha$ q- and CaMKII-mediated mitochondrial ROS generation and cell death, we generated an adenovirus expressing UCP3 (Ad-UCP3). Infection with Ad-UCP3 resulted in a 2.5-fold increase in expression of UCP3 and also normalized UCP3 expression in cells coinfecting with Ad-Q209L (Online Figure VIIB). Importantly, the ability of Ad-Q209L and Ad-CaMKII $\delta$  to induce mitochondrial ROS accumulation (Figure 7C) and cell death (Figure 7D) was significantly attenuated by coexpression of UCP3.



**Figure 7. Ca<sup>2+</sup>/calmodulin-dependent protein kinase II  $\delta$  (CaMKII $\delta$ )-induced mitochondrial reactive oxygen species and cell death are dependent on uncoupling protein 3 (UCP3).** **A**, Neonatal rat ventricular myocytes (NRVMs) were first transfected with scrambled small interfering RNA (siRNA) or UCP3 siRNA and subsequently infected with constitutively activated G $\alpha$ q (Ad-Q209L) or  $\beta$ -galactosidase (Ad-LacZ) and cultured in the presence or absence of the CaMKII inhibitor KN93. **A**, Average Mitox intensities in mitochondria from the experimental groups 8 hours after infection. **B**, Cell death determined by calcein/propidium iodide staining 72 hours after infection as in **A**. **C**, Mitox intensities in NRVMs 12 hours after infection with Ad-LacZ, Ad-Q209L, or constitutively activated CaMKII $\delta$ c (CaMKII $\delta$ ) and coinfecting with adenovirus expressing UCP3. **D**, Cell death determined by calcein/propidium iodide staining 72 hours after infection as in **C**. # $P$ <0.05 vs or Ad-LacZ; \* $P$ <0.05 vs Ad-Q209L or Ad-CaMKII $\delta$ .

In the above experiments, an adenovirus expressing CaMKII $\delta$ c was used. To assess whether the mitochondrial reprogramming was CaMKII $\delta$  subtype specific, we compared the response to adenoviral expression of the 2 major cardiac CaMKII $\delta$  isoforms, CaMKII $\delta$ c and CaMKII $\delta$ b. CaMKII $\delta$ -mediated downregulation of UCP3, PPAR- $\alpha$ , and other PPAR- $\alpha$ -dependent genes was observed after infection with constitutively activated CaMKII $\delta$ c, but these genes remained unchanged after infection with constitutively activated CaMKII $\delta$ b (Online Figures VE, VF, and VI).

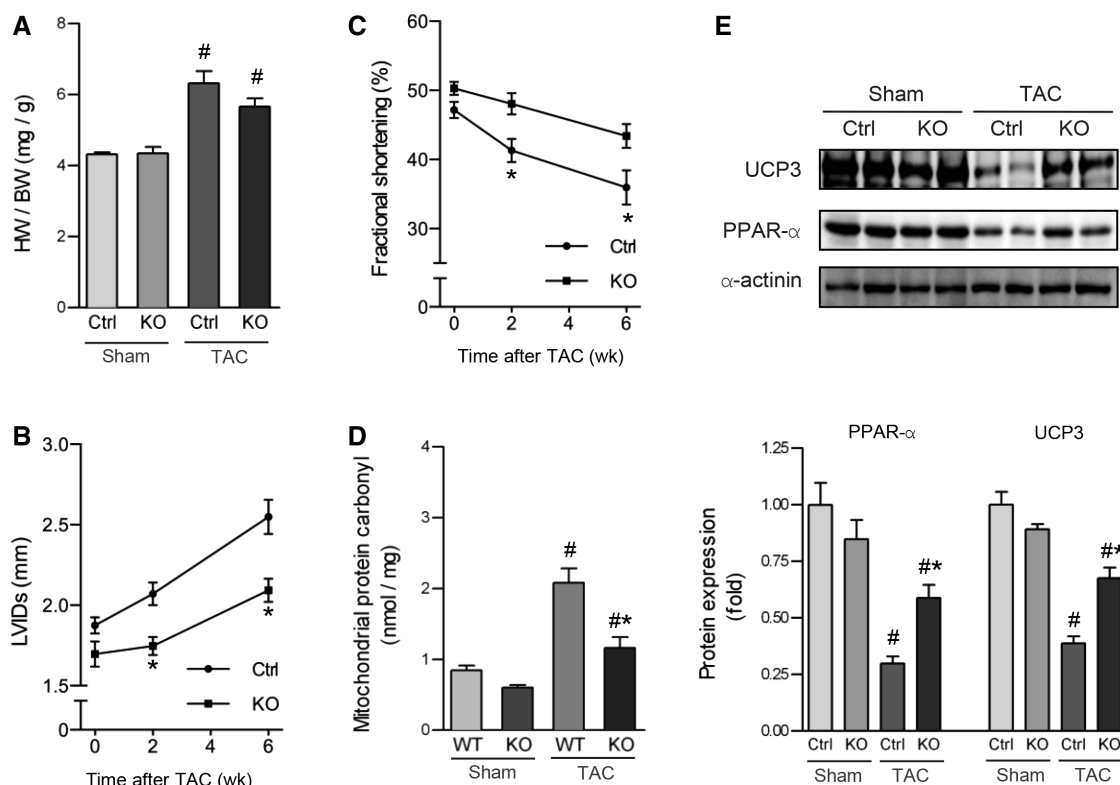
### CaMKII $\delta$ Deletion Prevents Mitochondrial Stress in Pressure Overload–Induced Heart Failure

