Foxo3 circular RNA promotes cardiac senescence by modulating multiple factors associated with stress and senescence responses

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Supplementary Information

Fig S1. Expression of circular RNA Foxo3 in young and old tissues

(a) Sections of young and old tissue were subject to staining for expression of β-Gal and circ-Foxo3. The young tissues expressed undetectable staining of β-Gal and circ-Foxo3, whereas the old tissues showed extensive β-Gal and circ-Foxo3 staining.

(b) Primary cardiomyocytes isolated from neonatal and 12 week heart tissues of strain C57 mice were treated with 25 μM H_2O_2 followed by β-Gal staining. Cells isolated from the mature tissues displayed increased number of β-Gal staining relative to the cells isolated from mature tissues.

(c) Upper, a siRNA was designed to specifically target circ-Foxo3. Lower, MEFs were transfected with the siRNA or a control oligo. RNAs isolated were subject to real-time PCR to confirm down-regulation of circ-Foxo3.

(d) Left, Cardiomyocytes isolated from adult mice were transfected with control oligos or circ-Foxo3 siRNA. RNAs were isolated and subject to real-time PCR showing that silencing circ-Foxo3 with siRNA decreased circ-Foxo3 expression in cardiomycytes. **, $p<0.01$. Error bars, SD ($n=4$). Right, Cardiomyocytes were transfected with control oligos or circ-Foxo3 siRNA, treated with 25 μM H_2O_2 for 2 hr, incubated in basal medium for 48 h, and subject to β-gal staining. Silencing circ-Foxo3 decreased cardiomyocytes β-gal staining.

(e) Typical pictures of TUNEL staining of heart tissues. Dox treatment induced mouse heart apoptosis, which could be enhanced by overexpression of circ-Foxo3 and repressed by circ-Foxo3 siRNA. It is anticipated that the death cells were mainly the inflammatory cells, cardiac myocytes, and interstitial cells.

(f) Left, Myocadium fibrosis was examined by Sirius red staining of collagen. Dox treatment induced mouse myocadium fibrosis, which was enhanced by overexpression of circ-Foxo3 and repressed by circ-Foxo3 siRNA. Right, Quantification of Sirius red staining showing

that Dox treatment induced mouse myocadium fibrosis, which was enhanced by overexpression of circ-Foxo3 and repressed by circ-Foxo3 siRNA.

Fig S2. Correlation of heart fibrosis with circ-Foxo3 levels.

(a) Expression of circ-Foxo3 detected by RNA in situ hybridization of heart tissues in each group. Typical photos of circ-Foxo3 expression are shown (left). The levels of circ-Foxo3 were quantified by Image J scanning of the tissues (right). *a*, $p<0.01$ *vs*. control group; *b*, $p<0.01$ *vs*. Dox treatment group. It was noted that the density of nuclei is lower in Dox and Dox-circ-Foxo3 groups compared to control and Dox-siRNA groups. It could be that circ-Foxo in situ hybridization took place primarily in the areas prone to cellular death. It could also be affected somehow by the lighting of circ-Foxo3 staining.

(b) Heart tissues were stained with DAPI (blue), circ-Foxo3 (red) and α-smooth muscle actin (α-SMA, green). Typical photos showed that expression of circ-Foxo3 was co-localized with α-SMA.

Fig S3. Interaction of Id1, E2F1, HIF-1α and FAK with circ-Foxo3.

(a) The lysates prepared from MEFs transfected with circ-Foxo3 or a control vector were mixed with the biotinylated probes and the mixtures were subject to Western blot analysis. Expression of Id1, E2F1, HIF-1α and FAK was not affected by circ-Foxo3 transfection.

(b) Upper, Cell lysates prepared from MEFs transfected with siRNA targeting circ-Foxo3 or a control oligo were mixed with the biotinylated probes followed by real-time PCR. Transfection with circ-Foxo3 siRNA decreased circ-Foxo3 levels. **,$p<0.01$, $n=4$. Lower, Similar assays were performed in primary cardiomyocytes.

(c) Left, The lysates were mixed with the biotinylated probes and the mixtures were subject to Western blotting. Expression of Id1, E2F1, HIF-1α and FAK was not affected by transfection with circ-Foxo3 siRNA. Right, Similar assays were performed in primary cardiomyocytes.

(d) A control oligo- or circ-Foxo3 siRNA transfected cardiomyocytes were cultured in basal medium or serum-free medium for 36 h, or treated with 100 μM H_2O_2 for 2 h, followed by Western blotting. Expression of HIF-1α was up-regulated in both control oligos and siRNA transfected cells cultured in serum-free medium or treated with H_2O_2.

Fig S4. Expression of circ-Foxo3 affected Id1 and E2F1 subcellular translocation.

(a) Full panel of sections showing expression of ID1 and E2F1 in the circ-Foxo3- or mock-transfected MEFs as shown in Fig 5d.

(b) Full panel of sections showing expression of ID1, E2F1, and circ-Foxo3 in the circ-Foxo3- or mock-transfected MEFs as shown in Fig 5e.

Fig S5. Expression and distribution of Id1 and E2F1 affected by Dox and circ-Foxo3.

(a). Heart tissues were stained with DAPI (blue), circ-Foxo3 (red) and E2F1 (green). Typical photos showed that overexpression of circ-Foxo3 repressed E2F1 expression in the nuclei whereas silencing circ-Foxo3 enhanced E2F1 translocation to the nuclei in Dox-treated mice.

(b) Heart tissues were stained with DAPI (blue), circ-Foxo3 (red) and ID1 (green). Typical photos showed that overexpression of circ-Foxo3 repressed ID1 expression in the nuclei whereas silencing circ-Foxo3 enhanced ID1 translocation to the nuclei in Dox-treated mice.

Fig S6. Expression and distribution of HIF-1α and FAK affected by Dox and circ-Foxo3.

(a) Heart tissues were stained with DAPI (blue), circ-Foxo3 (red) and HIF-1α (green). Typical photos showed that overexpression of circ-Foxo3 repressed HIF-1α expression in the nuclei and silencing circ-Foxo3 enhanced HIF-1α expression in the nuclei in Dox-treated mice.

(b) Heart tissues were stained with DAPI (blue), VADC1/Porin (red, for mitochondria) and FAK (green). Typical photos showed that overexpression of circ-Foxo3 repressed FAK translocation to the mitochondria while silencing circ-Foxo3 enhanced FAK translocation to the mitochondria in Dox-treated mice.

Fig S7. Co-localization of HIF-1α with circ-Foxo3 in response to stress

(a) circ-Foxo3- and vector-transfected MEFs were cultured in basal medium or treated with 75 μM H_2O_2 for 2 hours, followed by immunocytometry and confocal microscopic examination. Transfection with circ-Foxo3 retained HIF-1α in cytoplasm even after H_2O_2 treatment. This is the full panel of sections as shown in Fig 7a.

