p53 Mediates Autophagy and Cell Death by a Mechanism Contingent On Bnip3

Erika Yan Wang, Hongying Gang, Yaron Aviv, Rimpy Dhingra, Victoria Margulets, Lorrie A. Kirshenbaum

Abstract—Myocardial ischemia and angiotensin II activate the tumor suppressor p53 protein, which promotes cell death. Previously, we showed that the Bcl-2 death gene Bnip3 is highly induced during ischemia, where it triggers mitochondrial perturbations resulting in autophagy and cell death. However, whether p53 regulates Bnip3 and autophagy is unknown. Herein, we provide new compelling evidence for a novel signaling axis that commonly links p53 and Bnip3 for autophagy and cell death. p53 overexpression increased endogenous Bnip3 mRNA and protein levels resulting in mitochondrial defects leading to loss of mitochondrial ΔΨm. This was accompanied by an increase in autophagic flux and cell death. Notably, genetic loss of function studies, such as Atg7 knock-down or pharmacological inhibition of autophagy with 3-methyl adenine, suppressed cell death induced by p53—indicating that p53 induces maladaptive autophagy. Our previous work demonstrated that Bnip3 induces mitochondrial defects and autophagic cell death. Conversely, loss of function of Bnip3 in cardiac myocytes or Bnip3−/− mouse embryonic fibroblasts prevented mitochondrial targeting of p53, autophagy, and cell death. To our knowledge, these data provide the first evidence for the dual regulation of autophagy and cell death of cardiac myocytes by p53 that is mutually dependent on and obligatorily linked to Bnip3 gene activation. Hence, our findings may explain more fundamentally, how, autophagy and cell death are dually regulated during cardiac stress conditions where p53 is activated. (Hypertension. 2013;62:70-77.)

Key Words: autophagy • Bnip3 • cardiac myocytes • cell death • p53

The signaling pathways that coordinate cell growth and death in mammalian cells are poorly defined. The tumor suppressor protein p53 is best known for regulating G1-exit and cell-cycle entry in normal cells while abortive apoptosis in cells that have become damaged or genetically unstable.1 Indeed among the best characterized features of p53 is its ability to provoke apoptosis through transcription-dependent and transcription-independent mechanisms.2 Loss of p53 function is associated with proliferative disorders and cancers underscoring its importance as a tumor suppressor protein, reviewed in Chen et al3 and Prives et al.4 The inability of p53 to trigger cell death in response to chemotherapeutics or radiation therapy is purported as key underlying cause of cell growth and tumor progression.1,4,5 Moreover, the link between tumor suppression and p53 function is further complicated by the reported ability of p53 to promote autophagy in certain transformed and immortalized cell lines. Though autophagy is essential for recycling macromolecular proteins and removal of damaged organelles during cellular crisis, inappropriate or excessive autophagy beyond a certain threshold is maladaptive and incompatible with life.6,7 Hence, the differential outcomes of autophagy vis a vis life versus death may reflect the temporal activation of autophagy in a cell and context-specific manner.8 For example, in the context of ischemia-reperfusion injury, activation of autophagy during the early ischemic phase before reperfusion is considered protective, whereas late or delayed activation of autophagy during reperfusion is detrimental.8,9

Though the mechanisms that govern autophagy in cells remain poorly defined, there is growing evidence that the mitochondrion may be a central conduit for regulating autophagy and apoptosis.2,10–12 This notion is substantiated by the fact that certain members of the Bcl-2 gene family can trigger mitochondrial membrane permeability changes resulting in both autophagy and apoptosis.11 Hence, whether p53 induces autophagy in ventricular myocytes is adaptive, maladaptive, or involves cellular effectors common to apoptosis is unknown. Earlier work by our laboratory established that p53 localizes to mitochondria triggering mitochondrial perturbations, including loss of mitochondrial ΔΨm and mitochondrial permeability transition pore (mPTP) changes that were inhibited by Bcl-2.2,11 Though the mode by which p53 triggered mitochondrial perturbations was not determined, the fact that Bcl-2 suppressed the cytotoxic actions of p53, suggests p53 likely impinges on one or more Bcl-2 factors that regulate mitochondrial function.13 Previously, we demonstrated the hypoxia-inducible death protein Bnip3 (for Bcl-2/adenovirus E1B 19-kDa interacting
Bnip3 is uniquely distinguished from other BH3, only members of the Bcl-2 gene family for several salient and important reasons. First, the Bnip3 promoter is strongly repressed under normal basal conditions but highly induced during hypoxic or ischemic stress; second, Bnip3 disrupts mitochondrial function by a mechanism-dependent on its carboxyl-terminal transmembrane domain; third, Bnip3 activation triggers atypical cell death with features of autophagy and apoptosis. Notably and perhaps most compelling, our preliminary studies revealed a marked induction of Bnip3 gene expression by p53 in postnatal cardiac myocytes. The significance of this finding is unknown; however, given the reported increase in p53 expression during ischemic/hypoxic stress, we reasoned that Bnip3 may be a putative transcriptional target and down-stream effector of p53.

