Tumor Necrosis Factor Receptor–Associated Factor 2 Mediates Mitochondrial Autophagy
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Background—Tumor necrosis factor (TNF) signaling protects against ischemia/reperfusion–induced cardiomyocyte death, in vitro, ex vivo, and in vivo. TNF-receptor–associated factor 2 (TRAF2), an E3 ubiquitin ligase, coordinates cytoprotective signaling downstream of both TNF receptors, via unclear mechanisms. Noting that TRAF2 is recruited to mitochondria, and that autophagic removal of ubiquitin-tagged damaged mitochondria is cytoprotective, we tested the hypothesis that TRAF2 mediates mitochondrial autophagy.

Methods and Results—TRAF2 localizes to the mitochondria in neonatal rat cardiac myocytes, and TNF treatment transcriptionally upregulates TRAF2 abundance in the mitochondrial subfraction. TRAF2 colocalizes with ubiquitin, p62 adaptor protein, and mitochondria within LC3-bound autophagosomes; and exogenous TRAF2 enhances autophagic removal of mitochondria. TRAF2 knockdown with adenoviral shRNA transduction induces accumulation of depolarized mitochondria in resting neonatal rat cardiac myocytes, as well as in those treated with TNF or uncoupling agent carbonyl cyanide m-chlorophenyl hydrazine, suggesting an essential role for TRAF2 in homeostatic and stress-induced mitochondrial autophagy. TRAF2 also colocalizes and interacts with PARKIN, a previously described E3 ubiquitin ligase and mitophagy effector, on depolarized mitochondria in neonatal rat cardiac myocytes. Exogenous expression of TRAF2, but not its E3 ligase-deficient mutants, is sufficient to partially restore mitophagy in the setting of PARKIN knockdown, suggesting redundancy in their ubiquitin ligase roles. TRAF2 abundance increases in the mitochondrial subfraction of ischemia/reperfusion–modeled hearts; and exogenous TRAF2, but not its E3 ligase-deficient mutants, reduces depolarized mitochondria and rescues cell death in neonatal rat cardiac myocytes subjected to hypoxia/reoxygenation.

Conclusions—Taken together, these data indicate an essential role for TRAF2 in concert with PARKIN as a mitophagy effector, which contributes to TRAF2-induced cytoprotective signaling. (Circ Heart Fail. 2015;8:175-187. DOI: 10.1161/CIRCHEARTFAILURE.114.001635.)

Key Words: mitochondrial degradation ■ TNF receptor ■ TNF receptor–associated factor 2

Emerging evidence indicates that activation of innate immunity signaling is critical for myocardial adaptation to stress.1 One such highly evolutionary conserved pathway is activated by tumor necrosis factor (TNF), the prototypical member of the TNF superfamily of ligands.2 Indeed, TNF pre-treatment, or activation of either TNFR1 or TNFR2 receptor, prevents hypoxia/reperfusion-induced cell death in mammalian cardiomyocytes, in-vitro; and transgenic expression or exogenous administration of low doses of TNF attenuates ex vivo cardiac ischemia/reperfusion (I/R) injury.3,4 Also, absence of both TNFR1 and TNFR2 receptors increases I/R-induced cardiomyocyte death, ex vivo,4 and results in marked increase in infarct size with in vivo coronary ligation as compared with controls,5 pointing to a redundancy in cytoprotective signaling triggered by TNF via its cognate receptors. In this context, it is notable that TNF receptor–associated factor 2 (TRAF2), a scaffolding protein, is recruited to both TNF receptors on their activation,2 and its transgenic expression (at low levels) attenuates cardiomyocyte death with experimental ex vivo I/R injury.4 Although these data suggest that TRAF2 may facilitate cytoprotective signaling downstream of both TNF receptors, the underlying mechanisms remain largely unknown, despite extensive investigation.3-5

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I/R injury results in generation of reactive oxygen species, which provoke mitochondrial permeabilization leading to programmed cardiomyocyte death.5 Autophagy is an
evolutionarily conserved prosurvival pathway that sequesters damaged mitochondria within autophagosomes resulting in their intralysosomal degradation (by mitophagy), which is essential to protect against cardiomyocyte death in myocardial infarction. Activation of mitophagy also plays a central role in ischemia preconditioning. TNF signaling is implicated in induction of cardiomyocyte autophagy, which is cytoprotective against lipopolysaccharide-induced cell death. Whether TNF induces mitophagy or signals via TRAF2 to promote mitochondrial autophagy is not known.

Ubiquitination of mitochondrial proteins in response to mitochondrial damage is essential for their sequestration and degradation within the lysosomes. PARKIN, an E3 ubiquitin ligase, is recruited to damaged mitochondria via activation by PINK1 (PTEN-induced putative kinase 1, a serine-threonine kinase), and ubiquitinates mitochondrial proteins. However, although targeted ablation of PINK1 in cardiac myocytes results in mitochondrial abnormalities and cardiomyopathy, loss of PARKIN is well tolerated in the unstressed state, suggesting that other E3 ubiquitin ligases may be involved in removal of damaged mitochondria. Relevant to this discussion is the observation that TRAF2, an E3 ubiquitin ligase, is recruited to the mitochondria by mitochondrial antiviral signalosome, a mitochondrial localized protein with multiple scaffolding domains, after activation of innate immune signaling pathways. Therefore, we tested the hypothesis that TRAF2 mediates mitochondrial autophagy. Here, we show for the first time that TRAF2 is present on the mitochondria in resting cardiac myocytes, and functions in concert with PARKIN as an E3 ubiquitin ligase to facilitate autophagic removal of damaged mitochondria, raising the intriguing possibility that TRAF2-mediated mitophagy may be an important determinant of cytoprotective TNF signaling in I/R injury.

Methods

Neonatal rat cardiac myocyte (NRCM) cultures were prepared as described. MHCsTNF mice with cardiomyocyte-specific overexpression of wild-type (secreted) TNF were described previously. In vivo I/R injury was performed in adult male C57Bl6 mice, as described. All animal studies were approved by the Animal Studies Committee at Washington University School of Medicine and by the Institutional Animal Care and Use Committee at the John Cochran VA Medical Center. Hypoxia/reoxygenation modeling, generation of adenoviral constructs, immunofluorescence, and electron microscopy

Statistical Analysis

Post hoc pairwise comparison results are expressed as mean±SEM. Statistical differences were assessed with the unpaired 2-tailed Student t test for 2 experimental groups, 1-way ANOVA for multiple groups; and 2-way ANOVA for testing 2 variables across multiple groups, with the SPSS software. Bonferroni post hoc pairwise comparison adjustment was used after ANOVA for testing for significant differences between groups. Assumptions of normality were verified with visual examination of the residuals via histograms and the Shapiro–Wilk test; and variance was assessed by using the Levene test for equality of variances for t test and the Levene homogeneity of variance test for ANOVA, using SPSS software. Nonparametric tests (Mann–Whitney and Kruskal–Wallis) were used for data that was not normally distributed (in lieu of t test and 1-way ANOVA, respectively); and Dunnett T3 post hoc analysis was applied after 1-way ANOVA for data demonstrating unequal variance. A 2-tailed P value of <0.05 was considered statistically significant.

Results

TNF Induces Mitochondrial Autophagy With Increased TRAF2 Localization to Mitochondria

Previous studies indicate that TNF treatment is sufficient to induce autophagy in cardiomyocytes. We confirmed that TNF treatment induces autophagy in isolated NRCMs with progressive increase in levels of autophagosome-bound LC3-II (Figure 1A) and decline in p62 (an adaptor protein that gets consumed during autophagy; Figure 1A; Figure IA in the Data Supplement), without alteration in their transcript levels (Figure IB and IC in the Data Supplement). To evaluate the role of TRAF2 in TNF-induced autophagy, we examined TRAF2 expression and distribution in TNF-treated NRCMs. TNF treatment induced a time-dependent upregulation of TRAF2 protein (Figure 1A and IB) and transcripts (Figure 1C), paralleling the induction of autophagy. Guided by recent data demonstrating that TRAF2 is targeted to the mitochondria either as a part of the death-inducing signaling complex on TNF stimulation or via its interactions with a scaffolding protein, mitochondrial antiviral signaling, after activation of innate immune responses, we examined whether TRAF2 localizes to mitochondria in cardiac myocytes. We found that endogenous TRAF2 cosegregates with cytochrome oxidase IV (COX IV), which is an inner mitochondrial membrane-localized respiratory chain protein (Figure 1D) in unstimulated NRCMs, and colocalizes with translocase of outer mitochondrial membrane 20 homologue (Figure 1E; Figure IIA in the Data Supplement), an outer mitochondrial membrane protein, as well as with COX IV (Figure IIB and IIC in the Data Supplement) on confocal microscopy, indicating that it is present on the mitochondria in the resting state. Notably, TNF treatment increased the relative abundance of TRAF2 in the mitochondrial subfraction (by 30% versus diluent-treated group; n=3/group; P=0.026; Figure 1D), with increased colocalization of endogenous TRAF2 and mitochondrial proteins in punctate GFP-LC3-labeled autophagosomes (Figure 1E; Figure IIA to IIC in the Data Supplement). TRAF2 also colocalizes with ubiquitin (Figure 1F; Figure IID in the Data Supplement) and mitophagy adaptor protein, p62 (Figure 1G; Figure IIE in the Data Supplement), in GFP-LC3-labeled autophagosomes in unstimulated NRCMs; and this colocalization was further stimulated by TNF treatment, suggesting that TRAF2 may participate in ubiquitination of mitochondrial proteins and autophagic sequestration of damaged mitochondria in both resting and TNF-treated cells.