(b) HIF-1α was co-localized with circ-Foxo3 in cytoplasm. This is the full panel of sections as shown in Fig 7b.

Fig S8. Co-localization of FAK with circ-Foxo3 in response to stress

(a) Upper, distinct distribution of FAK and mitochondria was observed when the cells were cultured in normal medium. Lower, Treatment with H_2O_2 facilitated co-localization of FAK and circ-Foxo3 in the vector-transfected cells, but expression of circ-Foxo3 retained the distict distribution of FAK and circ-Foxo3. This is the full panel of sections as shown in Fig 7c.

(b) Primers used in this study.

(c) Probe sequences for binding assay.

Materials

The monoclonal antibodies against HIF-1α, Id1, E2F1, PCNA, VDAC1/Porin were purchased from Abcam. The monoclonal antibody against FAK was obtained from Santa Cruz Biochenology. The monoclonal antibodies against Foxo3, senescence-galactosidase staining kit were obtained from Cell signaling. Horseradish peroxidase-conjugated goat anti-mouse IgG and horseradish peroxidase-conjugated goat anti-rabbit IgG were obtained from Bio-Rad. RNA and DNA extract kits, RNA RT and PCR kits, were from Qiagen. The nuclear or cytoplasmic RNA purification kit was from Fisher Scientific. Northern blot kit was from Ambion. Biotin Chromogenic Detection kit was from Thermo Scientific. Protein A-Sepharose 4B Conjugate and Streptavidin C1 magnetic beads were obtained from Invitrogen.

Constructs and primers

Plasmid of mouse circular RNA Foxo3 (circ-Foxo3) and control plasmid were generated previously. Both plasmids contain a Bluescript backbone, a CMV promoter driving green fluorescent protein (GFP) expression and a human H1 promoter driving mouse circ-Foxo3 or a non-related sequence serving as a control. The control plasmid was inserted with a non-related sequence instead of the mouse circ-Foxo3 sequence.

All primer sequences used are listed in Fig S4b. Circ-Foxo3 siRNA, DNA oligo probes against endogenous or ectopic expression of circ-Foxo3, labelled with biotin or Cy5 were designed by us and synthesized by Integrated DNA Technology (Fig S4c). Human Foxo3 containing plasmid was obtained from Addgene (Cambridge, MA).

Senescence-associated β-galactosidase staining

β-gal staining was performed using senescence-galactosidase staining kit (Cell Signaling) according to the manufacturer's instructions. In brief, cells were cultured in 10% FBS/DMEM to sub-confluence and then fixed in 3.7% formaldehyde for 5 min, washed in PBS and stained in β-galactosidase solution (1 mg/ml 5-bromo-4-chloro-3-indolyl-β-gal) at 37°C until staining become visible in either experiment or control cultures. The number of positive cells was counted under a light microscope.

Tissue sections of young and old mouse hearts were freshly excised, rapidly frozen in liquid nitrogen, and mounted in OCT. Sections (5 μm) were placed on glass slides, fixed in 1% formalin in PBS at room temperature for 1 min, and immersed in β-gal staining solution at 37°C for 24 h. The samples were counterstained with eosin, and viewed under a bright field at 10-40x magnification.

Hydrogen peroxide inducing senescence

Cells in basal medium were treated with indicated concentrations of hydrogen peroxide for 2 h. After wash with PBS, the treated cultures were incubated in fresh growth medium without H_2O_2 for 48 h, and processed to β-gal staining.

Immunofluorescent microscopy

Cells growing on BD culture slides were fixed in 3.7% formaldehyde for 10 min, blocked with 10% goat serum for 30 min, and then incubated with primary antibody in TBS containing 10% goat serum albumin overnight. The slides were washed and stained with goat anti-mouse Alexa 594 or goat anti-rabbit Alexa 647 (Life Technologies) at room temperature for 1 hour. Fluorescent phalloidin, Acti-stain 488 (Cytoskeleton), was used to stain F-actin to view cellular structure. DNA staining was performed with DAPI. Confocal laser scanning microscopy was performed using an LSM 510 Meta microscope (Carl Zeiss).

Fluorescent in situ hybridization (FISH)

To detect circ-Foxo3 expression within cell or tissues, we used fluorescent in situ hybridization (FISH) with a mixture of DNA oligo probes labelled with Cy5, which was specific for either endogenous or ectopic expressed circ-Foxo3. A scramble sequence labelled with Cy5 was used as a negative control.

Tissues were freshly excised and fixed in 10% formalin overnight, immersed in 70% ethanol, embedded in wax, and sectioned. The sections were de-paraffinized with xylene and ethanol, boiled in a pressure cooker, and then permeabilized with 0.2% Triton X-100 for 15 min. For tissue culture, cells growing on BD culture slides were fixed in 3.7% formaldehyde for 10 min, and then permeabilized with 0.2% Triton X-100 for 15 min.

In situ Hybridization was carried out at 52°C for 3 h with 40 nM Cy5-labeled DNA oligo probes in hybridization buffer (Ambion), followed by serial washes with saline-sodium citrate (SSC) buffers. The samples were treated with 3% hydrogen peroxide to block endogenous peroxidase, washed with TN buffer (0.1 M Tris-HCl, pH 7.5 and 0.15 M NaCl) three times and then incubated in blocking buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5% blocking reagent and 0.5% BSA) at room temperature for 30 min. DNA staining was performed with DAPI. Confocal laser scanning microscopy was performed using an LSM 510 Meta microscope (Carl Zeiss). The Image J Scanning System was used to scan the color of interest in at least 3 representative photos for statistical analysis.

Preparation of nuclei

Cells were lysed in 1 ml lysis buffer (20 mM Hepes, pH 7.2, 10 mM KCl, 2 mM $MgCl_2$ and PI), and homogenized with a prechilled Dounce homogenizer with 20 strokes. Cell lysis was

centrifuged at 4200 rpm for 5 min. The pellet was washed with PBS 3 times, and resuspended in 100 μl lysis buffer containing 0.5 M NaCl. After centrifugation at 13200 rpm for 10 min, the supernatants containing nuclear extract were used for analysis.

Mitochondria preparation

Cells were lysed in 1 ml lysis buffer containing 1 mM DTT and 250 mM sucrose, and homogenized with a prechilled Dounce homogenizer with 20 strokes. Cell lysis was centrifuged at 1000 xg for 10 min. The supernatant was then centrifuged at 10,000 xg for 25 min. The pellet was resuspended in 10 mM Tris-HCl, pH 7.5, containing 250 mM sucrose and PI, and carefully loaded onto a discontinuous sucrose gradient (6 ml 1.0 M sucrose over 6 ml 1.5 M sucrose, dissolved in 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 1 mM DTT). After ultra-centrifugation at 60,000 xg for 60 min, the white band between the 1.0 M and 1.5 M sucrose interface was collected and resuspended in a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl and PI.

