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A novel therapeutic peptide targeting myocardial reperfusion injury.

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1 **Abstract**

2

3 **Aims**

4 Regulated cell death is a main contributor of myocardial ischemia-reperfusion injury during
5 acute myocardial infarction. In this context, targeting apoptosis could be a potent
6 therapeutical strategy. In a previous study, we showed that DAXX (death-associated protein)
7 was essential for transducing the FAS-dependent apoptotic signal during IR injury. The
8 present study aims at evaluating the cardioprotective effects of a synthetic peptide inhibiting
9 FAS:DAXX interaction.

10 **Methods and results**

11 An interfering peptide was engineered and then coupled to the Tat cell penetrating peptide
12 (Tat-DAXXp). Its internalization and anti-apoptotic properties were demonstrated in primary
13 cardiomyocytes. Importantly, an intravenous bolus injection of Tat-DAXXp (1 mg/kg) 5 min
14 before reperfusion in a murine myocardial ischemia-reperfusion model decreased infarct size
15 by 48% after 24 h of reperfusion. In addition, Tat-DAXXp was still efficient after a 30-min
16 delayed administration, and was completely degraded and eliminated within 24 h thereby
17 reducing risks of potential side effects. Importantly, Tat-DAXXp reduced mouse early post-
18 infarction mortality by 67%. Mechanistically, cardioprotection was supported by both anti-
19 apoptotic and pro-survival effects, and an improvement of myocardial functional recovery as
20 evidenced in *ex vivo* experiments.

21 **Conclusions**

22 Our study demonstrates that a single dose of Tat-DAXXp injected intravenously at the onset
23 of reperfusion leads to a strong cardioprotection *in vivo* by inhibiting ischemia-reperfusion
24 injury validating Tat-DAXXp as a promising candidate for therapeutic application.

25

1 **Translational Perspective**

2 Our study proposes a peptidic strategy targeting apoptosis far upstream in the FAS receptor
3 signalling cascade for limiting reperfusion injury in AMI patients. This new approach
4 displays features required for clinical applications.

5 A single injection of the therapeutic peptide could be associated with both thrombolysis or at
6 reperfusion during primary coronary angioplasty with possible delayed application, which
7 would open new perspectives in clinical setting for the management of myocardial infarction.

8

9

1 **1. Introduction**

2 Prompt reperfusion after acute myocardial infarction (AMI) using thrombolysis or
3 primary coronary angioplasty has improved functional myocardial recovery and increased
4 patient survival dramatically.^{1,2} Unfortunately, reperfusion also induces lethal injuries adding
5 on those related to prolonged ischemia. These are partly linked to the activation of regulated
6 cell death cascades upon abrupt restoration of oxygen supply. Lethal reperfusion injury results
7 from death of cardiac cells that were viable at the end of the ischemic period before
8 myocardial reperfusion.^{3,4} Targeting regulated cell death pathways after an ischemic event
9 appeared as a promising and innovative therapeutic strategy to prevent reperfusion injury and
10 to limit infarct size.⁵⁻⁷ Although several drugs targeting the mitochondrial intrinsic pathway
11 have shown cardioprotective effects in animal models they all failed to be efficient in clinical
12 trials.^{8,9}

13 The death associated protein DAXX (*Death-domain associated protein-6*; 120 kDa -
14 740 amino acids) appears to play a key role in ischemia-reperfusion (IR) injury in various
15 organs including the heart. The DAXX protein plays different roles depending of its
16 subcellular localization. Indeed, nuclear DAXX is reported to regulate transcription acting
17 mainly as an anti-apoptotic contributor.¹⁰ Upon cytosolic relocalization after ischemic and
18 oxidative stress, this same protein acquires a pro-apoptotic role.^{11,12} Upon the *Apoptosis*
19 *signal-regulating kinase 1* (ASK1)-shuttling, DAXX interacts with the intracellular region of
20 the *First Apoptosis Signal* (FAS) death-receptor pathway and triggers the downstream
21 apoptotic signalling pathway.¹³ Therefore, we have focused on the development of therapeutic
22 tools targeting the FAS:DAXX interaction as a new treatment for AMI patients.

23 Since protein:protein interactions are promoted by large surfaces lacking well-defined
24 binding pockets, a peptidic strategy appeared appropriate to uncouple the FAS:DAXX
25 interaction. The designed interfering peptide DAXXp was coupled to the Tat cell penetrating

1 peptide (CPP) to allow efficient cellular internalization.¹⁴ Tat was selected among various
2 available CPPs since it was previously used successfully to deliver different molecules in
3 cardiac tissues to reduce myocardial IR injury.^{5, 15-17}

4 Our study was designed to (i) evaluate the internalization and anti-apoptotic activity of
5 Tat-DAXXp (TD) *in vitro*, (ii) investigate its cardioprotective effect in a murine IR model *ex*
6 *vivo* and *in vivo* and (iii) define the time window of TD administration post reperfusion *in*
7 *vivo*. Altogether our results evidence the TD peptide as a promising candidate for therapeutic
8 application during myocardial reperfusion injury.

9

10

11 **2. Methods**

12 See details in supplementary material online.

13

14 **2.1. Peptide synthesis, peptide-array binding studies and peptide stability assays**

15 Tat, DAXXp, Tat-DAXXp (TD), Tat-scrDAXXp (TDS) as well as the deriving sequences
16 (see **Table S1**) were synthesized on resin by conventional Fmoc-chemistry (by the group of
17 Dr. Volkmer or by *Intavis AG*). Peptide libraries were generated by a MultiPep SPOTrobot
18 (*Intavis Bioanalytical Instruments AG*) on N-modified CAPE-membranes according to the
19 standard SPOT synthesis. Peptide stability was evaluated in the presence of 20% mouse
20 serum by HPLC (*Waters*) at various times (0 h, 1 h, 2 h, 4 h and 8 h).

21

22 **2.2. Cell cultures**

23 Mouse myoblast cells were commercially acquired (C2C12 – ATCC: CRL-1772™).

24 Primary neonate cardiomyocytes were isolated from 1- to 2-day-old C57BL/6J mice (*Charles*
25 *River Laboratories*) ventricles by enzymatic digestion.