In parallel studies, we also found that TRAF2 cosegregates with COX IV in mitochondria-enriched subfraction from wild-type mouse hearts (Figure 1H); and its abundance is increased in the cardiac mitochondrial fraction of transgenic mice with cardiac restricted overexpression of TNF (fold change in TRAF2/COX IV compared with control: 3.2±0.4-fold in MHCsTNF mice versus 1.0±0.1 in littermates; P=0.005; n=3/
group; Figure 1H). Indeed, similar to the observations with TNF treatment of NRCMs, total TRAF2 abundance is transcriptionally induced in MHCsTNF transgenic hearts (Figure 1I and 1J; Figure IIIA in the Data Supplement), along with increased autophagosome-bound LC3-II (Figure 1I; Figure IIIB in the Data Supplement). Interestingly, the increase in total LC3 and p62 protein abundance (Figure 1I; Figure IIIC and IIID in the Data Supplement) and their respective transcript levels (Figure IIIE and IIIF in the Data Supplement) points to a transcriptional stimulation of autophagy with sustained TNF signaling in MHCsTNF hearts, whereas a transcriptional response is not observed with short-term TNF treatment, in vitro (Figure IB and IC in the Data Supplement). Importantly, ultrastructural examination of cardiomyocytes from MHCsTNF transgenic myocardium demonstrated evidence of mitophagy with abnormal mitochondria manifesting swelling and rarefaction of cristae (Figure IVA in the Data Supplement) enclosed within autophagic structures. This was accompanied by significantly reduced mitochondrial DNA content (Figure IVB in the Data Supplement), and a trend toward reduced citrate synthase activity (Figure IVC in the Data Supplement) in the MHCsTNF myocardium as compared with littermate controls, indicating reduced mitochondrial mass, likely as a result of upregulated mitochondrial autophagy. Taken together, these data confirm that TNF induces TRAF2 expression with evidence of autphagic sequestration of mitochondria, which raises the interesting possibility that TNF and TRAF2-induced mitophagy may contribute to the reduced mitochondrial mass observed in MHCsTNF mouse hearts.

**Exogenous TRAF2 Is Sufficient to Induce Mitochondrial Autophagy**

To determine whether TRAF2 is sufficient to stimulate autophagy in cardiac myocytes, we adenovirally transduced NRCMs with TRAF2 and evaluated distribution of GFP-tagged LC3. As shown, exogenous TRAF2 expression induced autophagosome formation resulting in increased GFP-tagged LC3 puncta, as compared with control (Figure 2A). TRAF2 overexpression (~10-fold compared with endogenous levels; Figure 2B) induced a decline in endogenous LC3-II levels (Figure 2B and 2C), with a decline in total LC3 abundance (fold change in total LC3/GAPDH compared with control: 0.51±0.09 in Ad-TRAF2 versus 1.0±0.03 in Ad-LacZ-treated controls; P<0.001; N=7/group; Figure 2B). Taken together with the accumulation of LC3-II and p62 (Figure 2B to 2D) in the presence of Bafilomycin A1 (to inhibit lysosomal acidification), these data indicate that TRAF2 stimulates autophagy with intact flux. Exogenous TRAF2 colocalized with GFP-tagged punctate LC3 (Figure 2E to 2G; Figure IIF in the Data Supplement), p62 (Figure 2E), and ubiquitin (Figure 2F) in ring-shaped structures, many
of which enclose mitochondria (Figure 2G and Figure IIF in the Data Supplement). Interestingly, this colocalization was observed with increased frequency in TNF-treated NRCMs (Figure 2E to 2G; Figure IIF in the Data Supplement) wherein the mitochondria seem increasingly fragmented (Figure IIB and IIF in the Data Supplement), suggesting a role for exogenous TRAF2 in ubiquitination and autophagic sequestration of damaged mitochondria in TNF-treated cells. Ultrastructural analysis of TRAF2-treated NRCMs revealed multiple autophagic structures enclosing mitochondria (Figure 2H).

We also observed occasional amorphous dense deposits within TRAF2-transduced cardiac myocytes by electron microscopic analysis. Such deposits are observed in mice with transgenic expression of high levels of TRAF2 in the mouse myocardium, and may represent the aggregates of ubiquitinated cytoskeletal proteins observed in the MHCsTNF hearts. Although this observation suggests a more generalized role for TRAF2 downstream of TNF signaling in protein ubiquitination, these data, taken together, indicate that TNF-induced upregulation of TRAF2 is also involved in ubiquitination of mitochondrial proteins targeted for autophagic sequestration.

Exogenous TRAF2 provoked a decline in mitochondrial mass assessed by cardiolipin content (with expression of nonyl-acridine orange, NAO; Figure 3A and 3B), mitochondrial protein abundance (Figure 3C and 3D), and mitochondrial DNA content (Figure 3E); which was prevented by treatment with 3-methyl adenine (3MA, a PI3Kinase III inhibitor, which inhibits autophagosome formation). Also, TRAF2 overexpression led to reduced citrate synthase activity (100.7±7.8 versus 147.8±4.1 nmol/mg per minute in LacZ-treated controls; P=0.006; n=3/group), indicating that exogenous TRAF2 is sufficient to provoke autophagic removal of mitochondria.

Endogenous TRAF2 Is Essential for Removal of Mitochondria With TNF Treatment

TNF treatment in NRCMs induced a decline in mitochondrial mass assessed with NAO expression (Figure VA and VB in the Data Supplement) and mitochondrial DNA content (Figure VC in the Data Supplement) as compared with control; and concomitant inhibition of autophagy with 3MA prevented this decline (Figure VA to VC in the Data Supplement), indicating that TNF induces mitophagy, in vitro, mirroring the observations in MHCsTNF transgenic mice (Figure IV in the Data Supplement). To examine the role of endogenous TRAF2 in TNF-induced mitophagy, we adenovirally transduced NRCMs with shRNA targeting rat TRAF2 (or LacZ as nontargeting control; Figure 4A) and assessed total mitochondrial mass and relative content of depolarized mitochondria. Interestingly,
knockdown of endogenous TRAF2 provoked a significant increase in NAO expression and mitochondrial DNA content not only in TNF-treated but also in diluent-treated NRCMs (Figure 4B to 4D), with increased citrate synthase activity (310.6±20.1 with TRAF2shRNA versus 171.7±12.8 nmol/mg per minute in LacZshRNA control; *P*<0.001; *n*=3/group) in diluent-treated NRCMs, indicating that TRAF2 knockdown results in increased mitochondrial mass. To assess whether increased mitochondrial biogenesis was contributing to the observed increase in mitochondrial mass, we examined the transcript levels for PGC1α and PGC1β, 2 transcriptional coactivators essential for mitochondrial biogenesis in cardiac myocytes.29 TRAF2 knockdown was associated with a significant decline in the expression of PGC1α and PGC1β (Figure VI in the Data Supplement) as compared with controls, suggesting suppression of the mitochondrial biogenesis program, likely in response to accumulation of damaged mitochondria with TRAF2 knockdown. Indeed, TRAF2 knockdown resulted in a relative increase in depolarized mitochondria in diluent-treated NRCMs, evidenced by a significant increase in expression of JC-1 monomers (fluoresce green, right lower quadrant in Figure 4E and 4F) with concomitant loss of JC-1 aggregates (cells that fluoresce both red and green, right upper quadrant), indicating loss of mitochondrial inner membrane potential. These observations with TRAF2 knockdown mirror the effect of 3MA treatment to inhibit basal autophagy in resting cells, which also results in accumulation of depolarized mitochondria (Figure VII in the Data Supplement) and increased mitochondrial mass (Figure VA to VC in the Data Supplement).31