Western blot

Cells were lysed, and the protein samples were subject to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 5-12% acrylamide. Transblotting was processed onto a nitrocellulose membrane in 1xTris/glycine buffer containing 20% methanol at 66-V at 4°C for 2 h. The membrane was blocked in TBST (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) containing 5% non-fat dry milk powder (TBSTM) for 0.5 hour, and then incubated with primary antibodies at 4°C overnight. The membranes were washed with TBST (3×30 min) and then incubated with secondary antibodies for 1 hour. After washing, the bound antibodies were visualized with an ECL detection kit.

RT-PCR and real-time PCR

Total RNAs were isolated from patient specimens using Trizol (Invitrogen Life Technologies). The protocol is approved by the First Hospital, Sun Yet-sen University. Two micrograms of total RNAs were used to synthesize cDNA, a portion of which (1 μl, equal to 0.2 μg cDNA) was used in a PCR with two appropriate primers. Real-time PCR were performed with miScriptSYBR GreenPCR Kit (Qigen) using 1 μl cDNA as the template. The primers used as real-time PCR control were human-U6RNAf and human-U6RNAr.

Mouse heart tissues or 2×10^6 cells were harvested. Total RNA was extracted with the Qiagen RNeasy mini kit. Two micrograms of total RNAs were used to synthesize cDNA, followed by real-time PCR with miScriptSYBR GreenPCR Kit (Qigen) using 1 μl cDNA as the template. The primers used as real-time PCR control were mouse-U6RNAf and mouse-U6RNAr.

Immunoprecipitation

10^7 cells were washed with ice-cold phosphate-buffered saline, and lysed in 1 ml lysis buffer. Equal amounts of proteins were incubated with 5 μg primary antibody and 40 μl of 50% slurry of protein A-Sepharose at 4 °C for 4 h. The pellets were washed 3 times with PBS and resuspended in 2× Laemmli buffer (0.125 M Tris HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, pH, 6.8), followed by Western blot analysis.

RNA binding protein immunoprecipitation (RIP)

10^7 cells were washed with ice-cold PBS, lysed in 500 μl co-IP buffer (20 mM Tris-HCL, pH 7.5, 150 mM Nacl, 1 mM EDTA, 0.5 % NP-40, and 5 μg/ml aprotinin), and incubated with 5 μg primary antibody at 4 °C for 2 h. 40 μl of 50% slurry of protein A-Sepharose was added to each sample, and the mixtures were incubated at 4 °C for 4 h. The pellets were washed 3 times with PBS and resuspended in 0.5 ml Tri Reagent (Sigma-Aldrich). Eluted co-precipitated RNA

in the aqueous was subject to qRT-PCR analysis to demonstrate the presence of the binding using respective primers.

RNA pull-down assays

RNA pull-down assays were carried out as described [1, 2]. Briefly, 10^7 cells were washed in ice-cold phosphate-buffered saline, lysed in 500 μl co-IP buffer, and incubated with 3 μg biotinylated DNA oligo probes against endogenous or ectopic expressed circ-Foxo3 at room temperature for 2 h. 50 μl washed Streptavidin C1 magnetic beads (Invitrogen) were added to each binding reaction and further incubated at room temperature for another one hour. Beads were washed briefly with co-IP buffer for five times. The bound proteins in the pull-down material were analyzed by western blotting.

Northern blot

RNAs were isolated with RNA extract kit and Northern blot analysis was performed with Northern blot kit (Ambion). Briefly, the total RNAs (30 μg) were denatured in formaldehyde and then electrophoresed on a 1% agarose–formaldehyde gel. The RNAs were then transferred onto a Hybond-N+nylon membrane (Amersham) and hybridized with biotin-labeled DNA probes. Biotin Chromogenic Detection kit (Thermo Scientific) was used to develop the blots.

Animal model and Cardiac function assessment

All animal experiments were performed in accordance with relevant guidelines and regulations and approved by the Animal Care Committee of Sunnybrook Research Institute. The animal model of Doxorubicin-induced cardiomyopathy was induced in adult mice (6–8 weeks, BALB/c strain) by intraperitoneal injection of Doxorubicin (Dox, 3 mg/kg, 3 times per week with a total cumulative dose of Dox 18 mg/kg) [3]. Each group contained 10 mice. In the negative control group, mice were injected with an equal volume of saline. In the group treated with circ-Foxo3 plasmid or circ-Foxo3 siRNA (GenePharma, Shanghai), circ-Foxo3 plasmid (50 μg) or circ-Foxo3 siRNA (6 μg) was injected i.p. with the plasmid- or siRNA-PEG-Au NP complexes 24 hours before every Dox injection.

Synthesis of the deliver complexes (plasmid- or siRNA-PEG-Au NP) was performed as previously described. Briefly, 500 μg circ-Foxo3 plasmid or 60 μg nmol thiol modified was dissolved in 800 μl of RNase-free water. The mPEGSH (PG1-TH-2k, Nanocs) was mixed with plasmid or siRNA (1:20 molar ratio). Then 10-nm gold nanoparticles (AuNP, Cytodiagnostics) were mixed with plasmid or siRNA-PEG at a weight ratio of 1:1 (plasmid) or 1:20 (siRNA) for conjugation. The mixture was gently shaken at 60 °C for 30 min and transferred into a syringe.

Three weeks after Dox injection, all mice were anesthetized with 2% isoflurane inhalation to undergo transthoracic echocardiography and invasive hemodynamic assessment. Transthoracic echocardiography was performed and analyzed in a blinded manner, using a Vevo 2100 high-resolution imaging system equipped with a 30-MHz transducer (RMV-707B, VisualSonics, Toronto, ON, Canada) to measure left ventricular end diastolic diameter (LVEDD), left ventricular end systolic diameter (LVESD), left ventricular ejection fraction (LVEF), left ventricular fractional shortening (LVFS) and Rate of Rise of Left Ventricular Pressure (dp/dt). A 1.4-Fr high-fidelity microtip catheter connected to a pressure transducer (Millar Instruments, Houston, TX, USA) was inserted into the LV cavity via the carotid artery to evaluate left ventricular pressure (LVSP).

All mice were sacrificed following cardiac function assessment. Hearts were harvested and cut into two halves. The upper half of the heart was kept frozen for PCR or processed to obtain frozen sections, followed by β-gal, immunofluorescence and circ-Foxo3 hybridization staining, while the lower half was fixed with 10% buffered formalin and embedded in paraffin, then

sectioned to 5 μm slides. HE staining, TUNEL staining and Sirius red staining were performed as described [4-7].