1 Isolated adult murine cardiomyocytes were enzymatically dissociated on a Langendorff
2 apparatus from control hearts or hearts subjected to global ischemia and reperfusion.

3

4 **2.3 Animal experiments**

5 All experiments were carried out on C57BL/6J mice (*Charles River laboratories*) in
6 accordance with the European Communities Council directive of November 1986 and
7 conformed to the "Guide for the Care and Use of Laboratory Animals" published by the US
8 National Institutes of Health (NIH publication 8th Edition, 2011).

9

10 **2.4. Langendorff ex vivo studies**

11 For *ex vivo* experiments, C57Bl6 male mice were anesthetized with a first intramuscular
12 injection of an anesthetic cocktail comprising ketamine (14 mg/kg, Imalgène®; *Merial*) and
13 xylazine (14 mg/kg, Rompun®; *Bayer*), and by a second injection of pentobarbital (76.6
14 mg/kg; *Sanofi-Aventis*). The heart was excised after sternotomy, quickly cannulated through
15 the aorta and mounted on a Langendorff system to be perfused with an oxygenated Krebs
16 buffer and subjected to IR protocols.

17

18 **2.5. Surgical protocol of myocardial ischemia/reperfusion (IR)**

19 Investigation conformed to the 2010/63/EU Directive of the European Parliament. The
20 surgical protocols were approved by the "Comité d'éthique pour l'expérimentation animale
21 Languedoc-Roussillon" (CEEA-LR) with the authorization number CE-LR-0814.

22 Mice were anaesthetized with an intramuscular (IM) injection of ketamine (50 mg/kg),
23 xylazine (10 mg/kg) and chlorpromazine (1.25 mg/kg; Largactil® 5 mg/ml; *Sanofi-Aventis*).

24 After a second injection of ketamine and xylazine (same doses), a left lateral thoracotomy and
25 a coronary artery ligation were performed.

1 When reperfusion lasted 60 min, a third administration of ketamine/xylazine was performed
2 (same doses, IM) at the onset of reperfusion. For longer reperfusion (24h), mice underwent
3 subcutaneous administration of lidocaine (1.5 mg/kg) and a postoperative awakening in an
4 emergency care unit.

5 At the end of all *in vivo* protocols, mice were anesthetized with an intramuscular injection of
6 an anaesthetic mixture comprising ketamine (50 mg/kg), xylazine (10 mg/kg) and
7 chlorpromazine (1.25 mg/kg) and the heart was harvested for further analysis.

8

9 **2.6. Infarct size assessment**

10 Infarct size measurements were obtained after dual coloration of left ventricular sections by
11 phtalocyanin blue dye and 2,3,5-triphenyltetrazolium chloride.

12

13 **2.7. DNA fragmentation assay**

14 DNA fragmentation was quantified *in vitro* on cell lysates and *in vivo* in transmural samples
15 of non-ischemic or ischemic areas of left ventricle (LV). with an enzyme-linked
16 immunosorbent assay kit (Cell Death ELISA; *Roche Diagnostics*) designed to measure of
17 histone-complexed DNA fragments (mono- and oligonucleosomes) out of the cytoplasm of
18 cells after the induction of apoptosis.

19

20 **2.8. Immunoblotting**

21 Western blots were performed from LV protein extracts from *ex vivo* hearts using antibodies
22 as described in the supplemental materials.

23

24 **2.9. Immunostaining**

1 Fixated cells were incubated with an anti-DAXX antibody (*Santa Cruz Biotechnologies*) to
2 evaluate endogenous DAXX localisation after an ischemic stress. To follow TD
3 accumulation in the LV, carboxyfluorescein labelled TD peptide was injected 5 min before
4 the reperfusion. LV deparaffinized sections were imaged.

5

6 **2.10. SPECT-CT imaging**

7 Whole-body SPECT/CT images were acquired with a 4-head multiplexing multipinhole
8 NanoSPECT camera (*Bioscan Inc.*) at various times (0 h, 3 h, 6 h, and 24 h) after tail vein
9 injection of 10 MBq radiolabeled ^{125}I -Tat-DAXXp in control mice or at the time of
10 reperfusion in IR mice.

11

12 **2.11. Statistical analysis**

13 Statistical analysis was performed only for $n \geq 5$ independent experiments. Data (mean \pm SD)
14 were analyzed with nonparametric Kruskal-Wallis test for multiple comparison or Mann-
15 Whitney when appropriate. For repeated measures, data were analyzed with Two-way RM
16 ANOVA and the Tukey's or Sidak's post-test. Concerning **Figures 6B and C**, infarct size
17 data (mean \pm SD) were fitted by a linear regression model using the least squares method.
18 Data were analyzed with GraphPad Prism 6.0 (*GraphPad Software*).

19

1 **3. Results**

2 **3.1. Design of the interfering Tat-DAXXp peptide and evaluation of its cardioprotective**
3 **effect in vitro.**

4 The interfering DAXXpeptide (DAXXp) was engineered using peptide arrays generated by
5 SPOT technology (**Figure S1**). DAXXp was coupled to the Tat CPP resulting in the Tat-
6 DAXXp (noted TD, 3.4 kDa, 29 amino acids). Its anti-apoptotic potential and its
7 internalization mechanism via endocytosis were first demonstrated in C2C12 murine
8 myocytes cell line (see Supplemental Results and **Figures S2 and S3**). The minimal active
9 sequence (MAS) of the interfering peptide was investigated resulting in the 16-mer DAXXp
10 sequences (**Figure S4 and Table S1**), which was selected for further experiments.

11 In a second step, carboxyfluorescein (CF)-labelled TD internalization was confirmed
12 in primary neonate murine cardiomyocytes by flow cytometry ($87 \pm 3\%$ of the analyzed
13 cells) (**Figure 1A**). In contrast and as expected, the free DAXXp peptide was not internalized.
14 As observed in C2C12 cells (**Figure S3A**), CF-TD showed first a punctuated cytosolic pattern
15 (1 h-incubation) followed by a diffuse distribution in the cytosol at later times (4 h) (**Figure**
16 **1B**) in keeping with an endocytic uptake followed by efficient release from endocytic
17 compartments.