Importantly, the relative increase in NAO expression with TRAF2 knockdown (as compared with LacZshRNA-treated control) was significantly more in TNF-treated as compared with diluent-treated controls (3.4±0.4 versus 1.5±0.1-fold compared with respective LacZshRNA group; *P*<0.001; *n*=7/group; Figure 4B and 4C) with a trend toward increase in mitochondrial DNA content (2.7±0.2 versus 2.2±0.1-fold compared with respective LacZshRNA-treated group; *P*<0.07; *n*=6/group; Figure 4D). Also, TRAF2 knockdown resulted in significantly increased content of depolarized mitochondria in TNF-treated as compared with diluent-treated NRCMs (Figure 4E and 4F), indicating further accumulation of depolarized mitochondria with TRAF2 deficiency in TNF-treated cells. Remarkably, a gain of function strategy with exogenous TRAF2 expression had the opposite effect. Exogenous TRAF2 significantly reduced the relative proportion of depolarized mitochondria in TNF-treated cells (Figure VE and VE in the Data Supplement) with a trend toward a further reduction in NAO expression (as compared with Ad-LacZ control; Figure VA left and VB in the Data Supplement). And, although exogenous TRAF2 did not further reduce mitochondrial DNA in TNF-treated cells (Figure VC in the Data Supplement), examination of flux with simultaneous 3MA treatment to prevent mitochondrial autophagy32,33 revealed that TNF treatment increased flux through mitochondrial autophagy pathway which was further upregulated by concomitant exogenous TRAF2 expression, as evidenced by fold increase in NAO expression with 3MA treatment (1.2±0.1, 2.5±0.3, and 4.7±0.1-fold increase compared with the respective diluent-treated group in 3MA only, TNF+3MA, and TNF+TRAF2+3MA, respectively; *P*<0.05 by post hoc pairwise comparison for all comparisons; *n*=5/group; Figure VB in the Data Supplement) and fold increase in mitochondrial DNA content with 3MA treatment (1.2±0.1, 1.4±0.1, and 1.6±0.1-fold increase compared with respective diluent-treated group in 3MA only, TNF+3MA, and TNF+TRAF2+3MA, respectively; *P*<0.05 by post hoc pairwise comparison for all comparisons; *n*=5–11/group; Figure VC in the Data Supplement). Taken together, these data point to an essential role for TRAF2 in facilitating TNF-induced mitophagy.

**Endogenous TRAF2 Is Essential for Homeostatic Clearance of Depolarized Mitochondria**

The observation that TRAF2 knockdown increases mitochondrial mass and depolarization without inducing the mitochondrial biogenic program in resting cells (vide supra) indicates accumulation of damaged mitochondria with TRAF2 deficiency. To confirm this finding, we examined the mitochondrial ultrastructure in NRCMs transduced with TRAF2shRNA, and found a striking accumulation of degenerating structures containing mitochondria-like crista within...
Figure 4. Endogenous tumor necrosis factor (TNF) receptor–associated factor-2 (TRAF2) is essential for removal of damaged mitochondria. A, Immunoblot depicting knockdown of endogenous TRAF2 protein with increasing dose (multiplicity of infection [MOI]) of viral transduction after 72 hours. Adenoviral particles coding for shRNA targeting LacZ (as control) were added to equalize viral dose. B and C, Nonyl-acridine orange (NAO) expression with representative flow cytometric tracings (B) and quantification of mean fluorescence (C) in neonatal rat cardiac myocytes (NRCMs) adenovirally transduced with shRNA targeting rat TRAF2 or LacZ as nontargeting control (MOI=200; 72 h) and treated with TNF (200 ng/mL; 24 h). N=7/group. P values are by pairwise comparison after Kruskal–Wallis test. D, Mitochondrial DNA (n=5–6/group); and E and F, JC-1 expression (n=3/group) in NRCMs treated as in B. P values are by post hoc pairwise comparison after 1-way ANOVA. G, Representative transmission electron microscopic images of NRCMs transduced with shRNA targeting rat TRAF2 or LacZ for 72 hours. Black arrows point to membrane bound structures, often with multiple layers of membranes akin to onion skin appearance, enclosing remnants of mitochondrial cristae.
multiple membranes, often in an onion skin-like whorled appearance (Figure 4G). Conceivably, these structures represent damaged mitochondria in various stages of degeneration, when their well-orchestrated autophagic removal is prevented in the absence of TRAF2.

Previous studies have demonstrated a central role for PINK1 in sensing mitochondrial damage to facilitate recruitment of an E3 ubiquitin ligase, PARKIN, to ubiquitinate mitochondrial proteins and target damaged mitochondria for autophagic degradation. However, the lack of alteration in cardiac mitochondrial autophagy in cardiac myocytes. We treated NRCMs with carbonyl cyanide m-chlorophenyl hydrazone (CCCP), an ionophore, that provokes loss of mitochondrial membrane potential and targets depolarized mitochondria (Figure 4), we tested the hypothesis that TRAF2, which is also an E3 ubiquitin ligase, plays an important role in autophagic removal of depolarized mitochondria in cardiomyocytes. Given our observations with accumulation of depolarized mitochondria with TRAF2 knockdown (Figure 4), we tested the hypothesis that TRAF2, which is also an E3 ubiquitin ligase, plays an important role in autophagic removal of depolarized mitochondria in cardiomyocytes. We treated NRCMs with carbonyl cyanide m-chlorophenyl hydrazone (CCCP), an ionophore, that provokes loss of mitochondrial membrane potential and targets mitochondria for autophagic degradation. Treatment with CCCP provoked progressive mitochondrial fragmentation with loss of mitochondrial potential-dependent mitotracker staining (pink, Figure 5A), as described previously and increased colocalization of endogenous TRAF2 (red) with depolarized mitochondria (identified by COX IV, green; with colocalization in yellow, Figure 5A). Also, CCCP treatment provoked an increase in TRAF2 abundance in the mitochondria-enriched subcellular fraction, paralleling a relative increase in mitochondrial PARKIN abundance (Figure 5B). CCCP treatment provoked mitochondrial depolarization (Figure 6A and 6B) and a decline in mitochondrial mass, as evidenced by a reduction in NAO fluorescence (Figure 6C and 6D) with a trend toward reduced mitochondrial DNA content (Figure 6E). Notably, all parameters were markedly increased with concomitant 3MA treatment, indicating accumulation of CCCP-damaged mitochondria, when their autophagic removal is impaired. Importantly, TRAF2 knockdown also provoked an increase in depolarized mitochondria (Figure 6A and 6B) with increased mitochondrial mass (assessed by NAO and mitochondrial DNA content; Figure 6D and 6E) in CCCP-treated NRCMs, mimicking the effects of 3MA treatment. Taken together, these data indicate that TRAF2 plays an essential role in homeostatic removal of depolarized mitochondria in cardiomyocytes.

**TRAF2 Functions in Concert With PARKIN to Remove Damaged Mitochondria**

Analogous to the observation that TRAF2 abundance is increased in the mitochondrial compartment with TNF stimulation (Figure 1D and 1E; Figure IIB in the Data Supplement) and in MHCsTNF hearts (Figure 1H), TNF treatment also stimulates increased PARKIN abundance (Figure VIII A in the Data Supplement) and mitochondrial localization (Figure VIIIB in the Data Supplement) in NRCMs, and MHCsTNF hearts have increased PARKIN levels (Figure VIIIC in the Data Supplement) in the mitochondrial subfraction (Figure VIIID in the Data Supplement). Viewed together with the observation that both TRAF2 and PARKIN localize to the mitochondrial fraction with CCCP treatment (Figure 5B), these data indicate that as observed with PARKIN, TRAF2 is also recruited to damaged mitochondria. Indeed, TRAF2 colocalizes with PARKIN on mitochondria in resting cardiomyocytes, presumably on damaged mitochondria (Figure 7A, arrows; Figure IXA in the Data Supplement); and this colocalization is markedly enhanced with CCCP treatment, suggesting that TRAF2 and PARKIN are recruited together to depolarized mitochondria (Figure 7A; Figure IXA in the Data Supplement). Interestingly, previous studies suggest that PARKIN physically interacts with TRAF2 and ubiquitinates with it, independent of TRAF2’s endogenous E3 ubiquitin ligase activity, to activate TRAF2-mediated signaling. We have confirmed this physical interaction with a FRET (Forster resonance energy transfer) assay using a DsRed and AcGFP FRET pair-tagged TRAF2 and PARKIN, respectively (with controls in Figure IXB in the Data Supplement). The interaction between TRAF and...
PARKIN is minimal in the resting state (arrows, Figure 7B) and is markedly enhanced in presence of CCCP-induced mitochondrial depolarization (arrows, Figure 7B), suggesting increased interaction between these proteins on mitochondrial damage, which was confirmed via coimmunoprecipitation studies in the mitochondrial subfraction of CCCP-treated cells (Figure IXC and IXD in the Data Supplement).