In this report, we provide new compelling evidence that p53 is a putative transcriptional target and down-stream effector of p53. Preliminary studies and sequence analysis of the human Bnip3 promoter—supporting the notion that Bnip3 is transcriptionally activated by p53.

## Methods

### Cell Culture and Cell Viability

Postnatal ventricular myocytes from 1- to 2-day-old Sprague–Dawley rats were subjected to primary culture in serum-free media. Bnip3-/- mouse embryonic fibroblasts were cultured as previously reported. Cell viability was determined by epifluorescence microscopy using vital stains calcein-acetoxymethyl ester (2 μmol/L) to identify living cells (green) and ethidium homodimer-1 (2 μmol/L) to identify dead cells (red; Molecular Probes, Eugene, Oregon). Cells were analyzed from n=3 to n=4 independent myocyte isolations counting ≥200 cells for each condition. Data are expressed as mean±SE percent reduction from control.

### Western Blot Analysis

Cardiac myocytes cell lysates were subjected to Western blot analysis. The filter was probed with antibodies directed against p53 (Cat # 9282, Cell Signaling), p62/SQSTM1 (Cat # 5114, Cell Signaling), VDAC (voltage-dependent anion-selective channel; Cat # 4661, Cell Signaling), adenosine nucleotide translocator (SC-11433, Santa Cruz), p62/SQSTM1 (Cat # 5114, Cell Signaling), GAPDH (SC-32233, Santa Cruz), Bnip3 (developed in-house), as well as a cell death marker cleaved PARP (cat # 9282, Cell Signaling). The filter was probed with antibodies directed against p53 (Cat # 9282, Cell Signaling), p62/SQSTM1 (Cat # 5114, Cell Signaling), GAPDH (SC-32233, Santa Cruz), Bnip3 (developed in-house), as well as a cell death marker cleaved PARP (cat # 9282, Cell Signaling). Cells were observed in cells expressing p53. These findings support the notion that Bnip3 is transcriptionally activated by p53.

### Mitochondrial Membrane Potential $\Delta \Psi_m$

Mitochondrial membrane potential ($\Delta \Psi_m$) in cells was assessed by epifluorescence microscopy. Cells were preincubated with 50 μmol/L tetra-methylrhodamine methyl ester perchlorate (Molecular Probes, Eugene, OR).

### Autophagic Flux

To assess autophagy in cardiac myocytes, cells infected with an adenovirus encoding green fluorescent protein (GFP)-fused LC3 in the absence and in the presence of chloroquine to assess autophagic flux. The number of green puncta in cells was counted as an index for autophagic flux compared with control cells, as previously reported.

### Statistical Analysis

Multiple comparisons were determined by 1-way ANOVA or unpaired 2-tailed Student t test to compare mean differences between groups. Differences were considered statistically significant to a level of $P < 0.05$.

## Results

### Autophagy and Cell Death in Ventricular Myocytes Induced by p53

To begin to establish whether p53 activates autophagic processes in postnatal ventricular myocytes, we assessed autophagic flux in cardiac myocytes in cells expressing p53. For these studies, we monitored GFP-LC3 puncta in cells overexpressing p53 in the absence and in the presence of chloroquine. In contrast to vector control cells, a marked increase in GFP-LC3 puncta was observed in cells expressing p53. Notably, autophagosome formation was further increased in cells overexpressing p53 in the presence of chloroquine (Figures 1A–1D, 3E, and 3F). Moreover, an accompanied reduction in p62/SQSTM1 expression levels was observed in cells overexpressing p53 (Figure S1 in the online-only Data Supplement). These findings verify that p53 increases autophagic flux in cardiac myocytes. In addition, vital staining of cells revealed that p53-induced autophagy of ventricular myocytes was accompanied by a significant increase in cell death compared with vector control cells (Figure 1E–1H). Interestingly, inhibition of autophagy with either 3-methyl adenine or with shRNA to knock-down Atg7 suppressed autophagy and cell death of ventricular myocytes induced by p53.