We previously demonstrated that CaMKII $\delta$  is required for the transition from hypertrophy to heart failure following TAC-induced pressure overload.<sup>9</sup> To prove that the CaMKII signaling pathway responsible for decompensation to failure was cardiomyocyte autonomous, we generated cardiac-specific CaMKII $\delta$  KO mice.<sup>12</sup> We examined their response to pressure overload induced by TAC and determined that deletion of CaMKII $\delta$  did not prevent TAC-induced increases in hypertrophy (Figure 8A). Despite development of hypertrophy, the loss of CaMKII $\delta$  in cardiomyocytes significantly attenuated the development of ventricular dilation and the decrease in fractional shortening (Figure 8B and 8C). In addition, mitochondrial oxidative stress assessed by protein carbonylation was increased after TAC through a

CaMKII $\delta$ -dependent process (Figure 8D). Remarkably, as observed for the Gq mice, pressure overload resulted in decreases in PPAR- $\alpha$  and UCP3 expression, and these changes were attenuated in the absence of CaMKII $\delta$  (Figure 8E). Thus, the effect of CaMKII $\delta$  on mitochondrial oxidative stress and heart failure development extends beyond the Gq model of cardiomyopathy to also include the response to pressure overload.

### Discussion

Clinical therapeutics has aimed at preventing or reversing hypertrophy in patients with heart disease because conventional wisdom holds that reactive hypertrophy is maladaptive and perhaps dispensable for functional compensation to hemodynamic overload.<sup>26</sup> Hypertrophic growth is, however, an evolutionarily conserved cardiac response to reduce ventricular wall stress in pressure overloaded or dilated ventricles.<sup>1</sup> The problem is that this reactive hypertrophy ultimately progresses into a maladaptive cardiomyopathy with decreased survival. It would therefore seem most therapeutically beneficial to retain the physically advantageous features of cardiac hypertrophy that reduce wall stress, while preventing its subsequent decompensation. Our previous work suggests that CaMKII $\delta$ , while dispensable for hypertrophic growth, plays a key role in the transition from hypertrophy to heart failure.<sup>9</sup> How this occurs, and whether CaMKII $\delta$  activation is an essential component



**Figure 8. Ca<sup>2+</sup>/calmodulin-dependent protein kinase II  $\delta$  (CaMKII $\delta$ ) deletion prevents mitochondrial oxidative stress in pressure overload–induced heart failure.** Cardiomyocyte-specific CaMKII $\delta$  knockout mice (KO) or their littermate controls (Ctrl) were subjected to transverse aortic constriction (TAC). **A**, Heart weight/body weight ratio (HW/BW,  $n=6-9$ ) 2 weeks after TAC. **B** and **C**, Changes in echocardiographic indices of left ventricular (LV) function and LV dilatation after TAC. Shown are left ventricular internal systolic diameter (LVIDs, **B**) and fractional shortening (**C**;  $n=8$ ). **D**, Carbonylation of mitochondrial proteins in wild type (WT) and global KO mice as measured by ELISA of cardiac mitochondrial homogenates 6 weeks after TAC ( $n=5-8$  per group). **E**, Peroxisome proliferator–activated receptor  $\alpha$  (PPAR- $\alpha$ ) and uncoupling protein 3 (UCP3) protein expression normalized for  $\alpha$ -actinin measured in lysates from mouse ventricle 6 weeks after TAC. All values are expressed as mean $\pm$ SEM. <sup>#</sup> $P<0.05$  vs Ctrl-sham or WT-sham; <sup>\*</sup> $P<0.05$  vs Ctrl-TAC or WT-TAC.

of pathological hypertrophy downstream of Gq signaling, is not known. Here, we used combined genetic manipulation of Gq and CaMKII $\delta$  to dissociate growth-promoting and heart failure-inducing hypertrophy signals, in effect beneficially remodeling reactive hypertrophy. In so doing, we uncovered mitochondrial reprogramming as a key mediator of CaMKII $\delta$ -induced hypertrophy decompensation, further emphasizing the potential for CaMKII as a therapeutic target.

### Mitochondrial Reprogramming Through CaMKII $\delta$ as a Central Driver in Hypertrophy Decompensation

Mitochondrial reprogramming occurs during heart failure development and is characterized by a shift from fatty acid to glucose utilization, reduced ATP availability, and increased mitochondrial oxidative stress.<sup>27</sup> The relevance of mitochondrial reprogramming is underscored by the ability of mitochondrial-targeted antioxidants to prevent pathological hypertrophy and cardiac aging.<sup>15,28–30</sup> CaMKII is now known to be activated not only by calcium but also by oxidation.<sup>31</sup> Although it is clear that ROS generation and concomitant CaMKII oxidation contribute to the pathophysiology of heart disease,<sup>31–34</sup> the possibility that this maladaptive cascade is further accentuated by reciprocal effects of CaMKII on ROS production has not been previously considered. In the present study, we provide evidence that CaMKII $\delta$  induces altered mitochondrial gene expression associated with mitochondrial dysfunction, mitochondrial oxidative stress, and ROS-driven cell death. First, we show that CaMKII $\delta$  mediates mitochondrial reprogramming downstream of Gq signaling. Second, we demonstrate that CaMKII $\delta$ -induced mitochondrial reprogramming is associated with impaired respiration and increased mitochondrial oxidative stress. Third, we demonstrate that increased oxidative stress induced by CaMKII $\delta$  contributes to cardiomyocyte cell death and cardiomyopathy. Globally, these findings implicate CaMKII $\delta$  as a central mediator of the mitochondrial reprogramming and cardiomyocyte loss associated with heart failure development.