18 The anti-apoptotic activity of TD was evaluated in primary murine cardiomyocytes
19 submitted to an apoptotic stress (**Figure 1C**) using specific DNA fragmentation (a hallmark
20 of apoptosis) quantification. A 44%-decrease in DNA fragmentation was observed in
21 cardiomyocytes treated with TD ($p^* < 0.05$ versus Tat), which was not observed with the Tat
22 delivery vector alone or with a scrambled version (Tat-scrDAXXp, noted TDS) of the
23 interfering peptide (**Figure 1D**), both used as controls. As expected from its poor cell uptake
24 in this *in vitro* model, the free DAXXp did not induce any anti-apoptotic activity even at a 10
25 $\mu\text{mol/L}$ concentration.

1

2 **3.2. Effect of Tat-DAXXp on isolated perfused hearts**

3 The protective effect of TD was evaluated on isolated perfused hearts subjected to global IR
4 (**Figure 2A**). A significant 45%-decrease in infarct size was observed in the group of hearts
5 reperfused with TD ($p^*=0.012$ versus TDS; **Figure 2B**). TDS did not induce any
6 cardioprotective effect compared to the control (non-treated IR).

7 The developed tension assessed under constant frequency stimulation during the
8 perfusion protocol was greater in hearts perfused with TD than in those perfused with TDS or
9 buffer alone (**Figure 2C**). Interestingly, an improvement in the coronary flow was
10 demonstrated in the post-ischemic period in hearts treated by TD after a regional ischemia
11 with no perturbation of the electrocardiographic (ECG) parameters (**Figure 2D**).

12

13 **3.3. Evaluation of Tat-DAXXp tissue localization and degradation profile**

14 The tissue distribution of the CF-TD construct was evaluated in the heart of mice submitted to
15 an *in vivo* surgical protocol of myocardial infarction and injected 5 min before reperfusion
16 (1 mg/kg). Confocal images from left ventricle (LV) slices clearly reveal cardiac cells with a
17 green labeling indicating the peptidic presence in the cytoplasm after 1 h-reperfusion (**Figure**
18 **S5A**) in agreement with the *in vitro* intracellular distribution data in primary murine
19 cardiomyocytes (**Figure 1A**). Co-staining with DAPI showed few cases with a nuclear
20 peptide localization (**Figure S5A**, right panel).

21 Metabolic stability and elimination of the peptide are critical requirement to avoid
22 side-effects of the anti-apoptotic peptide. The peptide was rapidly degraded in a time-
23 dependent manner in the presence of mouse serum *in vitro*, with a total degradation after 24 h
24 (**Figure S5B**). Whole-body SPECT/CT recordings using ^{125}I -TD administration in control or
25 IR subjected animals revealed the presence of the peptide in the heart (**Figure 3A**) and a
26 nearly complete hepatic and renal clearance (80-100%) 24 h after its injection (**Figure 3B**).

1

2 **3.4. Cardioprotective effects of the interfering peptide in vivo**

3 Having established that the peptide was detected in the target tissue after systemic
4 administration, we determined the optimal peptide dose for treatment in an *in vivo* IR murine
5 model (details given in **Table S2**). Various doses of TD were administered as a single
6 intravenous bolus 5 min before reperfusion (**Figure 3C**). After 1 h-reperfusion, a 56%-
7 decrease in infarct size was obtained using a 1 mg/kg TD dose *versus* TDS ($p^{**}=0.006$). The
8 same efficiency was observed at a 10-fold higher dose (10 mg/kg, $p>0.99$). The
9 cardioprotective effect of TD was specific to the DAXX peptide sequence since the control
10 Tat and TDS peptides did not protect against IR injury (Tat *versus* TDS; $p>0.99$). In order to
11 verify that the cardioprotection was not transient, *in vivo* experiments were performed with 24
12 h-reperfusion protocols. Infarct size measurements (**Table S2**) revealed a significant
13 reduction in the presence of TD in particular at the 1 mg/kg TD dose (48% *versus* TDS;
14 $p^*=0.026$; **Figure 3D**). In all conditions (1 h and 24 h), there was no statistical difference
15 between AR/LV (area at risk/left ventricle mass) among groups (**Table S2; Figure S6**).
16 Relevant for clinical translation, a drastic 67%-decrease in mortality was observed during the
17 critical period of 24 h post-infarction in mice treated with 1 mg/kg TD (13%, $n=16$) *versus*
18 Tat (38%, $n=55$).

19

20 **3.5. Mechanisms of cardioprotection by Tat-DAXXp**

21 To evaluate if the cardioprotective effect of the TD peptide was correlated to a reduced
22 apoptosis, DNA fragmentation was quantified in hearts subjected to the same *in vivo* IR 1h-
23 reperfusion protocol. TD (1 mg/kg) inhibited apoptosis (reduction of 59% *versus* TDS;
24 $p^{**}=0.006$) while TDS did not exert any inhibitory effect *per se* (TDS *versus* Tat, $p>0.99$;
25 **Figure 4A**).

1 Similarly, anti-apoptotic effects of TD measured 24 h after IR were as potent than at
2 1h-reperfusion (reduction of 53% for TD *versus* TDS; $p^*=0.023$), and similar to that provided
3 by TD 10 mg/kg (TD 1 mg/kg *versus* TD 10 mg/kg, $p>0.99$; **Figure 4B**). TDS did not inhibit
4 apoptosis and did not decrease infarct size (TDS *versus* Tat, $p>0.99$; **Figure 4B**).

5 The potent anti-apoptotic effect of the TD peptide was further confirmed by Western
6 blot analysis. In hearts subjected to IR injury, TD treatment led to a down-regulation of
7 pJNK/JNK MAPKinase ratios and activation of caspase 3 (**Figure 4C**), FADD and cleaved
8 caspase 8 (FAS-FADD downstream pathway, **Figure 4D**), BAD and cleaved caspase 9 (key
9 components of mitochondria-dependent apoptosis) protein levels (**Figure 4E**). However,
10 neither DAXX nor FAS protein levels were modified upon TD treatment compared to non-
11 treated IR conditions (**Figures 4C and 4D**). Finally, TD treatment did not affect necroptosis
12 or the autophagy flux involved in IR-induced cell death¹⁸ (**Figures S7 and S8**).