### Bnip3 Is Transcriptionally Regulated by p53

Because earlier work by our laboratory demonstrated the ability of Bnip3 to trigger autophagy and cell death of ventricular myocytes, we reasoned that Bnip3 may be a transcriptional target of p53. Preliminary studies and sequence analysis revealed the presence of cis-acting elements for p53 within the human Bnip3 promoter—supporting the notion that Bnip3 may be transcriptionally regulated by p53. To test this possibility, we monitored Bnip3 expression in cardiac myocytes in the absence and in the presence of p53. As shown in Figure 2A and 2B, in contrast to vector control cells, a marked increase in endogenous Bnip3 mRNA and protein expression levels were observed in cells expressing p53. These findings suggest that Bnip3 is transcriptionally activated by p53.

### p53 Induces Mitochondrial Defects Dependent on Bnip3

Because earlier work by our laboratory demonstrated that p53 localizes to mitochondria in cardiac myocytes, we reasoned that mitochondrial perturbations induced by p53 may underlie autophagy and cell death. As shown by Western blot analysis (Figure 2E), p53 was detected in the mitochondrial fractions of cardiac myocytes. Notably, this was accompanied by a marked reduction in mitochondrial $\Delta \Psi_m$ (Figure 2C and 2D). These findings support the notion that mitochondrial perturbations induced by p53 promote autophagy and cell death of ventricular myocytes.

On the basis of these observations, we reasoned that p53 may provoke mitochondrial perturbations in a manner dependent on Bnip3. To test this possibility, we rendered cardiac myocytes defective for Bnip3 with shRNA directed against Bnip3. Previous work by our laboratory verified the specificity of the shRNA used to knock-down Bnip3 in ventricular myocytes for these studies. As shown in Figure 2C and 2D, a
Figure 1. p53 induces autophagy and cell death in ventricular myocytes. **A**, Epifluorescence microscopy of ventricular myocytes infected with autophagy reporter GFP-LC3 under control (CTRL) and p53 overexpression conditions with and without shRNA directed against Atg7. The number of green puncta were counted as an index of autophagosome formation compared with CTRL cells. **B**, Histogram represents quantitative data shown in **A**, data are expressed as mean±SE puncta/cell from CTRL, from n=3 to 4 independent myocytes isolations counting ≥180 cells for each condition tested; *P<0.01 compared with CTRL, †P<0.01 compared with p53. **C**, GFP-LC3 puncta in cardiac myocytes under CTRL and p53 overexpression conditions with and without 3-methyl adenine (3-MA; 10 mmol/L), using methodologies described in **A**. **D**, Histogram represents quantitative data for **C**, data expressed as mean±SE puncta/cell from CTRL, from n=3 to 4 independent myocytes isolations counting ≥180 cells for each condition tested; *P<0.01 compared with CTRL, †P<0.01 compared with p53. **E**, Epifluorescence microscopy of cardiac myocytes stained with vital dyes calcein-AM and ethidium homodimer-1 to identify the number of live cells (green) and dead cells (red), respectively, for conditions shown in **A**. **F**, Histogram represents quantitative data for **E**. Data are expressed as mean±SE percent change from CTRL, from n=3 to n=4 independent myocytes isolations counting ≥200 cells for each condition tested; *P<0.01 compared with CTRL, †P<0.01 compared with p53. **G**, Vital staining of cardiac myocytes by epifluorescence microscopy for conditions shown in **C**. **H**, Histogram represents quantitative data for **G**. Data are expressed as mean±SE percent change from CTRL, from n=3 to n=4 independent myocytes isolations counting ≥200 cells for each condition tested; *P<0.01 compared with CTRL, †P<0.01 compared with p53.
marked reduction in mitochondrial ΔΨm was observed in cells expressing p53; in contrast, however, knock-down of Bnip3 completely abrogated the loss in ΔΨm induced by p53. To verify these findings and the involvement of Bnip3 in p53-mediated mitochondrial defects, we tested the effects of p53 in the presence of a carboxyl-terminal transmembrane domain mutant of Bnip3 (Bnip3∆TM), previously reported by our laboratory to be defective for mitochondrial membrane integration (designated Bnip3∆TM). As shown in Figure 2C and 2D, in contrast to vector control cells, p53-mediated loss of mitochondrial ΔΨm was suppressed by the Bnip3∆TM mutant defective for mitochondrial targeting—a finding concordant with our shRNA knock-down data for Bnip3. Interestingly, we observed a marked reduction in mitochondrial-associated p53 in cells in the presence of the Bnip3∆TM (Figure 2E). Taken together, these findings strongly suggest the involvement of Bnip3 in mitochondrial defects induced by p53.