### Critical Role for UCP3 in CaMKII $\delta$ -Induced Mitochondrial ROS in the Heart

When activated,<sup>23,35</sup> uncoupling proteins can induce a proton leak by dissipating the proton motive force across the inner mitochondrial membrane, thereby reducing ROS generation at the cost of a reduction in coupling efficiency.<sup>36</sup> UCP3 is a major UCP isoform in the heart, and it seems to be critical for the cardiac response to multiple stresses.<sup>36–42</sup> Downregulation of UCP3 has been associated with increased cardiac ROS levels in a model of doxorubicin-induced heart failure.<sup>37</sup> The antioxidant effects of hexokinase-II and stannocalcin-I in cardiomyocytes have been suggested to involve UCP3.<sup>38,39</sup> Studies using UCP3 knockout mice demonstrate increased angiotensin-induced cardiac ROS levels and greater susceptibility to oxidative stress.<sup>39,42</sup> Furthermore, UCP3 knockout mice show impaired myocardial energetics and larger infarcts in response to cardiac ischemia/reperfusion.<sup>41</sup> These findings are consistent with and extended by our observation that Gq-induced mitochondrial ROS generation is mediated, at least in part, through transcriptional repression of UCP3. Here, we further demonstrate that CaMKII contributes to UCP3 downregulation in response to Gq and that

this downregulation is functionally important in mediating Gq and CaMKII-induced ROS accumulation and cell death. Our findings suggest that activation of CaMKII, which occurs in response to multiple agonists and stressors, is a nodal point for regulation of mitochondrial ROS generation which in turn sustains a maladaptive feed forward cycle in which ROS generation further activates CaMKII signaling.<sup>31</sup>

There is a broad precedent for transcriptional regulation of UCP3 by pharmacological interventions and physiological stress.<sup>36</sup> The role of CaMKII in transcriptional regulation has also been well documented.<sup>7</sup> Here, we demonstrate CaMKII-dependent decreases in expression of both UCP3 and the transcription factor PPAR- $\alpha$  in hearts of Gq mice and in NRVMs expressing activated Gq or CaMKII $\delta$ . siRNA-mediated knockdown of PPAR- $\alpha$  reduced UCP3 expression, and no further effects of Gq or CaMKII on UCP3 expression were observed in the absence of PPAR- $\alpha$ , supporting a role for PPAR- $\alpha$  in transcriptional control of UCP3. We also report decreases in both UCP3 and PPAR- $\alpha$  following pressure overload, which are rescued by CaMKII $\delta$  deletion. PPAR- $\alpha$  has been shown to be reduced in experimental models of heart failure and PPAR- $\alpha$  agonists shown to preserve cardiac function.<sup>22,36,43,44</sup> The observation that the CaMKII-regulated mitochondrial genes are significantly enriched for PPAR- $\alpha$ -binding sites suggests a mechanism by which CaMKII activation would induce mitochondrial reprogramming and associated decompensation to heart failure. How CaMKII downregulates PPAR- $\alpha$  and subsequently UCP3 remains to be determined. We used pharmacological inhibitors to test effects of both class I and class II histone deacylases on this process in NRVMs but failed to observe any block of CaMKII $\delta$ -mediated PPAR- $\alpha$  or UCP3 downregulation. Another possible mechanism for gene repression by CaMKII could be through effects on microRNAs, a topic that is of considerable interest but clearly beyond the scope of the present study.

### Clinical Implications

Using cellular and murine models of pathological hypertrophy induced by sustained Gq-signaling, we show that deletion of the predominant cardiac isoform (CaMKII $\delta$ ) does not block development of hypertrophy but prevents the transition from compensatory cardiac hypertrophy into dilated cardiomyopathy. Our study therefore shows that CaMKII $\delta$  is dispensable for Gq-induced hypertrophy but critical for the subsequent transition from hypertrophy to heart failure. Importantly, similar observations are made when pressure overload rather than Gq is used as a stressor. CaMKII is activated downstream of multiple neurohumoral agonists, including  $\beta$ -adrenergic agonists, angiotensin II, and aldosterone, and plays a crucial role in their pathophysiological effects in the heart.<sup>7,45,46</sup> Accordingly, pharmacological inhibitors of CaMKII may offer novel therapeutic opportunities for specifically blocking the maladaptive effects of hypertrophy induced by multiple insults.

### Limitations

CaMKII $\delta$  can localize to mitochondria, and a recent study determined that mitochondrial-localized CaMKII plays a central role in cardiac ischemia/reperfusion injury.<sup>18</sup> Our study was focused on CaMKII-induced changes in mitochondrial gene transcription, but we cannot exclude the possibility that

salutary effects of deleting CaMKII $\delta$  on mitochondrial function are also mediated, in part, by changes in the activity of CaMKII in mitochondria. In addition, a recent study suggested that CaMKII stimulates NADPH (nicotinamide adenine dinucleotide phosphate) oxidase-mediated ROS production, suggesting that CaMKII could also exert detrimental effects by increasing ROS levels through extramitochondrial mechanisms.<sup>47</sup> The observation that state 4 respiration and respiratory control ratios were comparable between the groups might appear inconsistent with there being a major role for UCP3 downregulation in the mitotoxicity induced by CaMKII. However, our assay conditions were not optimized for the purpose of detecting subtle differences in coupling, thus such changes would not have been observed under our assay conditions. Despite these possible limitations, our study provides compelling evidence for a role of CaMKII and UCP3 in controlling mitochondrial oxidative stress in the heart.

## Conclusions

Mitochondrial reprogramming by CaMKII $\delta$  contributes to the maladaptive transition from Gq-mediated hypertrophy to cardiomyopathy.

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## Disclosures

None.