Isolation of mouse cardiomyocytes

The enzymatic dispersion technique was used to isolate mouse cardiomyocytes from neonatal and adult male C57 mice as described in detail previously [8, 9]. Briefly, animals were heparinized, anaesthetized by inhalation of isoflurane and then sacrificed by cervical dislocation. The hearts were rapidly removed, washed in PBS solution (Sigma, P5244) containing 20 mM BDM, transferred into a drop of hepes-buffered Tyrode solution (130 mM NaCl; 5.4 mM KCl; 1 mM $CaCl_2$; 1 mM $MgCl_2$; 0.33 mM Na_2HPO_4; 10 mM Hepes; 5.5 mM glucose, pH 7.4, and minced into small pieces. Cardiac tissue fragments were transferred and incubated in 25 ml Tyrode solution containing 0.012 g Collagenase D (Roche Diagnostics, 1-088-882), 0.009 g Collagenase B (Roche Diagnostics, 1-088-823), and 0.001 g Protease XIV from Streptomyces griseus (Sigma, P-5147) at 37 °C for 20-30 min. Digested products were filtered and centrifuged at 600 rpm for 5 min. The cell pellet was re-suspend in DMEM/F12 medium containing 10% FBS and 20 mM BDM. The cell suspension was plated into 10 cm cell culture dish and incubated for 1-3 h in a cell culture incubator. This pre-plating step removed fibroblasts and endothelial cells, which adhered to the uncoated cell-culture dish. Non-adherent cardiomyocytes were transferred to cell culture dish coated with 1% gelatin solution (Sigma, G9391).

Statistical analysis

All experiments were performed in triplicate or as indicated and numerical data were subject to independent sample *t* test. The levels of significance were set at $*p<0.05$ and $**p<0.01$.

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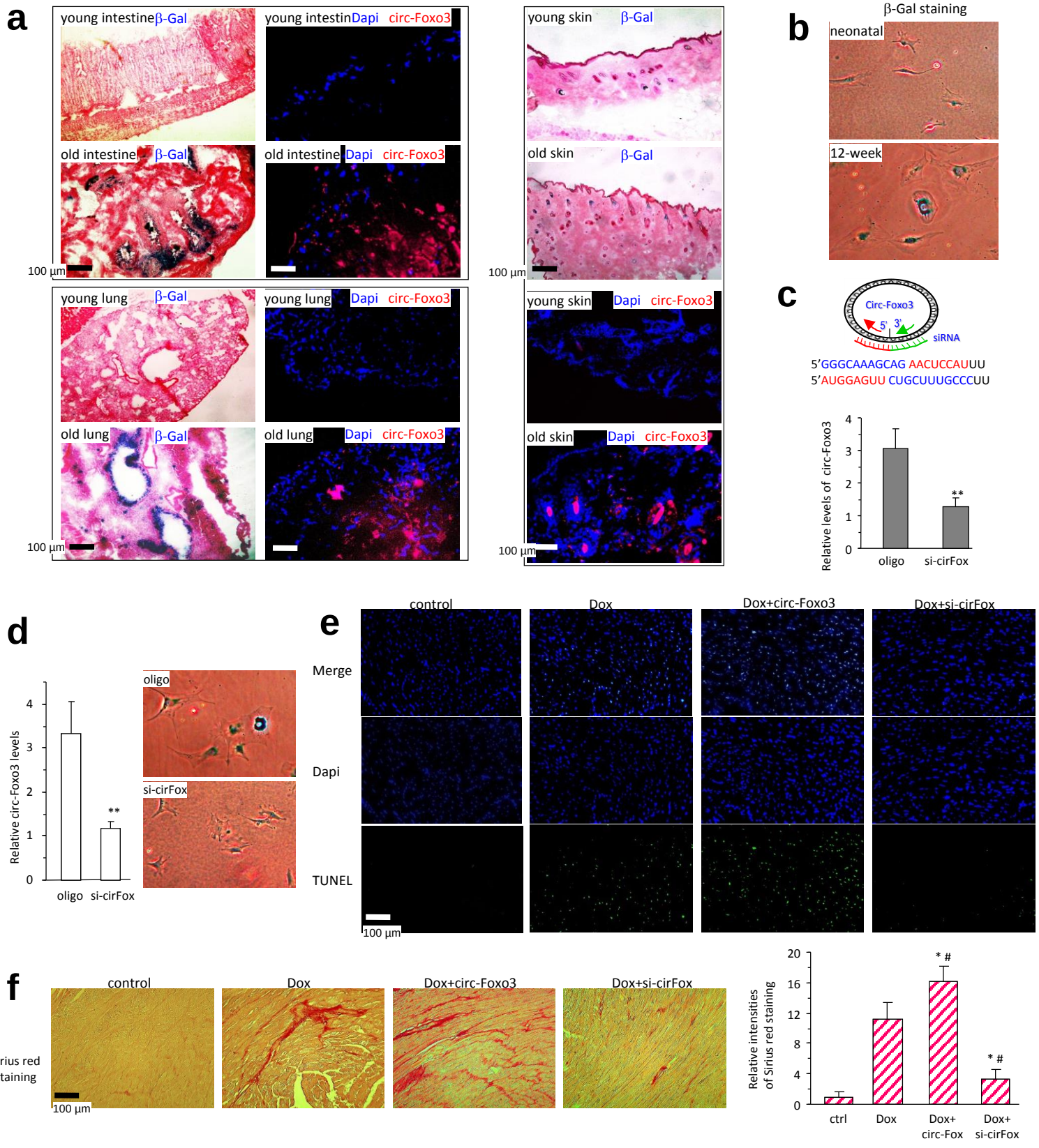
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Fig S1