13 HSP70 production, a hallmark of proteotoxic stress during IR injury,¹⁹ was also
14 drastically decreased upon TD treatment *versus* non-treated IR conditions (**Figure 5A**). In
15 addition, evaluation of the phosphorylation patterns of ERK1/2 and AKT pro-survival
16 kinases, showed a significantly increased in the phosphorylation ratio in TD treated *versus*
17 non-treated IR hearts (**Figures 5B,C**).

18 Anti-apoptotic effects have been correlated to the nuclear localization of the DAXX
19 protein.^{20, 21} Thus, nuclear *versus* cytoplasmatic DAXX protein levels were measured in cells
20 isolated from hearts subjected to IR protocol *ex vivo*. Upon IR or Tat conditions, very low
21 levels of nuclear DAXX protein were observed and quantified compared to the Sham
22 condition. TD treatment during reperfusion results in an increased percentage of cells with
23 nuclear DAXX protein compared to Tat treatment (**Figures 5D and 5E**). DAXX
24 phosphorylation, that triggers export from the nucleus to the cytoplasm,²¹ was increased upon
25 IR and Tat treatments compared to the Sham condition (p^{**} *versus* Sham). DAXX was

1 phosphorylated 1 h after TD treatment at an intermediate level between Sham and IR (p=ns
2 *versus* IR and *versus* Sham; **Figures 5F and 5G**) (see also Supplemental Results 2.5 and 2.6).

3

4 **3.6. Therapeutic time window of Tat-DAXXp administration and elimination**

5 In order to determine the therapeutic time window providing optimal cardioprotection, TD
6 administration was delayed after the onset of reperfusion. Delaying peptide administration by
7 5, 15 or 30 min after reperfusion (see protocol **Figure 6A**) did not abolish cardioprotection in
8 terms of infarct size or apoptosis. The protection was lost at 45 min (TD_{Δ45} *versus* TD,
9 p***=0.0002 for infarct size and DNA fragmentation evaluated at 1 h; **Figures 6B and 6D**)
10 consistently with our previous data on delayed ischemic postconditioning.²² Similar results
11 were obtained 24 h after the onset of reperfusion (**Figures 6C and 6E**). We thus found a linear
12 correlation between infarct size and time delay for TD injection after the onset of reperfusion
13 whether measured after 1 h-reperfusion ($r^2= 0.92$; **Figure 6B**) or 24 h-reperfusion ($r^2= 0.83$;
14 **Figure 6C**). There was no statistical difference between AR/LV among groups after 1 h or 24
15 h-reperfusion (**Table S2 and Figure S6**).

16

17 **4. Discussion**

18 Our study demonstrates the potent cardioprotective effects of the anti-apoptotic
19 peptide Tat-DAXXp (TD) targeting the FAS-dependent pathway during ischemia-reperfusion
20 injury. *In vitro*, this peptide was efficiently internalized in primary cardiomyocytes and
21 provided anti-apoptotic effects after an apoptotic stress. *In vivo*, a single bolus of TD injected
22 intravenously 5 min before reperfusion in a murine myocardial IR model decreased infarct
23 size by 48% after 24 h reperfusion without any side effects probably in relation with its rapid
24 degradation and elimination. Furthermore, cardioprotection was still efficient after delayed
25 administration up to 30 min after reperfusion. Mechanistically, cardioprotection was

1 supported by both anti-apoptotic and pro-survival effects, and an improvement of the
2 myocardial functional recovery was evidenced in *ex vivo* experiments. Importantly, TD
3 treatment reduced mouse early post-infarction mortality by 67%. Altogether, these results
4 suggest that TD treatment leads to a potent cardioprotection.

5 Prosurvival and apoptotic pathways are intricately and finely regulated with redundant
6 mechanisms to strongly control cell fate after IR stress (**Figure 7A**).^{18, 23} The death
7 receptor/extrinsic and mitochondrial/intrinsic apoptotic pathways are the most heavily studied
8 pathways of regulated cell death in myocardial IR injury. Once activated, these two apoptotic
9 pathways initiate a cascade of caspases converging on mitochondria and leading to cell
10 destruction. The involvement of the FAS receptor was reported to play a key role in
11 myocardial IR-induced cell apoptosis in keeping with high levels of circulating FAS Ligand
12 in the blood of AMI patients.²⁴⁻²⁶ However, initiation of the FAS-dependent apoptotic cascade
13 is induced by the binding of at least one of the two adaptor proteins, FADD and DAXX, to the
14 intracellular region of the FAS receptor. FADD (*Fas-Associated protein with Death Domain*)
15 then transduces death signals by activating caspase 8 within the DISC (*Death-Inducing Signal*
16 *Complex*). Independently of FADD, phosphorylated DAXX relocates upon oxidative stress
17 from the nucleus to the cytoplasm, interacts with FAS and activates the JNK pathway leading
18 to cell death.¹³ DAXX is able to activate the intrinsic pathway *via* the JNK-BAX-dependent
19 crosstalk.^{27, 28}

20 Our study shows that the interfering TD peptide provides a major cardioprotective effect
21 when injected at the onset of reperfusion (**Figure 7B**). The resulting anti-apoptotic effect was
22 evidenced first by a drastic decrease in specific DNA fragmentation in the myocardium after
23 IR, corroborating our previous studies on cardioprotection.^{5, 22, 29} As expected, because
24 FAS:DAXX interaction was prevented, the downstream activation of the JNK/caspase 3
25 pathway was down-regulated upon TD treatment. The expression of BAD protein (a *Bcl-2-*