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## Novelty and Significance

### What Is Known?

- Cardiomyocytes subject to neurohumoral stresses or expressing the signal transducer G $\alpha$ q transgenic (Gq) undergo a process of hypertrophy which ultimately decompensates into heart failure.
- Ca<sup>2+</sup>/calmodulin-dependent protein kinase II  $\delta$  (CaMKII $\delta$ ) is activated by neurohumoral and oxidative stress and is required for hypertrophy decompensation.
- Induction of heart failure by Gq is mediated through mitochondrial oxidative stress, but the link between these events is incompletely understood.

### What New Information Does This Article Contribute?

- CaMKII $\delta$  mediates heart failure development, mitochondrial dysfunction, and mitochondrial oxidative stress in response to pressure overload and Gq.
- CaMKII $\delta$  activation results in changes in expression of nuclear encoded mitochondrial genes that contribute to mitochondrial dysfunction and oxidative stress.
- The mitochondrial uncoupling protein 3 is downregulated by Gq/CaMKII $\delta$  and central to the mitochondrial oxidative stress and cell death that contributes to heart failure development.

Studies using CaMKII inhibitors and CaMKII $\delta$  knockout mice demonstrate its key role in the transition from hypertrophy to heart

failure induced by pressure overload and other stresses. The underlying mechanisms are unclear. The Gq transgenic mouse is a genetic model in which heart failure development is linked to mitochondrial oxidative stress. We crossed Gq transgenic and CaMKII $\delta$  knockout mice and determined that CaMKII $\delta$  deletion prevented Gq from inducing ventricular dilation and dysfunction, impairing mitochondrial respiration, increasing mitochondrial reactive oxygen species, and inducing cardiomyocyte apoptosis. Deep RNA sequencing revealed that there was extensive reprogramming of nuclear encoded mitochondrial genes in response to Gq which was prevented in the absence of CaMKII $\delta$ . We focused on a mitochondrial uncoupling protein, uncoupling protein 3, which was markedly downregulated by Gq signaling through CaMKII $\delta$ . Uncoupling protein 3 was shown by gain- and loss-of-function studies to contribute to mitochondrial reactive oxygen species production and depolarization as well as to cardiomyocyte cell death. We suggest that CaMKII $\delta$ -mediated suppression of uncoupling protein 3 is a central driver of the mitochondrial oxidative stress that underlies hypertrophy decompensation. These findings provide novel insights into the mechanistic underpinnings of hypertrophy decompensation and further emphasize the potential for CaMKII as a therapeutic target.

## Mitochondrial Reprogramming Induced by CaMKII $\delta$ Mediates Hypertrophy Decompensation

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## Supplemental material

### Online methods

#### Animal Models

The generation of  $G\alpha q$ -40 transgenic mice and CaMKII $\delta$  knockout mice has been described previously.<sup>1,2</sup> To study the role of CaMKII $\delta$  in  $G\alpha q$ -induced heart failure,  $G\alpha q$  transgenic mice were crossed with CaMKII $\delta$  knockout mice to generate CaMKII $\delta$  heterozygous mice with or without  $G\alpha q$  overexpression (CaMKII $\delta^{+/-} G\alpha q^+$  or CaMKII $\delta^{+/-} G\alpha q^-$ ). CaMKII $\delta^{+/+} G\alpha q^-$  (Wildtype, WT), CaMKII $\delta^{-/-} G\alpha q^-$  (CaMKII $\delta$  knockout, KO), CaMKII $\delta^{+/+} G\alpha q^+$  ( $G\alpha q$  transgenic, Gq) and CaMKII $\delta^{-/-} G\alpha q^+$  (Gq-TG mice in a CaMKII $\delta$  knockout background, TG/KO) were generated by the crosses of CaMKII $\delta^{+/-} G\alpha q^+$  and CaMKII $\delta^{+/-} G\alpha q^-$  mice. Between 6 and 14 male mice were included in each experimental group. Echocardiography, left ventricular hemodynamic measurements and cardiac histology were performed as previously described.<sup>1</sup> Studies involving pressure overload used cardiac specific CaMKII $\delta$  knockout mice generated as recently described<sup>3</sup>

Transverse aortic constriction was performed on 8-12 week old male mice under isoflurane anesthesia as previously described,<sup>1</sup> with minor modifications. Sham-operated mice were subjected to identical interventions except for the constriction of the aorta. After echocardiographic analysis at different time points, mice were anesthetized with ketamine/xylazine, and pressure gradients were measured to ensure similar pressure overload in WT and KO groups. KO and WT mice were sacrificed by

cervical dislocation, and hearts and lungs were removed and weighed promptly. All procedures were performed in accordance with NIH *Guide for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee.

### **RNA sequencing**

Left ventricular RNA was isolated from 4 mice from each group and processed for RNA sequencing studies as previously described.<sup>4</sup>

### **Mitochondrial bioenergetics**

Mouse heart mitochondria were isolated by differential centrifugation as described previously.<sup>5</sup> Respiration was measured using a Seahorse XF24 analyzer as previously described.<sup>6</sup> 2.5  $\mu\text{g}$  mitochondria were offered 10 mM pyruvate, 1 mM malate, and 4 mM ADP to drive state 3 respiration. When indicated (Fig 3A), the following were added: 0.4  $\mu\text{g}$  oligomycin/ $\mu\text{g}$  mitochondrial protein to measure state 4 respiration, 4  $\mu\text{M}$  FCCP to measure maximal uncoupler-stimulated respiration, and 2  $\mu\text{mol/L}$  rotenone plus 1  $\mu\text{M}$  antimycin A to measure non-mitochondrial oxygen consumption. Five technical replicates were conducted for each mitochondrial preparation.