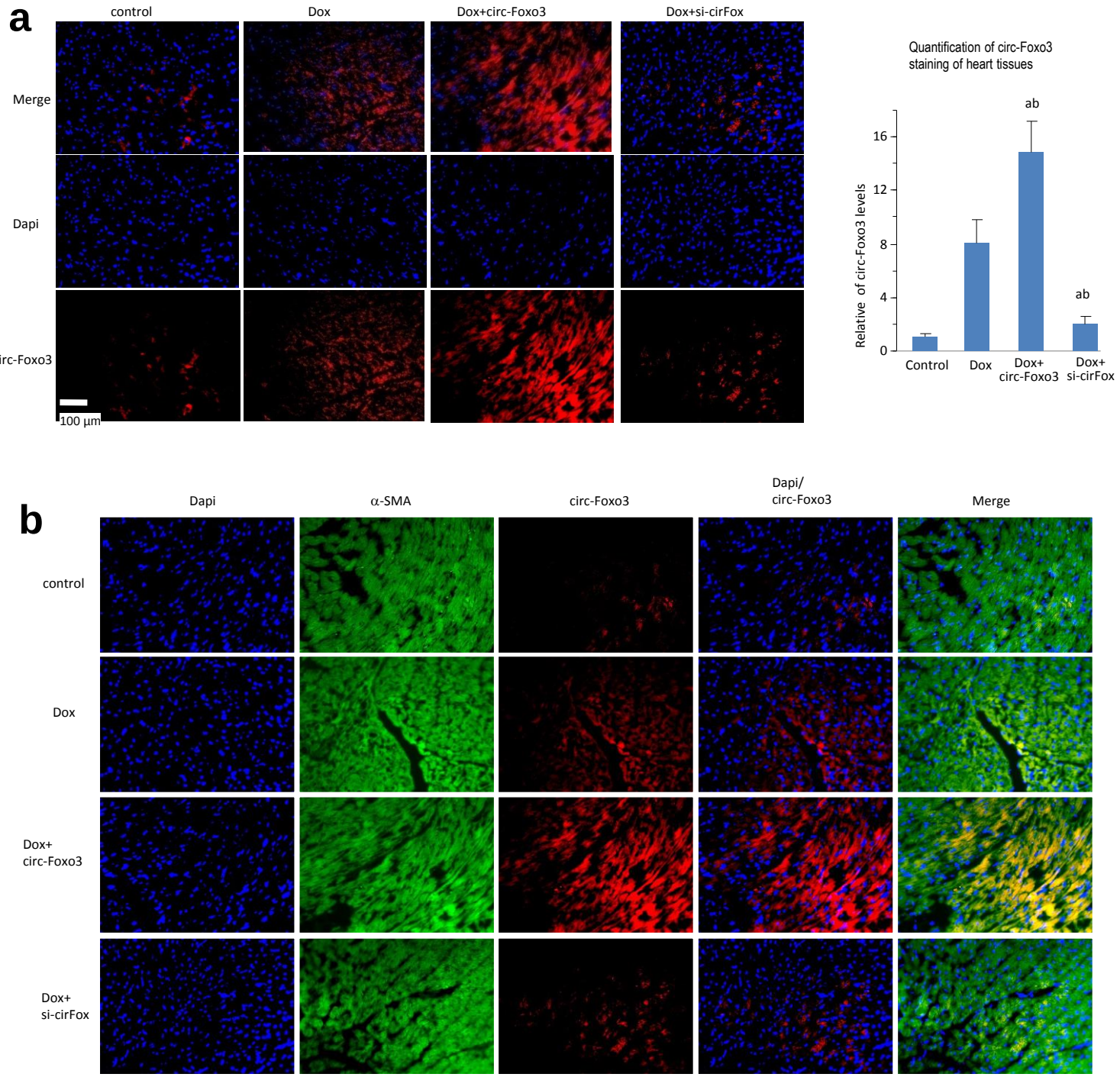
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Fig S2

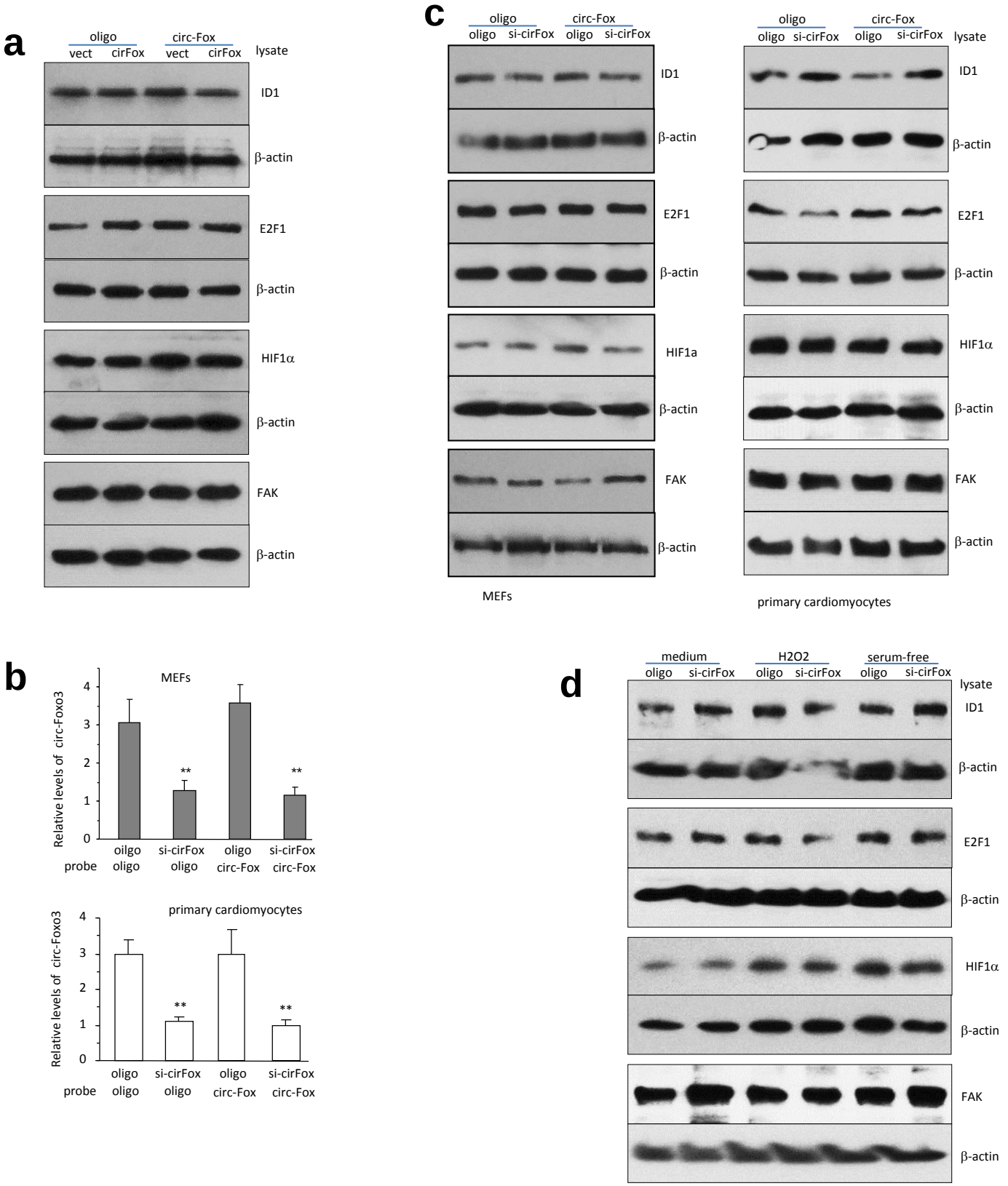
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Fig S3