p53 Induces Autophagy and Cell Death Dependent on Bnip3

To establish whether Bnip3 underlies autophagy and cell death of ventricular myocytes induced by p53, we assessed
Figure 3. p53 induces autophagy and cell death dependent on Bnip3. A, Epifluorescence microscopy of ventricular myocytes infected with autophagy reporter GFP-LC3 under control (CTRL) and p53 overexpression conditions with and without shRNA directed against Bnip3 or Bnip3ΔTM. B, Histogram represents quantitative data for A. Data are expressed as mean±SE puncta/cell from CTRL, from n=3 to n=4 independent myocytes isolations counting ≥180 cells for each condition tested; *P<0.01 compared with CTRL, †P<0.01 compared with p53. C, Vital staining of cells by epifluorescence for conditions in Figure 2C, using methodologies described in Figure 1. D, Histogram represents quantitative data for C. Data are expressed as mean±SE percent change from CTRL, from n=3 to n=4 independent myocytes isolations counting ≥200 cells for each condition tested; *P<0.01 compared with CTRL, †P<0.01 compared with p53. E, p53 induces autophagic flux in cardiac myocytes. Autophagic flux of GFP-LC3 puncta was measured in the presence of chloroquine (8 μmol/L) under CTRL and p53 overexpression conditions with and without shRNA directed against Bnip3. F, Histogram represents quantitative data for E. Data are expressed as mean±SE puncta/cell from CTRL, from n=3 to 4 independent myocytes isolations counting ≥180 cells for each condition tested; *P<0.01 compared with CTRL, †P<0.01 compared with p53.
autophagic flux and cell viability in cells in which Bnip3 was impaired or inhibited. As shown in Figure 3A, 3B, 3E, and 3F, in contrast to control cells, autophagic flux induced by p53 was completely suppressed in cells after Bnip3 knock-down or by Bnip3ΔTM. In addition, genetic knock-down of Bnip3 completely suppressed the degradation of p62/SQSTM1 protein induced by p53 (Figure S1). Further, inhibition of Bnip3 by shRNA knock-down or by dominant-negative inhibition independently suppressed cell death induced by p53 (Figure 3C and 3D), a finding concordant with our autophagy data. To conclusively prove the involvement of Bnip3 in autophagy and cell death induced by p53, we tested the impact of p53 in wild-type and Bnip3−/− mouse embryonic fibroblasts. As shown in Figure 4A–4D, in contrast to wild-type cells, which readily displayed the presence of autophagosomes and reduced cell viability in the presence of p53, p53 failed to induce autophagy or promote death in Bnip3−/− cells. Notably, repletion of Bnip3 into the Bnip3−/−-deficient background completely restored p53’s ability to provoke autophagy and cell death equivalent to that of wild-type cells. To prove Bnip3 is required for mitochondrial localization of p53, we prepared mitochondrial and cytoplasmic fractions from wild-type and Bnip3−/− mouse embryonic fibroblasts for Western blot analysis for p53. Our data reveal that p53 was expressed to compare levels in wild-type and Bnip3−/− cells. Notably, p53 protein was readily detectable in mitochondria mouse embryonic fibroblasts wild-type cells, concordant with its ability to provoke widespread cell death. In contrast, however, p53 was not detected in the mitochondria of Bnip3−/− cells—a finding concordant with p53’s inability to provoke mitochondrial defects and cell death in cells rendered defective for Bnip3 (Figure S2). Collectively, these findings strongly support our contention that Bnip3-dependent mitochondrial targeting of p53 promotes autophagy and cell death of ventricular myocytes.