### **Biochemical and histological analysis**

Tissue fractionation, RNA isolation and Western blot analysis were performed as described previously.<sup>1, 7</sup> Cardiac mitochondrial protein carbonyl was measured using OxiSelect protein carbonyl ELISA kit (Cell Biolabs, San Diego, CA). Apoptosis was



measured with the Roche DNA fragmentation ELISA. Cardiac ROS levels were measured with dihydroethidium (DHE) staining of cryopreserved myocardium.

### **Neonatal Ventricular Cardiomyocyte Culture and live cell imaging**

Neonatal rat ventricular myocytes (NRVMs) were isolated, cultured, infected and transfected as described previously.<sup>7</sup> Live cells were loaded with MitoSox (5  $\mu$ mol/L), tetramethylrhodamine ethyl ester (TMRE, 50 nmol/L) Mitotracker green (1 mmol/L) calcein-AM (1 mmol/L) or propidium iodide (5 mmol/L) and measured with a Zeiss observer, inverted microscope. Paraformaldehyde fixed cells were stained for Atrial Natriuretic Factor (ANF) or phalloidin, as described previously.<sup>8</sup>

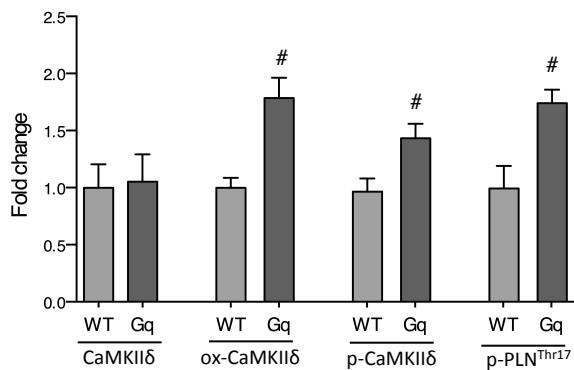
### **Statistical Analysis**

All data are presented as means  $\pm$  standard error of the mean (SE). Comparisons between groups were performed using the Student *t* test, the Mann Whitney U test, Kruskal-Wallis test or One-way ANOVA, followed by the Tukey post hoc test, where appropriate. A p value < 0.05 was considered statistically significant.

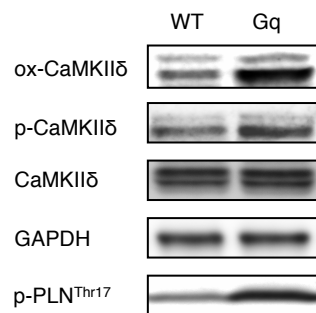
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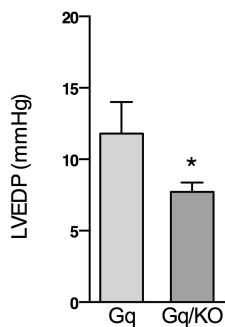


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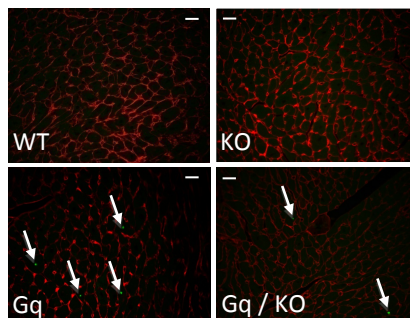


**Supplemental Fig 1. CaMKII $\delta$  activity is increased in Gq-transgenic mice.** A, Oxidized and phosphorylated CaMKII $\delta$  in whole cardiac lysates, corrected for total CaMKII $\delta$  or phosphorylation of phospholamban (PLN) at the CaMKII $\delta$  specific site in whole cardiac lysates, corrected for GAPDH. Results are expressed as fold difference compared to wildtype mice. B, Typical Western blot examples of figure 1A. The two bands visible with the total CaMKII $\delta$  antibody represent the two splice variants  $\delta_B$  and  $\delta_C$ , which may also account for the doublets seen in the ox-CaMKII $\delta$  and p-CaMKII $\delta$  blots. #,  $p < 0.05$  versus WT.