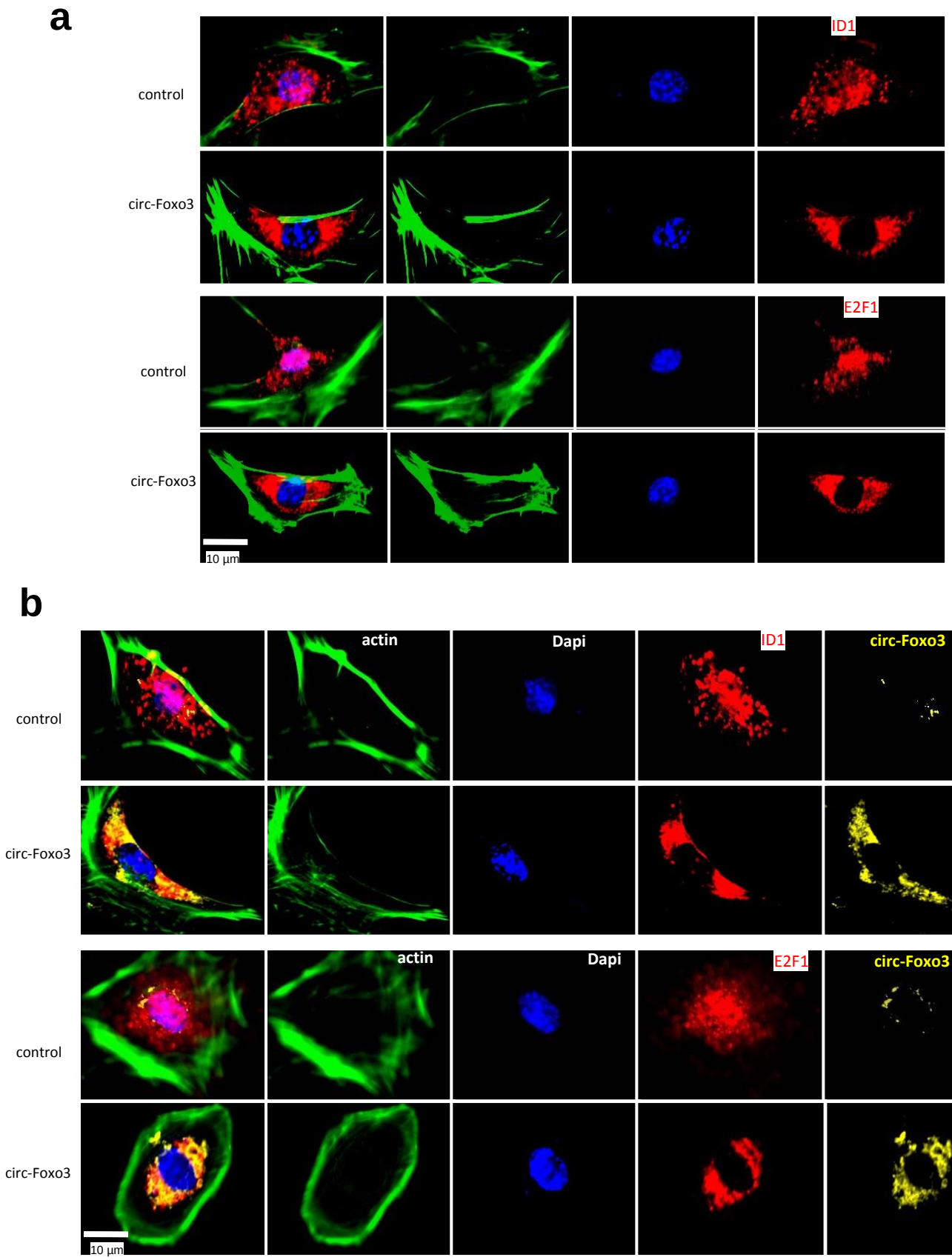
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Fig S4