Discussion

In this report, we provide the new compelling evidence that autophagy and cell death of ventricular myocytes induced by p53 are dependent on Bnip3. Autophagy is an adaptive process by which cells recycle damaged organelles or macromolecular proteins during nutrient or cellular stress. Although the removal of damaged mitochondria is viewed as an essential process for subverting cell death, excess or inappropriate autophagy is maladaptive and incompatible with life. At present, the mechanisms that underlie autophagic cell death in the heart remain unknown. It remains equally undetermined whether autophagy uses distinct cellular effectors to promote cell death, or impinges on overlapping pathways with apoptosis. Previous work by our laboratory identified the inducible protein Bnip3 sufficient to provoke autophagy and cell death of ventricular myocytes during ischemic and hypoxic stress. We attributed this to the loss of mitochondrial ΔΨm and mPTP opening. The fact that Bnip3 gene and protein expression were increased by p53 is compelling and identifies Bnip3 as putative down-stream effector of p53. Indeed, our data highlight a direct functional link between mitochondrial defects induced by p53 and Bnip3.

Another salient and important feature of our study highlights that cell death induced by p53 was suppressed by inhibiting autophagy—indicating that autophagy induced by p53 at least in this context is maladaptive. This notion is substantiated by our findings that cell death induced by p53 was suppressed by 3-methyl adenine or Atg7 knock-down. If autophagy induced by p53 was cytoprotective, we would have expected a greater incidence of cell death after autophagy inhibition in cells expressing p53; however, this was not the case. Moreover, the fact that baseline cell viability in the presence of 3-methyl adenine or Atg 7 knock-down was comparable with vector control cells, would further argue that autophagy induced by p53 is maladaptive. Another important finding of our study revealed that mitochondrial defects, autophagy, and cell death induced by p53 were completely abrogated by dominant-negative inhibition of Bnip3, Bnip3 knock-down, or in cells deficient for Bnip3. Indeed, we demonstrate by not 1, but by 4 independent approaches that autophagy and cell death induced by p53 is contingent on the Bnip3-dependent mitochondrial targeting of p53. Our data identify Bnip3 as a critical down-stream target of p53 crucial for inducing mitochondrial perturbations. Though earlier work by our laboratory and others demonstrated that the localization of p53 to mitochondrial membranes was sufficient to provoke loss of ΔΨm and cell death, the underlying mechanism was not determined. At present, it remains unclear how p53 localizes to mitochondria or triggers mitochondrial defects leading to autophagy and cell death. However, the fact that less p53 was associated with mitochondrial membranes in the presence of Bnip3ΔTM is intriguing and suggests the possibility that p53 disrupts mitochondrial function in a manner dependent on mitochondrial-associated Bnip3. Though protein–protein associations were not determined here, it is tempting to speculate that p53 anchors to mitochondrial membranes in a Bnip3-dependent manner. This notion is concordant with the reduction of mitochondrial-associated p53 in the presence of Bnip3ΔTM and the resistance of cardiac cells rendered deficient for Bnip3 or Bnip3−/− cells to the cytotoxic actions of p53. The dispensability of either Bax or PUMA for mediating mitochondrial defects by p53 coupled with the findings of the present study supports our contention that Bnip3 is a crucial down-stream effector of p53. This view is further supported by the fact that p53 had no direct effect on the expression levels of Atg5, Atg12, or Beclin-1 (H. Gang and L.A. Kirshenbaum, unpublished data, 2012). These findings suggest that autophagy induced by p53 likely arises from mitochondrial perturbations rather than autophagy gene activation per se. Hence, loss function of p53 is consistent with the resistance of certain cancers to Bnip3 gene activation and cell death. Hence, we speculate that by inducing autophagy/mitophagy, p53 may serve as a surveillance protein for maintaining mitochondrial quality control during cellular stress that beyond a certain threshold of injury triggers cell death.