To examine the role of TRAF2 vis-à-vis PARKIN in mitochondrial autophagy, we transduced NRCMs with adenoviruses coding for shRNA targeting PARKIN (Figure XA in the Data Supplement) in concert with exogenous TRAF2, and examined mitochondrial mass (by NAO expression) and prevalence of depolarized mitochondria (by JC-1). PARKIN knockdown provoked accumulation of depolarized mitochondria with increased NAO expression (Figure 7C and 7D) and of JC-1 monomers (Figure 7E) in the resting state, with further increase observed with CCCP-induced depolarization. Exogenous expression of full length TRAF2, but not TRAF2 mutants deficient in E3 ubiquitin ligase activity (TRAF2-Rm and ΔN-TRAF2; Figure XI in the Data Supplement), partially attenuated the increase in NAO expression (Figure 7C and 7D) and abundance of depolarized mitochondria (Figure 7E) in CCCP-treated NRCMs transduced with PARKINshRNA (by ~44% and 22%, respectively). Similarly, expression of exogenous TRAF2, but not the E3 ubiquitin ligase–deficient mutants, was sufficient to partially attenuate the accumulation of mitochondria in TNF-treated NRCMs transduced with PARKINshRNA (Figure XIIA and XIIB in the Data Supplement). Therefore, TRAF2 can partially complement PARKIN deficiency as an E3 ubiquitin ligase to promote mitochondrial autophagy.

We next evaluated whether PARKIN can substitute for TRAF2 in promoting mitochondrial autophagy. Exogenous PARKIN (Figure XB in the Data Supplement) is sufficient to provoke a decline in NAO expression (Figure 7C and 7D) and abundance of depolarized mitochondria (Figure 7E) in CCCP-treated NRCMs (Figure 7H), indicating that PARKIN overexpression is sufficient to facilitate autophagic removal of damaged mitochondria. Interestingly, exogenous PARKIN is also sufficient to partially restore mitochondrial autophagy in the

Figure 6. Endogenous tumor necrosis factor (TNF) receptor–associated factor-2 (TRAF2) is essential for removal of carbonyl cyanide m-chlorophenyl hydrazone (CCCP)-depolarized mitochondria. A and B, Representative flow cytometric analysis of JC-1 expression (A) with quantification (B) of cells with depolarized mitochondria (n=3/group) in neonatal rat cardiac myocytes (NRCMs) adenovirally transduced with shRNA targeting TRAF2 or LacZ (multiplicity of infection=200; 72 h) and treated with CCCP (50 μmol/L; 24 h) ±3-methyl adenine (3MA; 7 mmol/L; 24 h) or diluent (n=5–6/group). P values are by post hoc pairwise comparison after 1-way ANOVA. *P=0.033 vs control (white bar). C and D, Representative flow cytometric tracings (C) with quantification (D) of nonyl-acridine orange (NAO) expression (n=3–6/group) in NRCMs treated as in A. P values are by post hoc pairwise comparison after 1-way ANOVA. *P=0.048 and #P=0.025 vs control (open bars). E, Mitochondrial DNA content in NRCMs treated as in A (n=3–6/group). P values reported are by pairwise comparisons after Kruskal–Wallis test. *P=0.030 vs control (open bars).
setting of TRAF2 knockdown, as evidenced by a reduction in NAO expression (by ≈29%; Figure 7F and 7G) and depolarized mitochondria (by ≈40%; Figure 7H), when compared with TRAF2-deficient cells treated with Ad-LacZ as control. Notably, endogenous levels of TRAF2 are only modestly increased in the setting of PARKIN knockdown, whereas PARKIN levels are not significantly altered when TRAF2 is knocked down, in vitro in NRCMs (Figure XIII A and XIIIC). This observation suggests that at least in vitro regulation of endogenous TRAF2 and PARKIN may be unable to compensate for each other deficiency, resulting in a defect in mitophagy when either is knocked down. Interestingly, mitochondrial proteins accumulate differentially with deficiency of TRAF2 or PARKIN, suggesting independent roles in ubiquitination and degradation of specific mitochondrial proteins for these E3 ubiquitin ligases (Figure XIII A and XIIID to XIIIF). Taken together, these findings suggest that TRAF2 functions as an E3 ubiquitin ligase in concert with PARKIN to facilitate ubiquitination of proteins on damaged mitochondria and assist in their removal through the process of autophagy (Figure 7I).

**TRAF2 Facilitates Mitophagy to Confer Cytoprotection in Hypoxia/Reoxygenation Injury**

ROS-induced mitochondrial permeabilization is implicated in provoking cardiomyocyte death in I/R injury. To examine whether TRAF2 is recruited to the mitochondria with I/R-induced mitochondrial damage, we subjected mice to in vivo I/R modeling and performed subcellular fractionation on injured myocardial tissue. Interestingly, we observed a relative increase in TRAF2 abundance in the mitochondrial subfraction from the injured myocardium, early after I/R injury, paralleling that of PARKIN (Figure 8A and 8B). To determine the role of TRAF2 in mitochondrial autophagy in...
this setting, we examined subcellular location of TRAF2 and performed loss-of-function and gain-of-function modeling for TRAF2 in NRCMs subjected to hypoxia/reoxygenation injury, in vitro. Endogenous TRAF2 increasingly colocalized with depolarized mitochondria (with loss of mitotracker signal) in hypoxia/reoxygenation-injured NRCMs (Figure 8C). Knockdown of endogenous TRAF2 resulted in accumulation of depolarized mitochondria (Figure 8D) and increased cell death (Figure 8F) under normoxic conditions, which was markedly exacerbated with hypoxia/reoxygenation injury (Figure 8D and 8F). Conversely, exogenous TRAF2, but not its E3 ligase-deficient mutants (which are also mitophagy-deficient, vide supra), was sufficient to attenuate hypoxia/reoxygenation-induced cell death (Figure 8G) with a significant reduction in relative content of depolarized mitochondria (Figure 8E). Interestingly, exogenous expression of the E3 ligase-deficient mutants enhanced hypoxia/reoxygenation-induced cell death with increased content of depolarized mitochondria, paralleling our recent observations in ex vivo I/R injury,17 indicating a dominant negative effect. Taken together, these data suggest a central role for TRAF2 as an E3 ubiquitin ligase in facilitating mitophagy as a mechanism for its observed cytoprotection with I/R injury.

Discussion

The results of the present study suggest that TRAF2 mediates mitochondrial autophagy through an E3 ligase-dependent mechanism in both unstressed and stressed mammalian cardiomyocytes. The following lines of evidence support this conclusion. First, TRAF2 localizes to the mitochondria in quiescent cardiac myocytes and in native wild-type hearts. In the setting of TNF-induced mitochondrial damage, TRAF2 expression is transcriptionally upregulated with increased mitochondrial localization, both in vitro and in vivo (Figure 1). Second, knockdown of endogenous TRAF2 results in the accumulation of depolarized mitochondria in resting cells, as well as in cells treated with TNF (Figure 4), the uncoupling agent CCCP (Figure 6), and in cells subjected to hypoxia/reoxygenation injury (Figure 8), whereas exogenous TRAF2 is sufficient to facilitate autophagic removal of mitochondria in these settings (Figures 3, 4, and 8). Third, TRAF2 localizes to CCCP-depolarized mitochondria, where it interacts with PARKIN, a previously described mitophagy effector (Figures 5 and 7). Fourth, exogenous TRAF2 can partially restore mitophagy in the setting of PARKIN deficiency (Figure 7), whereas overexpression of PARKIN can partially overcome the effects of TRAF2 deficiency with respect to mitophagy, suggesting a partial redundancy in the E3 ligase activity of TRAF2 and PARKIN to facilitate ubiquitination and degradation of mitochondrial proteins. Fifth, endogenous TRAF2 increasingly localizes to the mitochondria in hearts subjected to in vivo cardiac I/R injury and in cardiac myocytes subjected to hypoxia/reoxygenation, in vitro (Figure 8). Finally, endogenous TRAF2 signaling is essential to prevent cardiomyocyte death with hypoxia/reoxygenation injury; and expression of exogenous TRAF2, but not its E3 ligase mutants, results in decreased abundance of depolarized mitochondria and enhanced cytoprotection with hypoxia/reoxygenation injury (Figure 8).