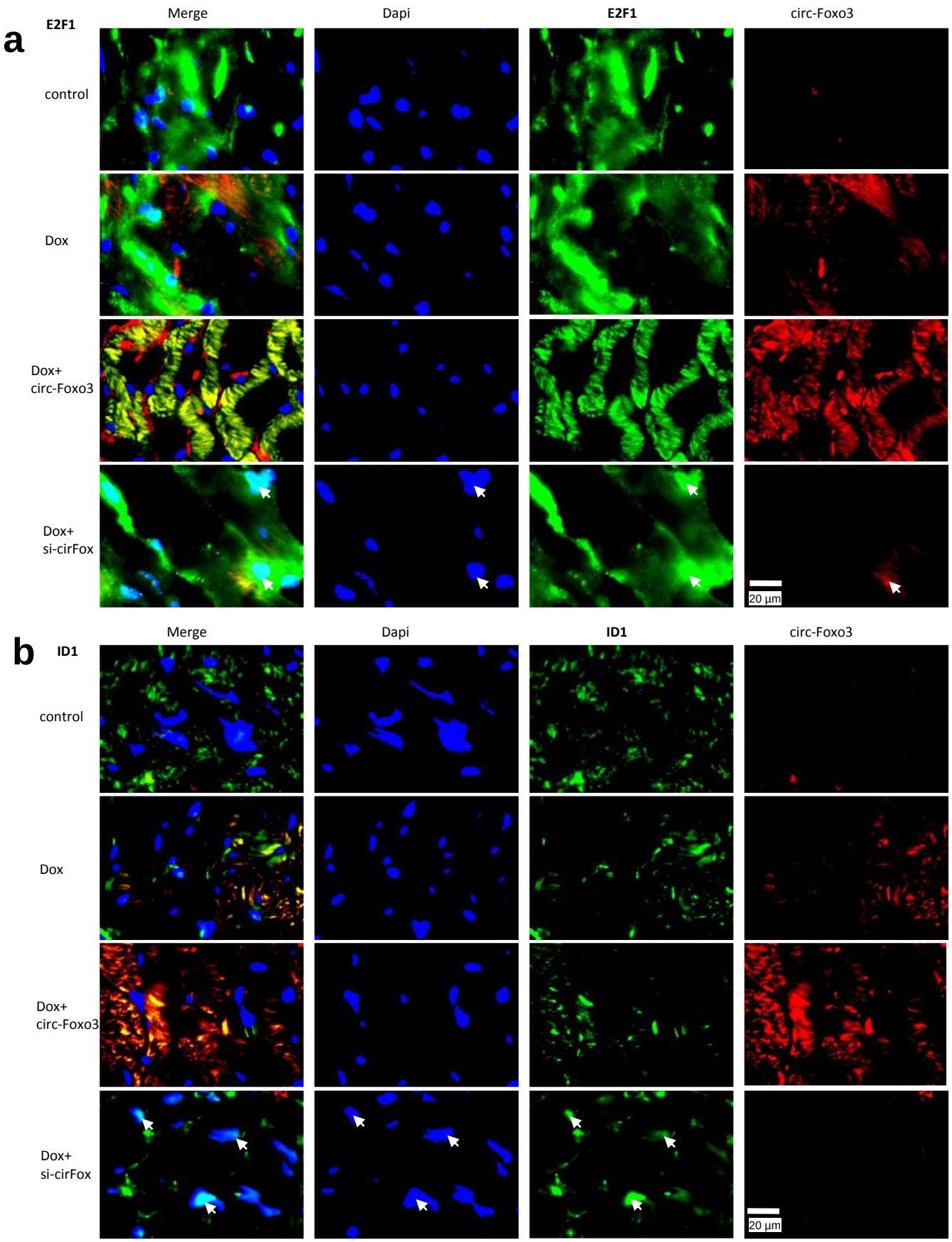
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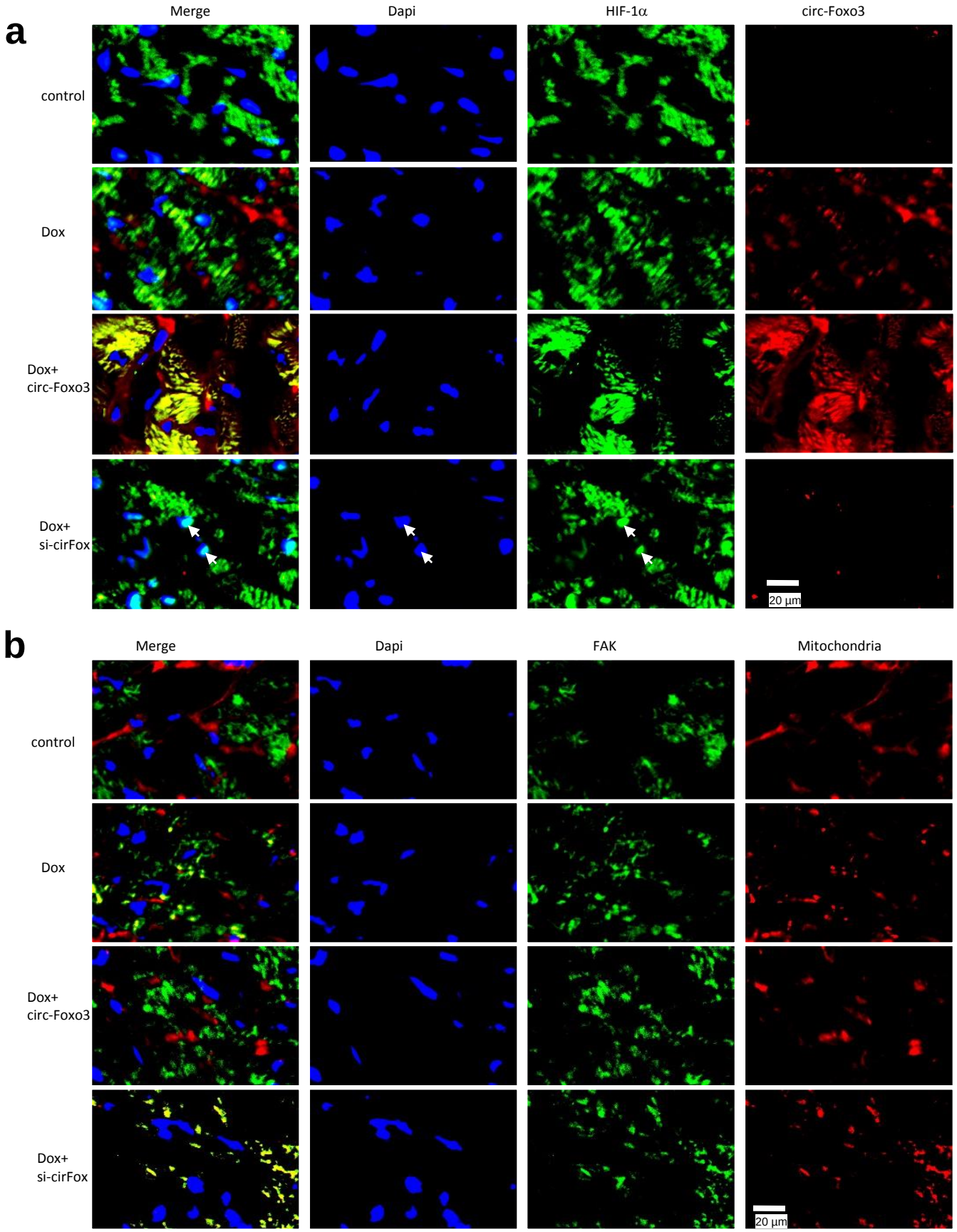
Fig S5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si-cirFox
20 μm