Though our data strongly support a model in which p53 provokes mitochondrial permeability changes dependent on Bnip3, we cannot exclude the possibility that p53 may in addition modulate permeability transition pore mPTP by directly or indirectly influencing mPTP components, such as cyclophilin D. This view is supported by a recent report demonstrating the inhibition of cyclophilin D with cyclosporine A was sufficient to suppress cell death induced by p53 and our own data demonstrating the resistance of cyclophilin DΔ cells to Bnip3-induced cell death (Figure S3). Taken
together, the findings of the present study are intriguing and provide the first direct evidence to mechanistically link mitochondrial perturbations induced by p53 to Bnip3 as an effector of autophagy and cell death of ventricular myocytes. Hence, our findings may explain more fundamentally how p53 dually mediates autophagy and death of ventricular myocytes.

**Perspectives**

In this report, we provide novel evidence that p53 induces autophagy of cardiac myocytes that is maladaptive. We show that p53 localizes to the mitochondrion and provokes mitochondrial defects in a manner dependent on the Bcl-2 death protein, Bnip3. Loss of Bnip3 function completely abrogated autophagy and cell death induced by p53. Hence, our findings reveal a novel signaling axis between mitochondrial localization of p53 and Bnip3 for autophagy and cell death that may explain more fundamentally how p53 dually regulates autophagy and cell death of ventricular myocytes during cardiac stress. Therapeutic interventions that modulate p53 expression may prove beneficial in curtailing maladaptive autophagy and cell death after injury.

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Disclosures

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References


Novelty and Significance

What Is New?

- We provide new compelling evidence for a novel signaling axis between p53 and the Bcl-2 death protein Bnip3 for the dual regulation of autophagy and cell death.
- The work herein is novel because it provides the first mechanistic evidence for a functional link between p53 and Bnip3 for autophagy and cell death, which has not been previously reported.

What Is Relevant?

- p53 is activated during cardiac stress by myocardial infarction and hypertension by angiotensin II.
- Inappropriate or excessive cell death induced by p53 underlies vascular remodeling and cardiac dysfunction.

Summary

To our knowledge, these data provide the first evidence for the dual regulation of autophagy and cell death of cardiac myocytes by p53. These findings may explain more fundamentally how autophagy and cell death are dually regulated during cardiac stress conditions, where p53 is activated.
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ON LINE SUPPLEMENT

p53 Mediates Autophagy and Cell Death by a Mechanism Contingent Upon Bnip3.

Erika Yan Wang‡, Hongying Gang‡, Yaron Aviv‡, Rimpy Dhingra‡, Victoria Margulets‡, and Lorrie A. Kirshenbaum††
S1. p53 Induces Autophagy in Ventricular Myocytes Shown by Degradation of p62

Western blot analysis of cardiac cell lysate under control and p53 over-expression conditions with and without shRNA directed against Bnip3. The filter was probed with an antibody directed against p62/SQSTM1. Antibody directed against α-Actin was used for loading control.
S2. p53-Mitochondria Localization is Abolished in Cells Deficient for Bnip3

Western Blot analysis of mitochondrial (mito) and cytoplasmic (cyto) fractions derived from wild type and Bnip3-/- mouse embryonic fibroblasts. The filter was probed with an antibody directed against p53. Antibody directed against VDAC (Voltage-dependent anion channel) and GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) were used as controls for mitochondrial and cytoplasmic fractions respectively.
S3. Cells Deficient for Cyclophilin D are Resistant to Mitochondrial Defects and Cell Death Induced by Bnip3.

Panel A: Vital staining of wild type and CypD-/- mouse embryonic fibroblasts by epifluorescence in the under control and Bnip3 over-expression conditions as described in Figure 4 panel A, Panel B: Mitochondrial permeability transition pore (mPTP) in wild type and CypD -/- for the conditions shown in panel A. mPTP was assessed by loading cells with calcien-AM in the presence of cobalt chloride to squelch the cytoplasmic signal. Loss of green fluorescence in mitochondria is an index of mPTP opening.
S4. p53 Gene Silencing in Basal Condition Doesn’t Affect Autophagic Flux in Ventricular Myocytes

Panel A, Autophagic flux of GFP-LC3 puncta was measured in the absence and presence of chloroquine with and without siRNA directed against p53; Panel B, Histogram represents quantitative data shown in panel A. Data are expressed as mean ±S.E. puncta/cell from control, from n=3-4 independent myocytes isolations counting > 180 cells for each condition tested; * denotes p<0.01 compared to control; † denotes p<0.01 compared to condition with siRNA-p53.