1 *Associated Death promoter* facilitating BAX/BAK activation) and the level of activated
2 caspase 9, both mediators of the intrinsic pathway, were down-regulated, probably due to the
3 mitochondrial crosstalk downstream JNK activation.²⁷ Consistently, co-administration of
4 cyclosporin A (inhibitor of the mitochondrial permeability transition pore opening by
5 interacting with cyclophilin D) and TD in our murine IR model did not provide any additive
6 cardioprotection (data not shown). Interestingly, we evidenced that TD treatment was able to
7 also decrease the FADD-caspase 8 extrinsic pathway probably due to its impact on DAXX-
8 FADD cooperativity within the DISC as already observed in T-cells overexpressing DAXX-
9 DN.³⁰ However and of great interest, TD peptide treatment was not associated in our study
10 with a deleterious reduction of DAXX protein level, which could result in unexpected pro-
11 apoptotic effects as reported in the case of siRNA DAXX silencing.¹⁰ Altogether, these data
12 show that TD treatment was able to decrease apoptosis upstream in the apoptotic signaling
13 cascade by acting on both the extrinsic and intrinsic cascades. Finally, activation of effector
14 caspase 3 was decreased resulting in a drastic decrease in DNA fragmentation and in HSP70
15 protein level, a hallmark of proteotoxic stress¹⁹ leading to a potent cardioprotection. Studies in
16 the literature have reported that HSP70 protein expression is strongly up-regulated during
17 FAS-induced apoptosis *in vitro* and during myocardial IR in order to protect the cells against
18 IR injury *via* a suppression of reactive oxygen species generation and by inhibiting cell
19 apoptosis.^{19, 31-33} Upon TD treatment, HSP70 protein level was reduced as compared to non-
20 treated IR hearts suggesting that TD plays a key role in early cardioprotection. These results
21 are of great interest because high levels of HSP70 are detected in the serum of patients with
22 heart failure.³⁴

23 Interestingly, cardioprotection afforded by TD treatment was also related to an activation
24 of survival pathways.^{35, 36} We investigated the expression of proteins involved in the RISK
25 (*Reperfusion Injury Salvage Kinase*) pathway including ERK1/2 and PI3kinase-AKT, which

1 both play crucial roles in preventing reperfusion injury in the myocardium. In both cases, our
2 results show an activation of these survival kinases after TD treatment. ERK1/2 activation is
3 associated with a protection against apoptosis in cardiac myocytes and to a reduction of IR
4 injury in the heart *in vivo*.^{35, 36} These results are also consistent with those already obtained in
5 DAXX-DN mice that are cardioprotected against reperfusion injury.²⁹

6 As already mentioned, DAXX is described as a dual protein with both anti-apoptotic
7 and pro-apoptotic roles depending on its subcellular localization, nucleus *versus* cytoplasm,
8 respectively^{21, 27, 37-39}. Such a dual role requires a fine tuning of its nucleo-cytoplasmic ratio
9 by phosphorylation, nuclear export, positive ASK1-SEK1-JNK³⁸ and negative ASK1-JIP
10 (*JNK Interacting Protein*)⁴⁰ feedback loops in order to control the balance between survival
11 and death, making complex mechanistic studies (See Supplementary Results 2.6). Our study
12 performed in hearts subjected to IR *ex vivo* shows that, upon TD treatment, DAXX
13 phosphorylation was 37%-decreased compared to IR condition. This is in accordance with the
14 observed higher nuclear localization of DAXX after TD treatment compared to IR. These
15 results were further confirmed by evaluating pDAXX levels 15 min after the onset of
16 reperfusion. Our data show that, at this time point, pDAXX levels represents 63% of that
17 measured in the 1 h-IR group (**Figure S9**) despite an obvious delayed cardioprotection
18 characterized by a 42% decrease in both infarct size and soluble nucleosome (TD_{Δ15} *versus*
19 Tat; see **Figures 6B,D**). This suggests that DAXX export from the nucleus to the cytoplasm
20 after phosphorylation occurs within the first minutes of the wavefront of IR injury²² even in
21 the presence of the peptide. Blocking DAXX trafficking was identified as a potential
22 therapeutic target for ischemic injury in hippocampal neurons.^{20, 41} Our data suggest that
23 DAXX nuclear trafficking may be indirectly influenced by TD treatment as a result of its
24 impact on both apoptotic and survival pathways as well as on the feedback loops controlling
25 DAXX nucleo-cytoplasmic ratio (see **Figure 7**).

1 In conclusion, TD peptide treatment appears as a particularly relevant therapeutic
2 strategy in view of the key and specific role played by DAXX in the upstream events of
3 reperfusion-induced apoptotic pathways. Targeting FAS:DAXX interaction from the extrinsic
4 pathway also indirectly reduced the activation of the intrinsic (JNK-BAX crosstalk) cascade
5 thus allowing a complete inhibition of reperfusion injury with a single pharmacological agent.
6 Indeed, a single bolus administration of the TD anti-apoptotic peptide at low dose was
7 sufficient to trigger an effective cardioprotection mainly *via* a reduction of apoptosis during
8 the requested time window. Our data indeed indicate that TD decreases both apoptosis and
9 infarct size when administered up to 30 min after the re-opening of the occluded coronary
10 artery. This perspective of delayed application, which is exactly in agreement with the
11 delayed post-conditioning previously reported,²² is clinically important since it offers the
12 possibility to treat a larger number of patients including those already reperfused with
13 thrombolytic agents before hospital admission. However, for further clinical translation, a
14 detailed evaluation of the mechanism by which TD interacts with the FAS:DAXX protein
15 complex as well as a determination of TD long-term cardioprotection in an IR murine model
16 as well as in clinical relevant animal models will be important to fully understand the
17 molecular effect of the peptide during reperfusion.

18

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25

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9

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11

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7 hippocampal neurons against ischemia/reperfusion injury via the RIP3/DAXX signaling
8 pathway in rats. *Neurosci Lett* 2017;651:207-215.