Fig S6

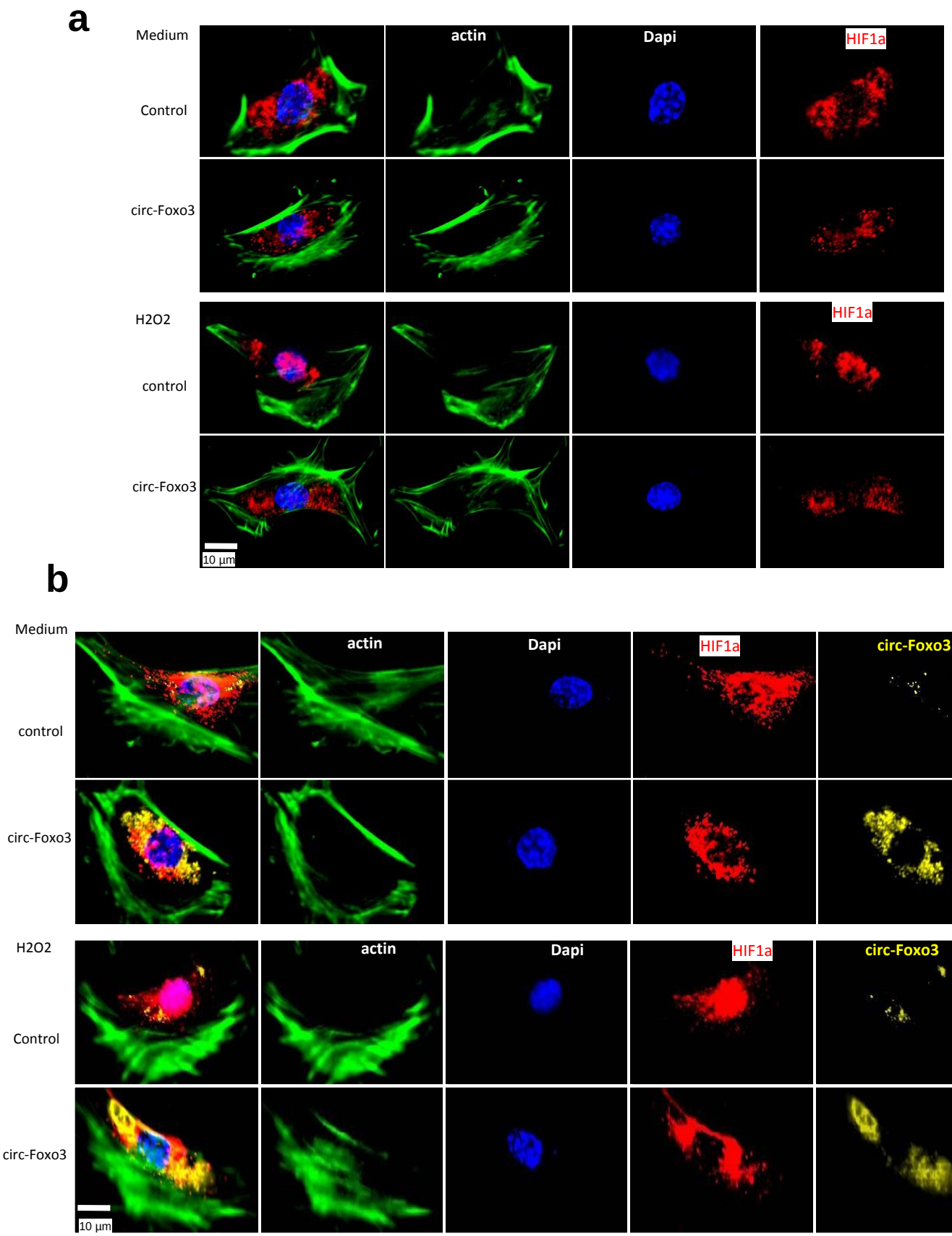
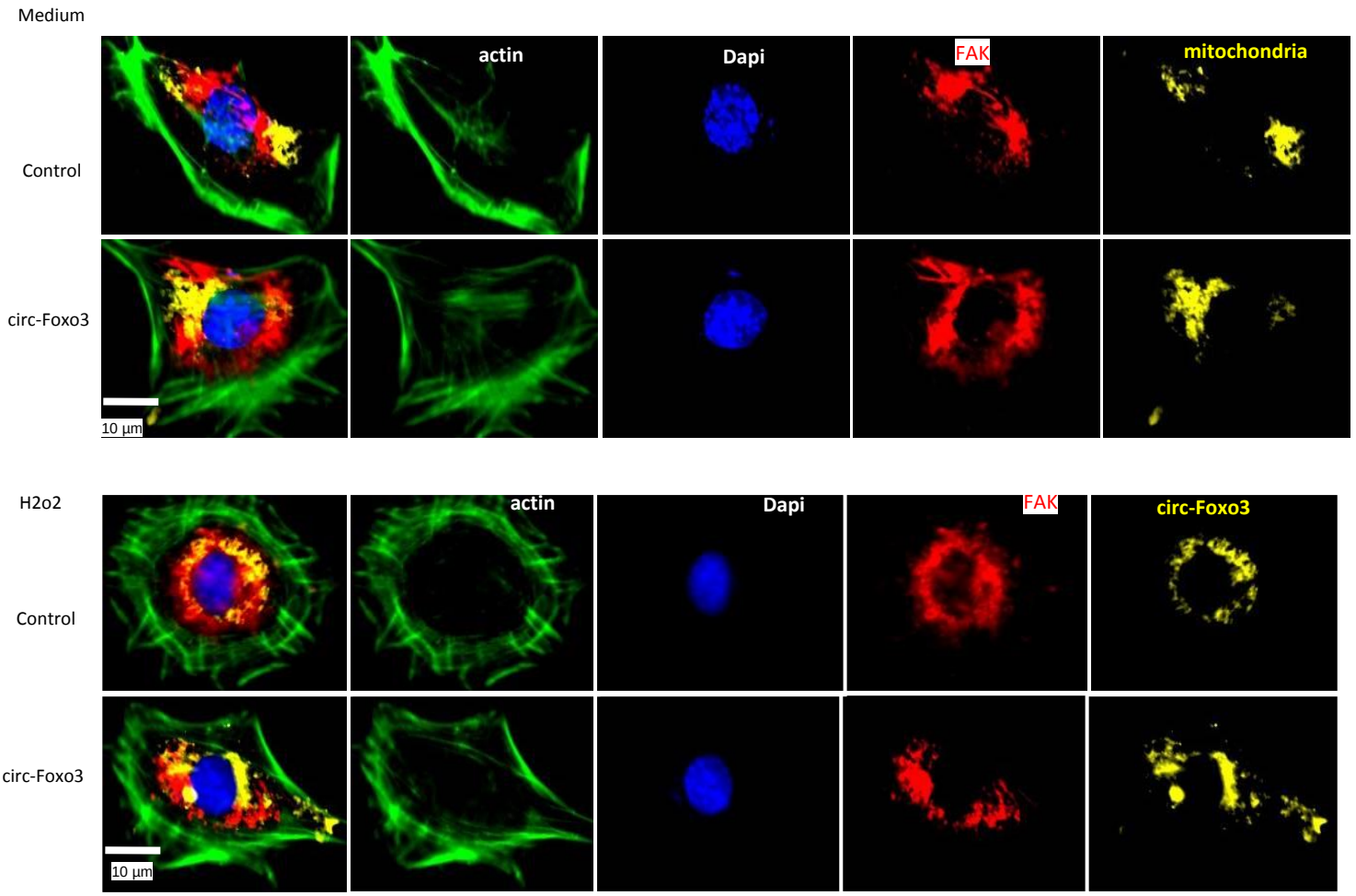

a
Medium
Control
circ-Foxo3
H2O2
control
circ-Foxo3
actin
Dapi
HIF1a
HIF1a
10 μm
b
Medium
control
circ-Foxo3
H2O2
Control
circ-Foxo3
actin
Dapi
HIF1a
circ-Foxo3
actin
Dapi
HIF1a
circ-Foxo3
10 μm

Fig S7

a



b

Primers used in this study

name	sequence	product	
Cir.FOXo3-F	5’ gtggggaacttcactggtgctaag	}	Endogenous and ectopic circ-Foxo3
Cir.FOXO3-R2	5’ ggg ttg atga tcc acc aag agc tctt		
Foxo3-PCR-F	5’ gcaagagctcttggtggatcatcaa	}	Linear Foxo3 mRNA
Foxo3-PCR-R	5’ tgggg ct gcc agg ccac ttgg agag		
Foxo3-F	5’ cttggacctggacatgttcaatgg	}	Specific for ectopic circ-Foxo3
ectopic-Foxo3-R	5’ tttc ggatcc ttc agcc tgg cac c		

c

Biotinylation probe detecting circ-Foxo3. **5’ (B) aaaTGTGCCGGATGGAGTTCTGCTTTGCCCACTTC**
(B): biotinylation

Cy5 labelled probe detecting circ-Foxo3. **5’ (Cy) aaaTGTGCCGGATGGAGTTCTGCTTTGCCCACTTC**
(Cy): Cy5 labelling

Fig S8