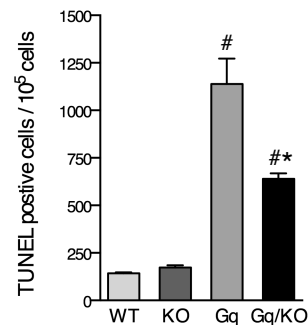
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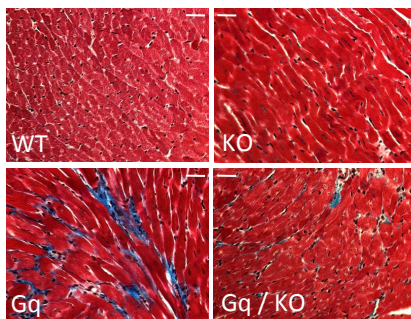
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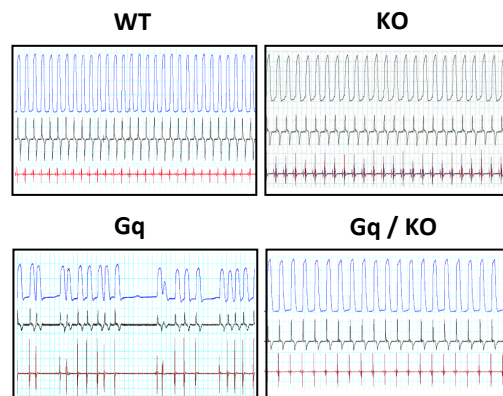
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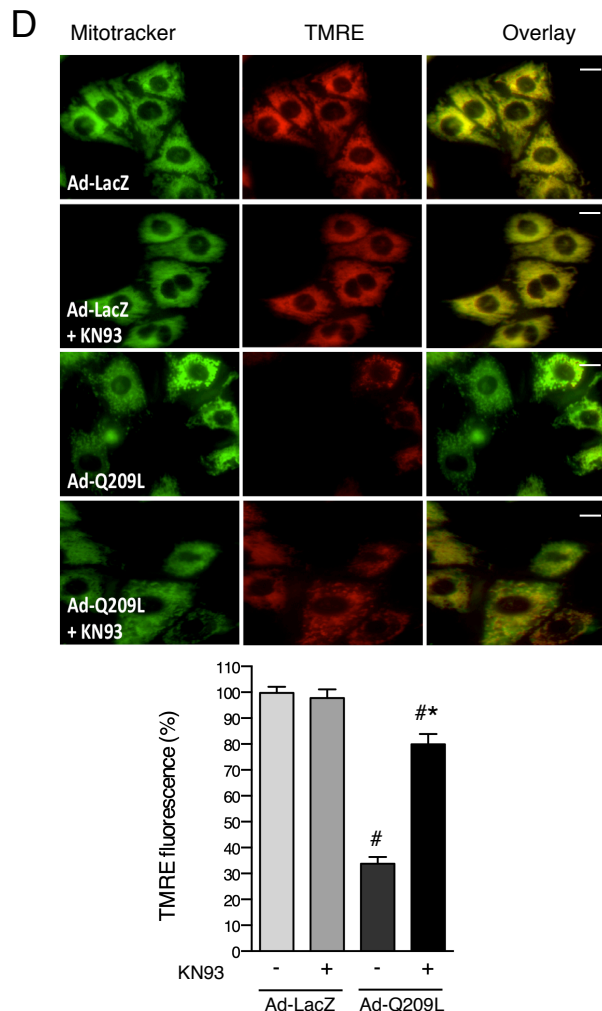
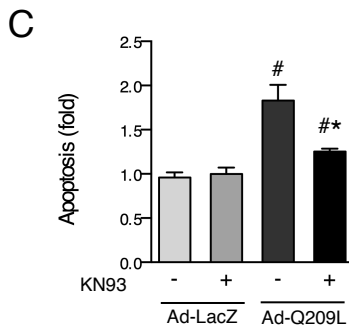
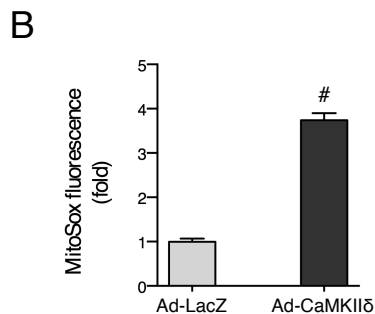
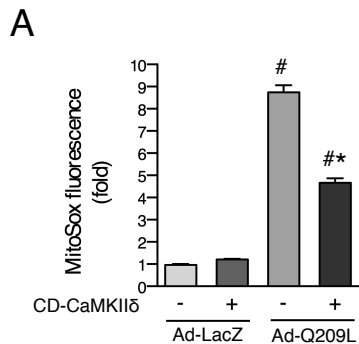
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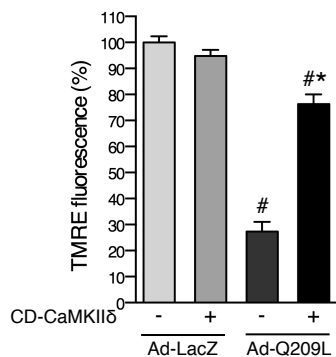


**Supplemental Fig II. Knockout of CaMKII $\delta$  prevents Gq-induced cardiac apoptosis, fibrosis and arrhythmias.** A, Left ventricular end diastolic pressure. N=4 per group. B, Apoptotic nuclei stained with the TUNEL in-situ apoptosis detection kit (green) and counterstained with Rhodamine-labeled wheat germ agglutinin (red). Apoptotic nuclei are indicated with an arrow. Scale bar is 20  $\mu$ M. B, C Average number of TUNEL positive cardiomyocytes. D, Cardiac sections stained with Masson trichrome staining. Scale bar is 40  $\mu$ M. E, Representative cardiac pressure tracings of the experimental groups showing more frequent extra systole in Gq mice compared to Gq/KO mice. LVEDP, left ventricular end diastolic pressure; WT, wildtype; KO, CaMKII $\delta$ -knockout; Gq, Gq-transgenic; Gq/KO, Gq-transgenic in a CaMKII $\delta$ -knockout background. #,  $p < 0.05$  versus WT, \*;  $p < 0.05$  versus Gq.