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11

12 **10. Figure legends**

13 **Figure 1: Internalization and anti-apoptotic properties of Tat-DAXXp in** 14 **cardiomyocytes.**

15 (A) Cellular internalization was assessed by flow cytometry in cardiomyocytes incubated with
16 carboxyfluorescein (CF)-labeled TD or CF-DAXXp (1 $\mu\text{mol/L}$) for 1 h. Untreated cells
17 represent the negative control (Ctrl). Data plotted as scatter dot blots and mean \pm SD with n=3
18 independent cultures. (B) Representative image of cultured cardiomyocytes incubated with 1
19 $\mu\text{mol/L}$ CF-TD incubated for 1 h or 4 h. Cell nuclei were stained with Hoechst-dye (blue).
20 Bar scale = 10 μm ; (C, D) Specific DNA fragmentation was measured in cardiomyocytes
21 treated with staurosporin (STS) for 6 h with or without peptides (concentrations in $\mu\text{mol/L}$
22 noted in parentheses) and allowed to recover during 40 h. Data (plotted as scatter dot blots
23 and mean \pm SD with n=5 independent cultures) were compared using Kruskal-Wallis (Dunn's
24 *post hoc* test) and were noted * for $p < 0.05$ versus Tat and # for $p < 0.05$ versus TDS.

25

1 **Figure 2: Improved functional recovery upon Tat-DAXXp treatment *ex vivo*.**

2 (A) Mouse hearts were perfused on a Langendorff system and subjected to 30 min of global
 3 (B,C) or 40 min of regional (D) ischemia followed by 1 h-reperfusion. 1 μ mol/L TD or TDS
 4 were perfused during the first minute of reperfusion; (B) Scatter dot blots and mean \pm SD
 5 were plotted for infarct size (% LV). A drastic decrease in infarct size was observed after TD
 6 treatment *versus* TDS (21.34 ± 3.48 for TD, n=6 *versus* 38.51 ± 7.62 for TDS, n=9;
 7 $p^*=0.012$) which did not provide any cardioprotection *versus* IR (38.51 ± 7.62 for TDS, n=9
 8 *versus* 40.68 ± 2.85 for IR, n=6, ns for $p>0.999$). Statistical analysis was performed using
 9 Kruskal-Whallis with the Dunn's post test for multiple comparison. Representative pictures of
 10 tissue samples after phtalocyanin blue and TTC-dye dual coloration for IR, TD and TDS
 11 administered at 1 mg/kg. Scale bar: 2 mm. (C) Developed tension evaluated with the DMT
 12 myograph during ischemia (grey rectangle) and reperfusion (TD, n=6 *versus* TDS, n=5;
 13 $p^{***}=0.0009$). For repeated measures, data (plotted as mean \pm SD) were compared using two-
 14 way RM ANOVA and the Tukey's post-test. (D) Coronary flow measured on the EMKA
 15 system in hearts treated by TD (n=6) *versus* IR (IR; n=6); $p^{**}=0.0072$. For repeated
 16 measures, data (plotted as mean \pm SD) were compared using two-way RM ANOVA and the
 17 Sidak's post-test.

18

19 **Figure 3: Effect of TD in mice subjected to an IR protocol *in vivo*.**

20 (A) Whole-body SPECT/CT *in vivo* imaging of 125 I-TD in IR (40 minutes ischemia - 3 h
 21 reperfusion) *versus* non operated Ctrl mice at 3 h post-injection. From left to right,
 22 representative images with coronal, sagittal, and transverse views of tracer activity. Please
 23 note 125 I-TD localization in the heart (white arrow) with a more pronounced signal in the
 24 ischemic condition (IR mouse) *versus* control (Ctrl). L for left side and R for right. Scale bar:
 25 5 mm. (B) Quantification of *in vivo* images of 125 I-TD whole-body distribution at indicated

1 time points (0 h, 3 h, 6 h and 24 h) post-injection in control (Ctrl; n=3) and IR mice treated
2 (n=2).
3 **(C,D)** Scatter dot blots and mean \pm SD for infarct size (% of area at risk) was measured after
4 1 h **(C)** or 24 h **(D)** of reperfusion in mice subjected to 40 min-ischemia and treated with Tat,
5 TDS or TD intravenously 5 min before reperfusion at the indicated doses (in mg/kg).
6 Representative pictures of tissue samples after phtalocyanin blue and TTC-dye dual
7 coloration for Tat, TD and TDS administered at 1 mg/kg. A drastic decrease in infarct size at
8 1 h of reperfusion was observed after TD treatment *versus* Tat (15.54 ± 6.91 for TD, n=11
9 *versus* 38.37 ± 8.46 for Tat, n=14; p****<0.0001). Note that n= 6 for TD (0.1 mg/kg) and TD
10 (10 mg/kg). **(D)** Same results at 24 h-reperfusion: 17.19 ± 5.16 for TD, n=8 *versus* $33.29 \pm$
11 6.07 for Tat, n=17; p**=0.001. Note that n= 7 for TD (0.1 mg/kg) and n=6 for TD (10
12 mg/kg). Data were compared using Kruskal-Whallis with the Dunn's post test. Bar scale: 2
13 mm.

14

15 **Figure 4: Anti-apoptotic effect of Tat-DAXXp treatment**

16 Internucleosomal DNA fragmentation was determined by ELISA in IR mice treated with Tat,
17 TDS and TD (at indicated doses in mg/kg) after 1 h **(A)** or 24 h **(B)** of reperfusion. Scatter dot
18 blots and mean \pm SD show the I/NI ratio corresponding to the ratio of soluble nucleosomes in
19 the ischemic *versus* the non-ischemic portion of LV tissues. A drastic decrease in soluble
20 nucleosome ratio at 1 h of reperfusion was observed after TD treatment *versus* Tat ($1.61 \pm$
21 0.44 for TD, n=6 *versus* 4.09 ± 0.75 for Tat, n=11; p****<0.0001). Note that n=7 for TDS,
22 n=10 for TD (0.1 mg/kg) and n=6 for TD (10 mg/kg). Same results at 24 h-reperfusion: 1.57
23 ± 0.37 for TD, n=6 *versus* 4.01 ± 1.33 for Tat, n=13; p****<0.0001. Note that n=10 for TDS,
24 n=6 for TD (0.1 mg/kg) and n=6 for TD (10 mg/kg). Data were compared using Kruskal-
25 Whallis with the Dunn's post test.