Mitophagy removes damaged mitochondria to protect against cell death in myocardial infarction10 and neurodegeneration,28 and is required for ischemic preconditioning in the myocardium.11 Our findings indicate that TRAF2-mediated mitophagy may be a mechanism whereby endogenous
TRAF2 signals downstream of either TNF receptor activation\(^7\) to prevent cardiomyocyte death with hypoxia/reoxygenation injury, in vitro,\(^1\) and with myocardial infarction, in vivo.\(^6\) Also, TRAF2-mediated upregulation of mitophagy may further attenuate I/R-induced cardiomyocyte death, as observed in transgenic mouse hearts with cardiomyocyte-specific expression of TRAF2 and TNF (at low levels) subjected to ex vivo I/R injury,\(^4\) and facilitate the preconditioning effects of TNF administration on the myocardium, in vivo.\(^3\) Conversely, the observed critical role for TRAF2 in mediating homeostatic mitophagy may explain the early lethality in TRAF2 null mice in the neonatal period, which is preceded by marked runting and elevated circulating TNF levels, with markedly increased cell death in various cell types.\(^39,40\)

Our data point to an essential role for TRAF2 in parallel with PARKIN in facilitating mitophagy in cardiac myocytes. Activation of PARKIN, an E3 ubiquitin ligase, by PINK1 (a serine-threonine kinase) on damaged mitochondria has been ascribed an essential role in orchestrating their autophagic removal, and impaired homeostatic mitophagy has been implicated as the underlying mechanism for development of Parkinson disease in individuals harboring mutations in their respective genes.\(^13\)–\(^15\) However, studies in the heart reveal that although mice with cardiomyocyte-specific ablation of PINK1 demonstrate cardiomyopathy with markedly abnormal mitochondria,\(^16\) loss of PARKIN (with PARK2 ablation) does not alter cardiac structure or function in young adult mice,\(^10\) or increase susceptibility to in vivo I/R injury.\(^11\) These observations suggest that PARKIN deficiency may be complemented by other proteins in the heart, and its deficiency becomes apparent only with a surge in mitochondrial damage as occurs with prolonged ischemia,\(^10\) or cumulatively with aging.\(^17\) Our data demonstrate that TRAF2 is present on the mitochondria in resting cardiomyocytes. In addition, TRAF2 has also been demonstrated to localize to the mitochondria on TNF receptor signaling associated with other proteins, namely TRADD, FADD, RIP1, and procaspase-8, in the death-inducing signaling complex\(^20\) or complex II\(^41\); and recent studies indicate that TRAF2 interacts with mitochondrial scaffolding proteins, such as mitochondrial antiviral signalosome,\(^19\)–\(^21\) whereby it would be positioned to participate in mitochondrial autophagy. Also, PARKIN interacts with TRAF2 (Figure 7B), and PARKIN-mediated ubiquitination has been demonstrated to activate TRAF2 in fibroblasts.\(^34\) Given our observation that damaged mitochondria accumulate with TRAF2 deficiency, mimicking the effects of PARKIN knockdown, we posit that TRAF2 functions in concert with PARKIN in ubiquitinating mitochondrial proteins on mitochondrial damage (as proposed in Figure 7I). Notably, although both exogenous PARKIN and TRAF2 are sufficient to partially restore mitophagy with each other deficiency, in vitro (Figure 7C to 7H), mitochondrial proteins accumulate differentially with individual deficiencies of TRAF2 and PARKIN (Figure XIII in the Data Supplement), suggesting both unique and redundant roles for TRAF2 vis-a-vis PARKIN. Further studies are required to clarify the molecular mechanism for recruitment of TRAF2 to damaged mitochondria, and determine whether PARKIN-mediated ubiquitination of TRAF2 is necessary to activate TRAF2 signaling; or TRAF2 functions independently of and in parallel to PARKIN in cardiomyocyte mitophagy. In addition, whether TRAF2-mediated ubiquitination is also required for removal of nonmitochondrial proteins via autophagy or ubiquitin-proteasome pathway needs to be explored.

Recent studies indicate that preventing removal of damaged mitochondria and mitochondrial DNA induces proinflammatory signaling.\(^42,43\) In this context, it is notable that TRAF2 null mice die of colitis with widespread bowel wall inflammation within the first 3 weeks of life,\(^39\) and treatment with antibiotics or concomitant TNF receptor 1 ablation only partially rescues the inflammatory colitis\(^39\) with a modest prolongation of lifespan.\(^44\) In addition, concomitant ablation of TNF does not attenuate the chronic inflammatory state observed in various organ systems of the TRAF2 null mice,\(^45\) indicating that the primary driver of the pathogenesis is downstream of the inflammatory mediators activated with loss of TRAF2. Intriguingly, mutations in genes that play critical roles in autophagic signaling have been implicated in loss of autophagy in enterocytes, leading to development of colitis and Crohn disease.\(^46\)–\(^48\) Therefore, impairment in homeostatic removal of damaged mitochondria may be the underlying mechanism for development of colitis and multiorgan inflammation in TRAF2-deficient mice, a premise which requires further experimental validation.

TRAF2 plays a prosurvival key role as an adaptor protein to transduce activation of kinases and transcription factors downstream of multiple innate immunity receptors.\(^1\)–\(^4\)\(^,41\) The results of the present study extend these prior observations\(^4\) and suggest a novel cytoprotective role for TRAF2 in mediating mitochondrial autophagy through an E3 ligase–dependent mechanism. Unraveling the role for innate immunity pathways with respect to regulation of mitochondrial mass and mitochondrial quality control may provide important insights in understanding the molecular mechanisms of human disease, as well as developing targeted therapies to counteract untoward effects of sustained inflammatory signaling.

**Acknowledgments**

We wish to thank Jeffrey E. Saffitz, MD, PhD, Chairman, Department of Pathology at Beth Israel Deaconess Medical Center for assistance with interpretation of electron micrographs. We thank Eric Novak, MS, Cardiovascular Division at Washington University for expert input on statistical analyses methodology. We also acknowledge Joan Avery and Lora Staloch from Center of Cardiovascular Research for technical assistance.

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**Disclosures**

None.
References


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**CLINICAL PERSPECTIVE**

Activation of innate immunity pathways protects the heart against stress, whereas sustained signaling downstream of multiple innate immunity mediators results in development of cardiomyopathy and heart failure. Understanding the cellular mechanisms of action of key players in these signaling cascades is therefore critical to develop targeted strategies to prevent and treat heart failure. Tumor necrosis factor–associated factor 2 (or TRAF2), a scaffolding protein, is one such nodal integrator of signaling downstream of tumor necrosis factor, a highly evolutionarily conserved prototypical innate immunity mediator. We have previously described that endogenous TRAF2 signaling protects against cardiomyocyte death with ischemia/reperfusion injury, and this cytoprotection can be enhanced with modest TRAF2 overexpression, via hitherto unclear mechanisms. In the present study, we find that endogenous TRAF2 localizes to mitochondria in cardiomyocytes, with increased localization on mitochondria damaged by hypoxia/reoxygenation injury, TNF signaling, and protonophore exposure. Damaged mitochondria provoke cell death in these settings; and ubiquitination of mitochondrial proteins targets them for removal by autophagy, an evolutionarily conserved lysosomal degradative pathway, to ensure cell survival. Our findings indicate that TRAF2 plays an essential role as a ubiquitin-ligating enzyme to facilitate mitochondrial autophagy, whereby loss-of-function of TRAF2 results in accumulation of damaged mitochondria and gain-of-function of TRAF2 is sufficient to enhance removal of damaged mitochondria and prevent cell death during hypoxia/reoxygenation injury. Interestingly, TRAF2 interacts with PARKIN, a previously described mitochondrial autophagy mediator; and they both play partially redundant roles in this process. TRAF2-mediated mitochondrial autophagy merits further evaluation as a therapeutic target in heart disease.
Tumor Necrosis Factor Receptor–Associated Factor 2 Mediates Mitochondrial Autophagy
Kai-Chun Yang, Xiucui Ma, Haiyan Liu, John Murphy, Philip M. Barger, Douglas L. Mann and Abhinav Diwan