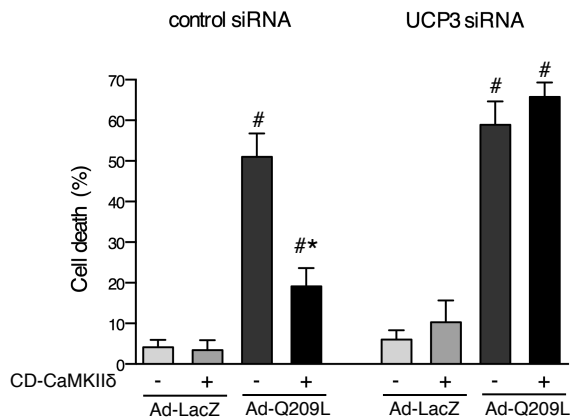


**Supplemental Fig III. Effect of CaMKII $\delta$  on mitochondrial ROS, apoptosis and mitochondrial membrane depolarization downstream of Gq.** A, MitoSox intensities in neonatal rat ventricular myocytes (NRVMs), 12 hours after infection with an adenovirus expressing constitutively activated Gq $\alpha$  (Ad-Q209L) or  $\beta$ -galactosidase (Ad-LacZ) and co-infected with and adenovirus expressing catalytically dead CaMKII $\delta$ c (CD-CaMKII $\delta$ ) or Ad-LacZ. B, Average MitoSox intensities in mitochondria from NRVMs after infection with constitutively activated CaMKII $\delta$ c (Ad-CaMKII $\delta$ ) or Ad-LacZ. C, Apoptosis in NRVMs measured with the POD assay, 72 hours after infection with Ad-Q209L or Ad-LacZ and cultured in the presence or absence of the CaMKII inhibitor KN93. D, Live cell imaging of NRVMs stained with the non-toxic mitochondrial specific marker, mitotracker-green (green) and TetraMethylRhodamine, Ethyl ester (TMRE), an indicator of mitochondrial membrane potential (red), cultured as in C. Scale bar is 10  $\mu$ M. Bar graph represents average TMRE intensities in mitochondria from the different experimental groups. #,  $p < 0.05$  versus or Ad-LacZ, \*  $p < 0.05$  versus Ad-Q209L.

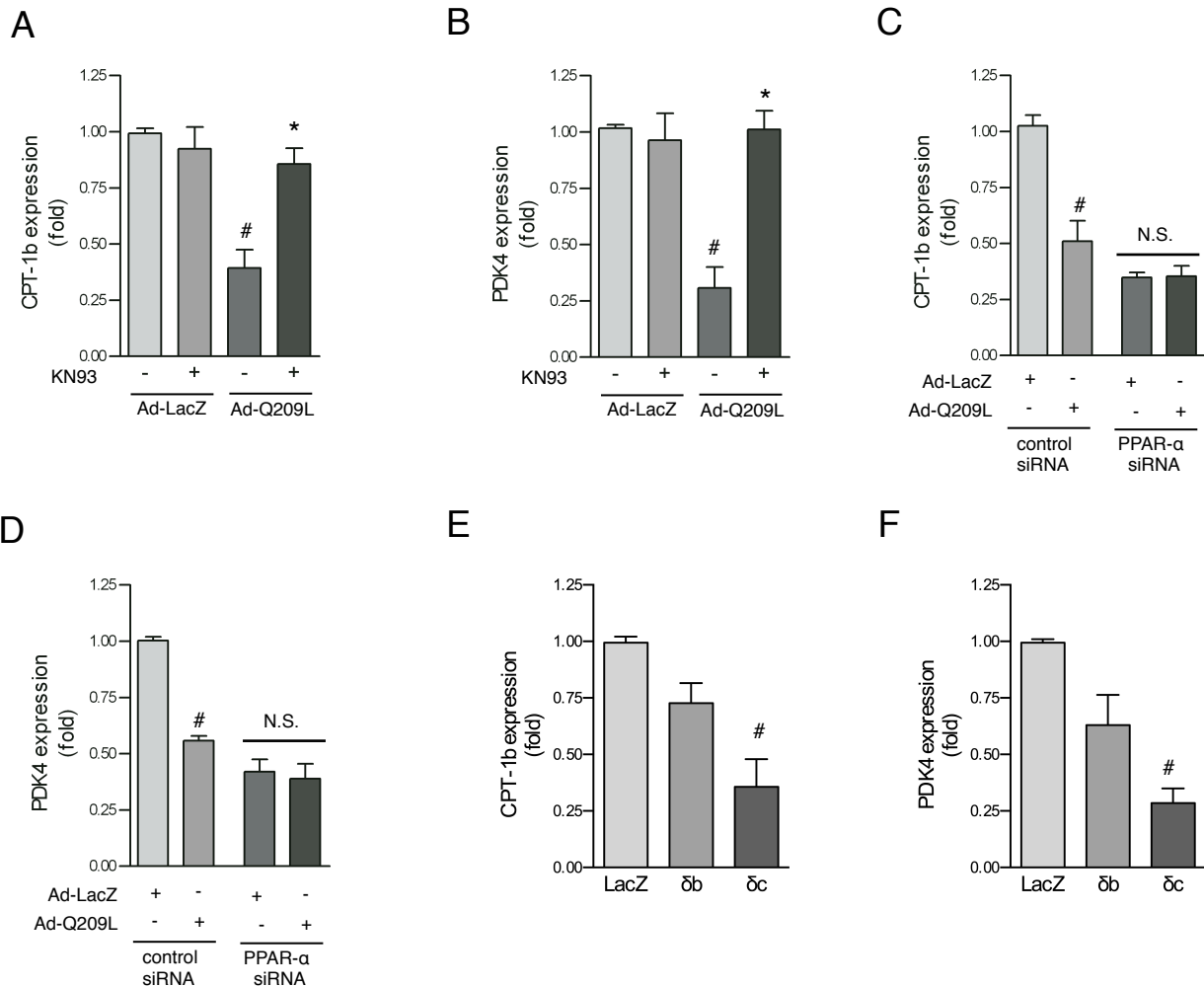
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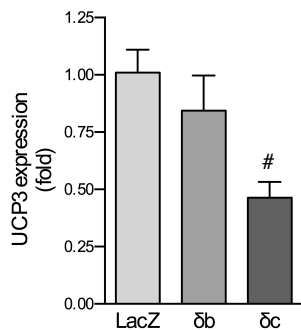