1 (C-E): *Western blot* analysis was performed from LV protein extracts from non-treated or TD
2 (1 μ M/L) murine IR hearts (*ex vivo*). Scatter dot blots and mean \pm SD were plotted for (C)
3 DAXX (p=0.1775; n=6 for each group), pJNK/JNK ratio (p**=0.0022; n=6 for each group)
4 and pro-caspase 3 (p*=0.0173; n=6 for each group); (D): FADD (p*=0.0260; n=6 for each
5 group), FAS (p=0.3939; n=6 for each group) and cleaved caspase 8 (p**=0.0022; n=6 for
6 each group); (E), BAD (p**=0.0022; n=6 for each group), pro- (p*=0.0221; n=6 for IR and
7 n=7 for TD) and cleaved caspase 9 (p**=0.0047; n=6 for IR and n=7 for TD). Representative
8 gel blots are presented for each protein for the two conditions (IR *versus* TD). Tubulin,
9 vinculin or α -actinin were used as protein loading control. Statistical analysis was performed
10 using non-parametric Mann-Whitney test.

11

12 **Figure 5: Mechanisms of TD cardioprotection**

13 (A-C) *Western blot* analysis was performed from LV protein extracts from non-treated or TD
14 (1 μ mol/L; n=6) murine IR (n=6) hearts (*ex vivo*). Scatter dot blots and mean \pm SD were
15 plotted for (A), HSP70 (p*=0.0260) (B) and pERK_{1/2} / ERK_{1/2} (p*=0.0173) and (C)
16 pAKT/AKT (p*=0.0130) ratios. Representative gel blots are presented for each protein for the
17 two conditions (IR *versus* TD). Vinculin or α -actinin were used as protein loading control.
18 Statistical analysis was performed using non-parametric Mann-Whitney test.

19 (D) Representatives images of cultured cardiomyocytes isolated from hearts subjected *ex vivo*
20 to global IR without or with TD or Tat treatment compared to basal conditions. Bar scale=50
21 μ m for DAXX, DAPI and merge images; bar scale=10 μ m for enlargement inset.

22 (E) Percentages (mean \pm SD) of cells expressing exclusively nuclear DAXX (ratio to DAXX
23 positive cells) in cardiomyocytes isolated from hearts in basal conditions (n=3 independent
24 cultures) or subjected *ex vivo* to global IR (n=5) in the presence or not of TD (33 \pm 2 for TD,
25 n=3 *versus* 9 \pm 3 for Tat, n=3).

1 (F) Representative gel blots are presented for each protein for the two conditions obtained in
 2 *Western blot* analysis performed on LV protein extracts from Sham (n=6), IR (n=6), Tat (n=7)
 3 and TD (n=6) hearts. (G) Scatter dot blots and mean \pm SD were plotted for pDAXX protein
 4 levels. Data were compared using non parametric Kruskal-Whallis test (Dunn's *post hoc* test)
 5 and statistical significance was noted for Sham $p^{**}= 0.0039$ or ns for TD *versus* Tat.

6
 7 **Figure 6: Time window of cardioprotection.**

8 (A) Mice were subjected to 40 min ischemia and reperfused for 1 h (B, D) or 24 h (C, E). TD
 9 (1 mg/kg) was injected 5 min before, or 15, 30 and 45 min ($\Delta 15$, $\Delta 30$ and $\Delta 45$, respectively)
 10 after the onset of reperfusion. At the end of the protocol, infarct size (in % of area at risk) and
 11 internucleosomal DNA fragmentation were quantified in LV. (B, C) Mean \pm SD for infarct
 12 size/AR were plotted as a function of Δt (delay of TD injection, in min) and fitted as linear
 13 regressions ($r^2= 0.92$ in (B) and 0.83 in (C)). A dotted line for infarct size mean of Tat-treated
 14 animals was reported. A drastic decrease in infarct size at 1 h-reperfusion was observed when
 15 TD was injected 30 min post-reperfusion *versus* Tat treatment (25.12 ± 4.95 for $TD_{\Delta 30}$, n=10
 16 *versus* 38.37 ± 8.46 for Tat, n=14; $p^{***}=0.003$). Please note that n=11 for TD, n=6 for $TD_{\Delta 15}$
 17 et n=9 for $TD_{\Delta 45}$. Same results at 24 h-reperfusion: 23.62 ± 4.69 for $TD_{\Delta 30}$, n=14 *versus* 33.29
 18 ± 6.07 for Tat, n=17; $p^{****}<0.0001$. Note that n=8 for TD, n=6 for $TD_{\Delta 15}$ et n=9 for $TD_{\Delta 45}$.
 19 Infarct size data (mean \pm SD) were fitted by a linear regression model using the least squares
 20 method.

21 (D, E) Scatter dot blots and mean \pm SD shown the DNA fragmentation for all groups
 22 described in (A). A drastic decrease in soluble nucleosome ratio at 1 h-reperfusion (D) was
 23 still observed when TD was injected up to 30 min post-reperfusion *versus* Tat treatment (2.58
 24 ± 0.51 for $TD_{\Delta 30}$, n=11 *versus* 4.09 ± 0.75 for Tat, n=11; $p^*=0.016$). Note that n=6 for TD,
 25 n=6 for $TD_{\Delta 15}$ and n=8 for $TD_{\Delta 45}$. (E) Same results were observed at 24 h-reperfusion: $2.34 \pm$

1 0.71 for TD_{Δ30}, n=10 *versus* 4.01 ± 1.33 for Tat, n=13; p*=0.031. Please note that n=6 for TD,
2 n=6 for TD_{Δ15} and n=8 for TD_{Δ45}. Data (plotted as mean ± SD) were compared using non
3 parametric Kruskal-Whallis test (Dunn's *post hoc* test). Statistical significance *versus* Tat
4 noted with * and *versus* TD noted with #.

5

6 **Figure 7: Schematic representation of apoptotic and survival cascades impacted by Tat-**
7 **DAXXp treatment.**

8 (A) Schema presenting the signaling apoptotic cascades activated during IR involving DAXX
9 in the myocardium.

10 (B) Recapitulative schema presenting the impact of TD treatment administered at the onset of
11 reperfusion and leading to the inhibition of both the extrinsic and intrinsic pathway as well as
12 the activation of the survival kinases.