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Detailed Methods

Cardiac myocyte culture: Neonatal rat cardiac myocyte (NRCM) cultures were prepared as described.\textsuperscript{1,2} Briefly, hearts were removed from 1-day-old Sprague-Dawley rats, the atria and great vessels were trimmed off, and tissue was finely minced followed by sequential digestion with 0.5 mg/ml collagenase (Worthington Biochemical, NJ). Ventricular cardiomyocytes were separated from fibroblasts by differential plating in media containing Dulbecco's modified Eagle's medium (DMEM), 10% horse serum, 5% fetal calf serum, 100 μmol/L bromodeoxyuridine, penicillin, streptomycin, and L-glutamine; followed by tissue culture in gelatin-coated plates in media containing modified DMEM, 100 μM bromodeoxyuridine, penicillin, streptomycin, L-glutamine, 10ng/mL insulin, 10ug/mL transferrin and 0.5mg/mL bovine serum albumin.

Generation of adenoviral constructs: The coding sequence for mouse TRAF2 (from mRNA sequence NM_009422.2) with N-terminal FLAG tag, and for rat PARKIN (from mRNA sequence NM_020093.1) with N-terminal HA-tag, were cloned into the pAd-CMV/V5-DESTTM vector (Invitrogen). Site directed mutagenesis was performed using the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene, 210518-5) to generate E3 ligase deficient mutants, termed Flag-TRAF2-Rm (with RING domain mutations, namely C49A, H51A, C54A and C57A) and Flag-TRAF2-ΔN (with deletion of 87 N-terminal amino acids including the RING domain), as previously described.\textsuperscript{3-5} Respective adenoviral particles were generated with the Virapower\textsuperscript{TM} system. Adenoviral particles coding for shRNA targeting rat TRAF2 (NM_001107815.2) and rat PARK2 (coding for PARKIN; as previously described \textsuperscript{6}) were generated with BLOCKiT-TM adenoviral system (Invitrogen). Specific oligo sequences employed (with targeted coding sequence underlined) were as follows: 1) TRAF2shRNA: top strand oligo: 5’-
CACCGAATACGAGAGCTGCCACGAAGCGAACTTCGTGGCAGCTCTCGTATT-3'; bottom strand oligo: 5’- AAAAAATACGAGAGCTGCCACGAAGTTTCGCTTCGTGGCAGCTCTCGTATTC-3’. 2) PARKINshRNA: top strand: 5’-
CACCGGAACAACAGATATCGTTCCACATAATGCAGAATTACTATGTGAACGATACTCTGGTTCC-3’; bottom strand: 5’-
AAAAGGAACAACAGATATCGTTCCACATAATGCAGAATTACTATGTGAACGATACTCTGGTTCC-3’. Adenovirus coding for GFP-tagged LC3 has been described previously ². Viral particles were generated in HEK293A cells and titered per manufacturer’s instructions.

**Immunofluorescence imaging:** Imaging for GFP-LC3, TRAF2 (FLAG tagged and endogenous TRAF2), mitochondria (COX-IV, TOM20, Mitotracker Red and Deep RedTM expression), p62 and ubiquitin, were performed on fixed and permeabilized NRCMs, as previously described² on a Zeiss Confocal LSM 700 Laser Scanning confocal using a 63x Zeiss Plan-Apochromat 63x/1.4 oil immersion objective. Co-localization was assessed with line scans using the Zen 2010B SP1 software.

**Flow cytometry:** NRCMs were incubated with nonyl-acridine orange (NAO, 10nM for 15 min at 37°C), or JC-1, a ratiometric mitochondrial polarization indicator dye (10µg/ml for 10 min at 37°C), and subjected to flow cytometry on FACScan instrument (Becton-Dickinson) as described¹. Cytlogic software (CyFlo) was employed to analyze 20,000 events per run.

**Assessment of mitochondrial DNA content:** Mitochondrial DNA content was assessed with quantitative PCR analysis as previously described.⁷ Briefly, DNA was prepared from mouse heart tissue and NRCM pellets. Real-time PCR was performed for mitochondrially encoded genes: ND2 or NADH2 and for the nuclear encoded genes: MX1 or RCAN1, in mouse and rat tissues, respectively. The ratio of copies of
mitochondrial to nuclear genes represents the relative mitochondrial copy number. Specific primers employed are listed in Supplementary Table 1.

Assessment of citrate synthase activity: Cardiac tissue was homogenized in 50mM Tris pH 7.4, and citrate synthase activity was measured with following the protocol described in the citrate synthase assay kit (CS0720, Sigma). Citrate synthase activity was normalized to total protein content measured by Bradford assay.

Immunoblotting: Immunoblotting was performed on cellular extracts using previously described techniques 28. Antibodies employed were as follows: FLAG (Sigma, F3165); LC3 (Novus Biologicals, NB100-2220); p62 (Abcam, ab5416); TRAF2 (Epitomics, 5525-1), HA (Sigma, H6908), COX IV (Abcam, ab14744); TOM20 (Sigma, WH0009804M1); VDAC (Cell Signaling, 4661); PARKIN (Abcam, ab15954); GAPDH (Abcam, ab22555) and α-Sarcomeric actin (Abcam, ab52219). Image J software was employed for quantitative analysis. Protein abundance was normalized to GAPDH or α-sarcomeric action expression and reported as fold change versus control. Chemicals employed were 3-methyladenine (EMD4Biosciences, 189490); Bafilomycin A1 (Sigma, B1793) and CCCP (Sigma, C2759).

Quantitative PCR analysis: Total RNA was extracted from mouse hearts and NRCMs using RNAeasy Fibrous Tissue Minikit (Qiagen, 74704) and RNAeasy Minikit (Qiagen, 74104), respectively. Quantitative PCR analysis was performed as described,1 using primers detailed in Supplementary table 2.

Electron Microscopy: Left ventricular myocardium from MHCsTNF and littermate wild type mice; and NRCMs (plated and treated on chamber slides) were fixed in a modified Karnovsky's fixative comprised of 3% glutaraldehyde, 1% paraformaldehyde in 0.1M sodium cacodylate buffer; followed by post-fixation in 2% osmium tetroxide in 0.1M sodium cacodylate buffer for 1 hour. The tissue or cell layer was then
stained with 2% aqueous uranyl acetate for 30 minutes, dehydrated in series of graded ethanol concentrations and embedded in PolyBedTM (Polysciences, 08792-1). Blocks were sectioned at 90nm thickness, post stained with Venable's lead citrate and viewed with a JEOL model 1200EX electron microscope (JEOL, Tokyo, Japan). Digital images were acquired using the AMT Advantage HR (Advanced Microscopy Techniques, Danvers, MA) high definition CCD, 1.3 megapixel TEM camera.

**Assessment of FRET interaction**: Constructs coding for DsRed-TRAF2 and GFP-PARKIN were created by cloning the respective coding sequences in frame with the fluorophores at the N-terminus, as described.² The constructs were co-transfected in HEK293 cells. FRET interaction was imaged as previously described.² A construct encoding for DsRed-monomer-GFP fusion protein was employed as positive control.²

**Sub-cellular fractionation**: Fresh cardiac tissue and NRCMs were subjected to biochemical fractionation to recover mitochondrial enriched and cytoplasmic fractions, as previously described.⁸ Briefly, tissue or cells were homogenized in a buffer containing 10 mM HEPES, pH 7.2, 320 mM sucrose, 3 mM MgCl₂, 25 mM Na₂P₂O₇, 1 mM DTT, 5 mM EGTA, 1 mM PMSF, and Complete Mini Protease Inhibitor Cocktail Tablet (Roche) and subjected to centrifugation at 3,800 g for 15 minutes to remove nuclei and myofibrils. The supernatant was subjected to centrifugation at 10,000 g for 20 minutes, and the resultant pellet was labeled mitochondrial fraction. The supernatant was then subjected to centrifugation at 100,000 g for 1 hour. The supernatant from this was labeled as the cytoplasmic fraction. Expression of proteins localized to the mitochondria (COXIV) and cytoplasm (GAPDH) was examined to confirm relative enrichment.