**Supplemental Fig IV. Effect of catalytically dead CaMKII $\delta$  on mitochondrial membrane depolarization and cell death.** A, Average TetraMethylRhodamine, Ethyl ester (TMRE) intensities in neonatal rat ventricular myocytes, 72 hours after infection with constitutively activated Gq $\alpha$  (Ad-Q209L) or  $\beta$ -galactosidase (Ad-LacZ) and co-infection with adenovirus expressing catalytically dead CaMKII $\delta_C$  (CD-CaMKII $\delta$ ) or Ad-LacZ. B, NRVMs were transfected with scrambled siRNA or UCP3 siRNA for 48 hours before infection with Ad-Q209L or Ad-LacZ and co-infected with adenovirus expressing CD-CaMKII $\delta$  or Ad-LacZ. Graphs depict average cell death determined by calcein / propidium iodide staining, 72 hours after infection. #,  $p < 0.05$  versus or Ad-LacZ, \*  $p < 0.05$  versus Ad-Q209L.

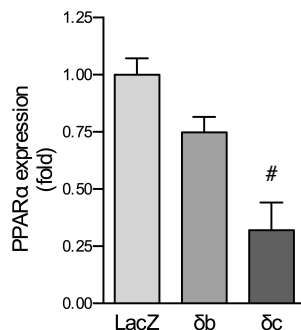


**Supplemental Fig V. Transcription of PPAR-α dependent genes is inhibited by Gαq in a CaMKIIδ-dependent fashion.** Shown are mRNA levels of A, carnitine palmitoyltransferase 1B (CPT-1b) and B, pyruvate dehydrogenase kinase 4 (PDK4) normalized for 36B4 in neonatal rat ventricular myocytes (NRVMs) infected with constitutively activated Gq (Ad-Q209L) or β-galactosidase (Ad-LacZ) and cultured in the presence or absence of the CaMKII inhibitor KN93. C, CPT-1b mRNA expression normalized for 36B4 in NRVMs which were first transfected with control siRNA or PPARα siRNA and subsequently infected with Ad-Q209L or Ad-LacZ. and D, PDK4 mRNA expression normalized for 36B4 in NRVMs cultured as in C. E, CPT-1b and F, PDK4 mRNA expression normalized for 36B4 after infection with constitutively activated CaMKIIδb (δb), constitutively activated CaMKIIδc (δc) or Ad-LacZ. #, p < 0.05 versus or Ad-LacZ, N.S., non-significant.

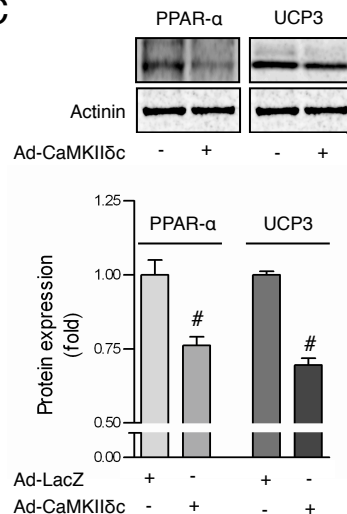
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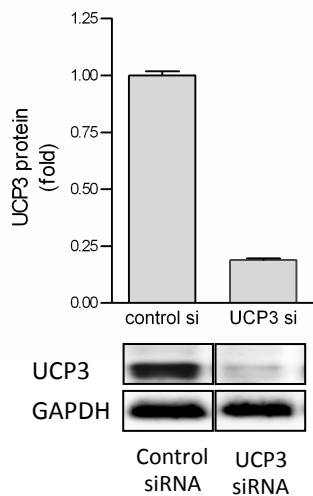
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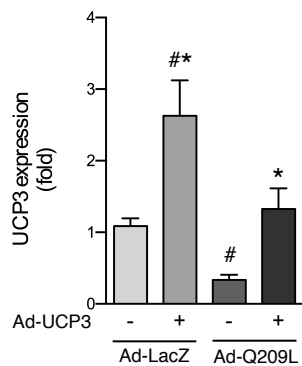
**Supplemental Fig VI. Effects of CaMKII $\delta$  splice variants on PPAR- $\alpha$  and UCP3 expression.** Neonatal rat ventricular myocytes were infected with constitutively activated CaMKII $\delta b$ , constitutively activated CaMKII $\delta c$  or  $\beta$ -galactosidase (Ad-LacZ) for 24 hours. Shown are A, UCP3 mRNA expression normalized for 36B4. B, PPAR- $\alpha$  mRNA expression normalized for 36B4. C, PPAR- $\alpha$  and UCP3 protein expression normalized for actinin. #,  $p < 0.05$  versus LacZ. LacZ, Ad-LacZ;  $\delta b$ , adenovirus expressing constitutively activated CaMKII $\delta b$ ;  $\delta c$ , adenovirus expressing constitutively activated CaMKII $\delta c$



A



B



**Supplemental Fig VII. Overexpression and silencing of UCP3 in neonatal rat ventricular myocytes.** A, Expression of UCP3 normalized for GAPDH in whole cell lysates of neonatal rat ventricular myocytes (NRVMs), 48 hours after transfection with control or UCP3 siRNA (Bioneer). Typical Western blot of samples for figure 4B are shown. The lanes were run on the same gel but were noncontiguous. B, expression of UCP3 mRNA normalized for 36B4 in NRVMs, 24 hours after infection with an adenovirus expressing UCP3 (Ad-UCP3), constitutively activated Gαq (Ad-Q209L) or β-galactosidase (Ad-LacZ). #, p < 0.05 versus or Ad-LacZ, \* p < 0.05 versus Ad-Q209L.