**Co-immunoprecipitation studies**: Murine embryonic fibroblasts (mefs) from C57BL/6 wild type mice were transduced with adenoviruses to express FLAG-TRAF2 or HA-PARKIN (100 MOI); and
subcellular fractionation performed to prepare mitochondria-enriched fraction. Three hundred micrograms of protein was incubated with anti-FLAG (Abcam, ab0290), anti-HA or normal rabbit IgG, and immunoprecipitation performed using DynaBeads (Invitrogen, 100.07D).

In vivo ischemia-reperfusion modeling: Adult male C57Bl/6 mice (8 weeks of age) were subjected to reversible left anterior descending artery coronary ligation, as described. Briefly, mice were anesthetized with a mixture of ketamine (80mg/kg) and xylazine (5mg/kg); surgically prepped and ventilated. After thoracotomy, the left anterior descending artery (LAD) was identified and a 9-0 polypropolene suture was passed under the LAD. A knot was tied over a 1-mm section of PE-10 tubing placed directly over the vessel to create the occlusion. Ischemia was confirmed by absence of blood flow verified visually and presence of ST elevations on EKG. Reperfusion was induced releasing the occlusion and confirmed by resolution of ST segment elevation. The apical section was subjected to sub-cellular fractionation, as the ischemia-reperfusion-injured region. Sham mice underwent the entire surgical procedure without occlusion of the LAD artery. All animal studies were approved by the Animal Studies Committee at Washington University School of Medicine and the Institutional Animal Care and Use Committee at the John Cochran Veterans Affairs Medical Center.

Hypoxia-reoxygenation modeling in vitro: Cells were subjected to hypoxia in vitro in an oxygen control cabinet (Coy Laboratories, Grass Lake, MI) mounted within an incubator and equipped with oxygen controller and sensor for continuous oxygen level monitoring, as described. A mixture of 95% nitrogen and 5% CO2 was utilized to create hypoxia and oxygen levels in the chamber were monitored and maintained at <1%.
Assessment of mitochondrial fragmentation: Mitochondrial morphology was ascertained as fragmented or tubular by visual inspection of individual cells at high magnification, as described. N=50 cells were evaluated per experimental group and results expressed as percent of cells showing predominantly fragmented mitochondria.

Assessment of cell death: Cell death assays were performed in 96 well plates (Nunc, Fisher) with the Live-Dead Cytotoxicity Viability kit for Mammalian cells (Invitrogen), using TECAN Infinite M200 Pro microplate reader (Tecan) following manufacturer’s instructions, as described.
**Supplementary Tables**

**Supplementary Table 1**

Primer sequences employed for mitochondrial DNA content analysis by quantitative PCR.

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<th>Species</th>
<th>Gene</th>
<th>Target</th>
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<th>Reverse (5’ – 3’)</th>
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<tr>
<td>Mouse</td>
<td><em>ND2</em></td>
<td>Mito</td>
<td>CCCATTCCACTTCTGAT</td>
<td>ATGATAGTAGAGTTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TACC</td>
<td>AGTAGCG</td>
</tr>
<tr>
<td>Mouse</td>
<td><em>Mx1</em></td>
<td>Nuclear</td>
<td>GACATAAGGTTAGCAG</td>
<td>TCTCGATATTACAGGG</td>
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<td></td>
<td></td>
<td></td>
<td>CTAAAGGATCA</td>
<td>CTAGCTAT</td>
</tr>
<tr>
<td>Rat</td>
<td><em>NADH2</em></td>
<td>Mito</td>
<td>ATCACCACCATTCTCGC</td>
<td>TCCTATGTTGGCAATT</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>AAT</td>
<td>GATG</td>
</tr>
<tr>
<td>Rat</td>
<td><em>RCAN</em></td>
<td>Nuclear</td>
<td>GGTGGTGTAGCCTCGA</td>
<td>CTTCATCCCTCCTTG</td>
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<td></td>
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<td>AG</td>
<td>TAAC</td>
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Supplementary Table 2

Primer sequences employed for detecting individual transcripts in mouse heart or NRCMs by quantitative PCR.

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<td>Mouse SQSTM1</td>
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<td>CGCCTTCATCCGAGAAAC</td>
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<tr>
<td>Mouse MAP1LC3b</td>
<td>CGTCCTGGACAAGACCAAGT</td>
<td>ATTGCTGTCGCCGAGATGCTTC</td>
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<tr>
<td>Mouse TRAF2</td>
<td>CCTACTGCTGAGCTATTCT</td>
<td>CAATCTTGTCTGGTCTAGC</td>
</tr>
<tr>
<td>Mouse GAPDH</td>
<td>ACTCCCACTCTTCCACCTTC</td>
<td>TCTTGCTCAGTGCTCTTTGC</td>
</tr>
<tr>
<td>Rat SQSTM1</td>
<td>GCTGCCCTGTACCCACATCT</td>
<td>CGCCTTCATCCGAGAAAC</td>
</tr>
<tr>
<td>Rat MAP1LC3b</td>
<td>TTTGTAAGGGCGGTTCTGAC</td>
<td>CAGGTAGCAGGAAGCAGAGG</td>
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<tr>
<td>Rat PPARGC1a</td>
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<td>AACAATGGCAGGGTTTTGTTC</td>
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<tr>
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<tr>
<td>Rat GAPDH</td>
<td>GGCCGAGGGCCACTA</td>
<td>TGTTGAAGTCACAGGAGACAACCT</td>
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Supplementary Figure S10

A

LacZ shRNA

PARKIN shRNA

MOI: 1 10 100 1 10 100

50kD

PARKIN

37kD

αSA

B

Ad-LacZ Ad-HA-PARKIN

IB: 50kD

PARKIN

HA

HSA

IB: 50kD

PARKIN

37kD

37kD

αSA
Supplementary Figure S11

IB: FLAG
50kD

IB: TRAF2 (N-terminal antibody)
50kD

37kD

Ad-LacZ
Ad-FLAGmTRAF2
Ad-FLAGmTRAF2 Rm mutant
Ad-FLAGmTRAF2 ΔN mutant

TRAFF2
TRAFF2
ns
TRAFF2
ΔN

GAPDH
Supplementary Figure Legends.

**Figure S1: TNF induces autophagy in neonatal rat cardiac myocytes (NRCMs).**  
A) Quantitation of p62 abundance in NRCMs treated with TNF (200ng/ml) for the indicated duration or diluent (n=4/time point). See Figure 1A for representative immunoblot. P value is by post-hoc pairwise comparison test after one-way ANOVA.  
B, C) Relative expression of MAP1LC3b (B) and SQSTM1 (C) transcripts in NRCMs treated with TNF (200ng/ml) for the indicated duration or diluent (n=4/time point). No statistically significant differences were noted by post-hoc pairwise comparison test after one-way ANOVA.

**Figure S2: Sub-cellular localization of TRAF2 in TNF-treated NRCMs.**  
A) Representative line scans (left) demonstrating overlap (arrows) of fluorescence peaks for expression of LC3, TRAF2 and TOM20 in NRCMs from images (right) in Figure 1E.  
B) Representative confocal images demonstrating localization of endogenous TRAF2 with GFP-LC3 and mitochondria (COX IV; arrows) in NRCMs treated with TNF (200ng/ml) or diluent for 24 hours. Scale bar =10µm. TNF-treated cells demonstrated increased mitochondrial fragmentation (73% of TNF-treated cells versus 10% of those treated with diluent).  
C) Representative line scans (left) demonstrating overlap (arrows) of fluorescence peaks for expression of LC3, TRAF2 and COXIV in NRCMs from images (right) in B.  
D) Representative line scans (left) demonstrating overlap (arrows) of fluorescence peaks for expression of LC3, TRAF2 and Ubiquitin in NRCMs from images (right) in Figure 1F.  
E) Representative line scans (left) demonstrating overlap (arrows) of fluorescence peaks for expression of LC3, TRAF2 and p62 in NRCMs from images (right) in Figure 1G.  
F) Representative confocal images demonstrating co-localization of exogenous TRAF2 (FLAG) with mitochondria (mitotracker red) and autophagosomes (GFP-tagged LC3) in NRCMs adenovirally transduced with TRAF2 or LacZ as control (for 48 hours), and treated with TNF (200ng/ml) or diluent for 24 hours. TNF-treated NRCMs demonstrate fragmentation of mitochondrial network (78%
of TNF-treated cells versus 13% of those treated with diluent), with increased co-localization of mitochondria with punctate GFP-LC3 (i.e. with autophagosomes, arrows in Ad-LacZ+TNF treated cells). Exogenous TRAF2 is observed to co-localize with both mitochondria and autophagosomes (arrows in Ad-TRAF2 treated cells). Magnified images are presented in individual channels to demonstrate co-localization between TRAF2, LC3 and Mitotracker Red in the boxed area.

**Figure S3: Effects of TNF signaling on TRAF2, LC3 and p62, in vivo.** A) Quantitation of TRAF2 transcripts (normalized to GAPDH and expressed as fold over littermate wild types) in MHCsTNF and littermate control myocardium at 12 weeks of age. P value is by t-test. B-D) Quantitation of LC3-II (B), p62 (C) and total LC3 (D); all normalized to GAPDH and expressed as fold over littermate wild type, in cardiac extracts from MHCsTNF mice and littermate controls (n=5-6/group). P values are by t-test. See Figure 1I for representative immunoblot. E, F) Relative expression of MAP1LC3b (E) and SQSTM1 (F) transcripts, normalized to GAPDH and expressed as fold over control, in MHCsTNF mice and littermate control myocardium, at 12 weeks of age. N=5-8/group. P values are by t-test.

**Figure S4: MHCsTNF mice demonstrate increased mitochondrial autophagy with decline in mitochondria mass.** A) Representative transmission electron micrographs of MHCsTNF and littermate control myocardium (at 12 weeks of age) demonstrating numerous autophagosomes (arrows, also see magnified image bottom right for visualization of double membrane bound structures), some of which enclose mitochondria (see arrowheads). Representative of n=3 mice/group. B, C) Ratio of mitochondrial to nuclear DNA (B) and citrate synthase activity (C) in myocardial tissue from MHCsTNF mice and littermates (n=7-9/group). P value is by t-test.

**Figure S5: Exogenous TRAF2 facilitates autophagic removal of mitochondria in TNF-treated cells.** A, B) Analysis of NAO expression with representative flow cytometric tracings (A) and quantitation of
mean fluorescence (B); and C) Mitochondrial DNA content in NRCMs adenovirally transduced with TRAF2 or LacZ control (MOI=100, 48hrs) and treated with TNF (200ng/ml, 24hrs) in the presence of 3MA (7mmol/l, 24hrs) or diluent. N=8-24/group for B; and N=5-20/group for C. D, E) Representative flow cytometric tracings (D) with quantitation of cells with depolarized mitochondria (E) in NRCMs adenovirally transduced with TRAF2 or LacZ (MOI=100, 48hrs) and treated with TNF (200ng/ml, 24hrs) or diluent (n=3/group). P values shown are by post-hoc pairwise comparison test after one-way ANOVA.

**Figure S6:** Knockdown of endogenous TRAF2 in NRCMs does not induce the mitochondrial biogenesis program. Quantitative PCR analysis for relative expression of *PPARGC1α* (A); and *PPARGC1β* (B) relative to GAPDH in NRCMs adenovirally transduced with shRNA targeting endogenous TRAF2 or LacZ (non-targeting) control (both at MOI=200) for 72 hours (expressed as fold over control). N=4/group. P values shown are by t-test.

**Figure S7:** Inhibition of autophagy with 3MA results in accumulation of depolarized mitochondria. Representative flow cytometric analysis (A) with quantitation of cells with predominance of depolarized mitochondria (B; JC-1 monomers with green fluorescence in right lower quadrant in A) in NRCMs treated with 3MA (7mM) or diluent for 24hrs. N=3/group. P value shown is by t-test. Representative tracing of a ‘no dye added’ control is shown in A (left).

**Figure S8:** TNF upregulates PARKIN abundance and triggers it localization to the mitochondria. A) Immunoblot depicting PARKIN abundance in NRCMs treated with TNF (200ng/ml) for the indicated duration (in hours). Representative of n=2 experiments. B) Immunoblot demonstrating subcellular localization of PARKIN in mitochondria-enriched fraction (COXIV expressing) and cytoplasmic fraction (GAPDH expressing) in NRCMs treated with TNF (200ng/ml) or diluent for 24 hours. Representative of n=2 experiments. C, D) Immunoblot (C) with quantitation (D) of PARKIN abundance
in cardiac extracts from MHCsTNF and littermate WT mice at 12 weeks of age. N=3/group. P value is by t-test.

**Figure S9: Interaction between TRAF2 and PARKIN.** A) Representative line scans demonstrating overlap (arrows) of fluorescence peaks for TRAF2 with PARKIN in CCCP and diluent-treated cells (see image on the right) from images in Figure 7A. B) Representative confocal images for imaging FRET interaction between DsRed and AcGFP FRET pair, demonstrating a lack of FRET interaction (negative control) between pAcGFP-C1 and pDsRed-monomer expression vectors (co-transfected); or a detectable FRET interaction (positive control) in HEK293A cells transfected with a construct expressing DsRed-GFP dual fluorescent fusion protein for 24 hours. Scale bar =10µm. See Figure 8B for FRET interaction between PARKIN and TRAF2. C, D) Mefs were transduced with FLAG-TRAF2 or HA-PARKIN (each at MOI=100 for 48 hours) followed by treatment with CCCP (30µM) for 2 hours, and mitochondria enriched fraction prepared by subcellular fractionation (see supplementary methods). Immunoprecipitation was performed with anti-FLAG (αFLAG; B), anti-HA (αHA; C) and control IgG antibodies followed by immunodetection for PARKIN or TRAF2. Representative of n=2 experiments.

**Figure S10: Modulation of PARKIN expression with adenovirus expressing HA-PARKIN and shRNA targeting rat PARKIN in NRCMs.** A, B) Immunoblots depicting PARKIN abundance in NRCMs transduced with increasing doses of shRNA targeting PARKIN (or a non-targeting control) for 72 hrs (A); and with adenoviruses coding for expression of HA-tagged rat PARKIN (or LacZ as control) at MOI=100 for 24 hours (B).

**Figure S11: Expression of TRAF2 mutants in NRCMs.** Immunoblot depicting expression of N-terminal FLAG tagged full length murine TRAF2, ring domain mutant- TRAF2-Rm, and mutant lacking 87 amino acids at the N-terminus- TRAF2-∆N; all adenovirally transduced (at MOI=10) in NRCMs for
24 hours with Ad-LacZ as control. Top blot demonstrates exogenous TRAF2 with anti FLAG antibody, and middle blot demonstrates endogenous TRAF2 with anti-TRAF2 antibody generated against an N-terminal peptide (which does not detect the TRAF2-ΔN mutant protein).

Figure S12: TRAF2 facilitates removal of damaged mitochondria in PARKIN-deficient cells. A, B)
Flow cytometric assessment of NAO expression with representative tracings (A) and quantification (B) in NRCMs adenovirally transduced with shRNA targeting rat PARKIN (at MOI=10) or non-targeting control (LacZshRNA) for 72 hours, and TNF (200ng/ml) for 24 hrs, in the presence of adenoviruses expressing TRAF2, its mutants, namely TRAF2-Rm and TRAF2-ΔN (tracing not shown) and LacZ as control (all at MOI=10) for 24 hours. N=3-5/group. P values shown are by post-hoc pairwise comparison test after one-way ANOVA. ‘*’ indicates P=0.005 vs. control (open bar); ‘#’ indicates P<0.001 vs. TNF+PARKINshRNA-treated group (4th bar from the left); and ‘+’ and ‘$’ indicate P<0.001 and P=0.01, respectively, vs. PARKINshRNA group (3rd bar from the left).

Figure S13: Knockdown of endogenous TRAF2 and PARKIN differentially affect mitochondrial protein abundance. A) Immunoblot depicting abundance of PARKIN and TRAF2; and selected mitochondrial proteins in NRCMs adenovirally transduced with shRNA targeting rat TRAF2 (MOI=200), rat PARKIN (MOI=10) or LacZ (as non-targeting control) for 72 hours. Additional shLacZ adenoviral particles were added to equalize total viral particles delivered to each treatment (MOI=210). Expression of α-Sarcomeric actin (αSA) is shown as loading control. B-F) Quantitation of TRAF2 (B), PARKIN (C), VDAC (D), TOM20 (E) and COXIV (F) in NRCMs treated as in A. N=3-5/group. P values are by post-hoc pairwise comparison test after one-way ANOVA.
Reference List