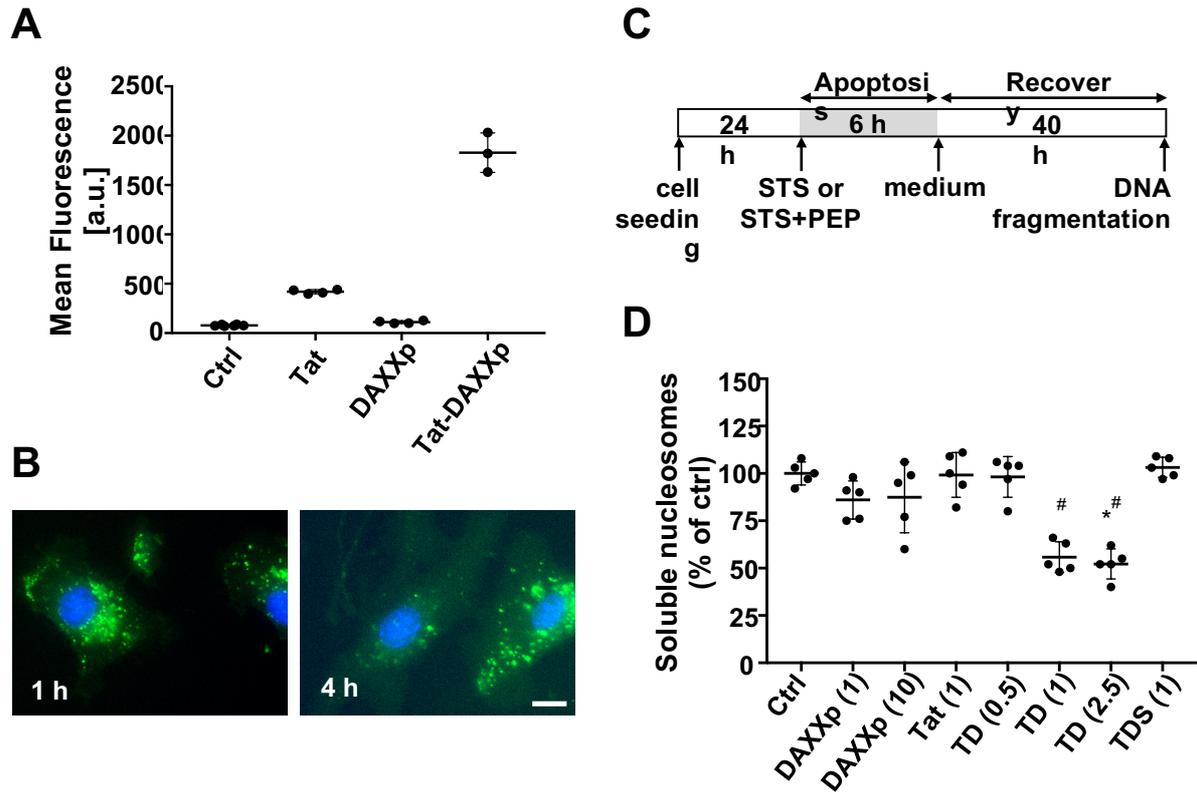
13 In both schemas the feedback loops regulating DAXX nucleo-cytoplasmic ratio are
14 highlighted.

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16

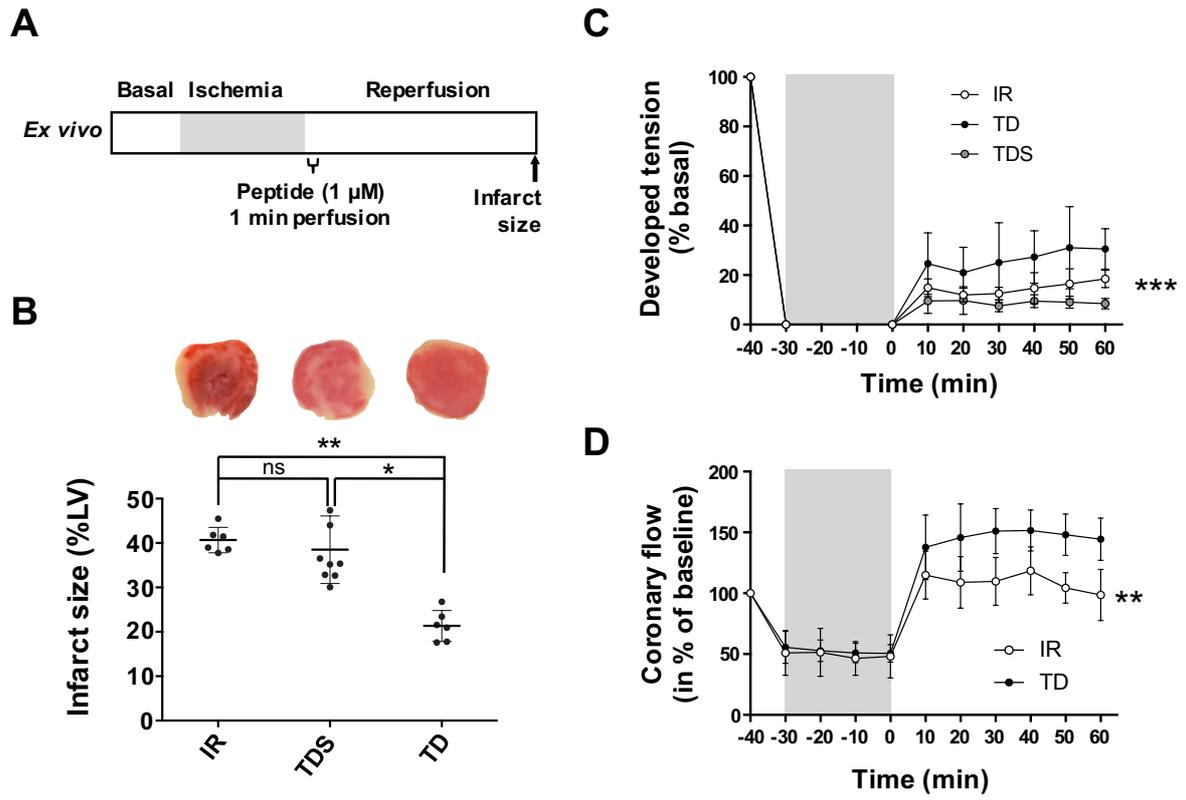
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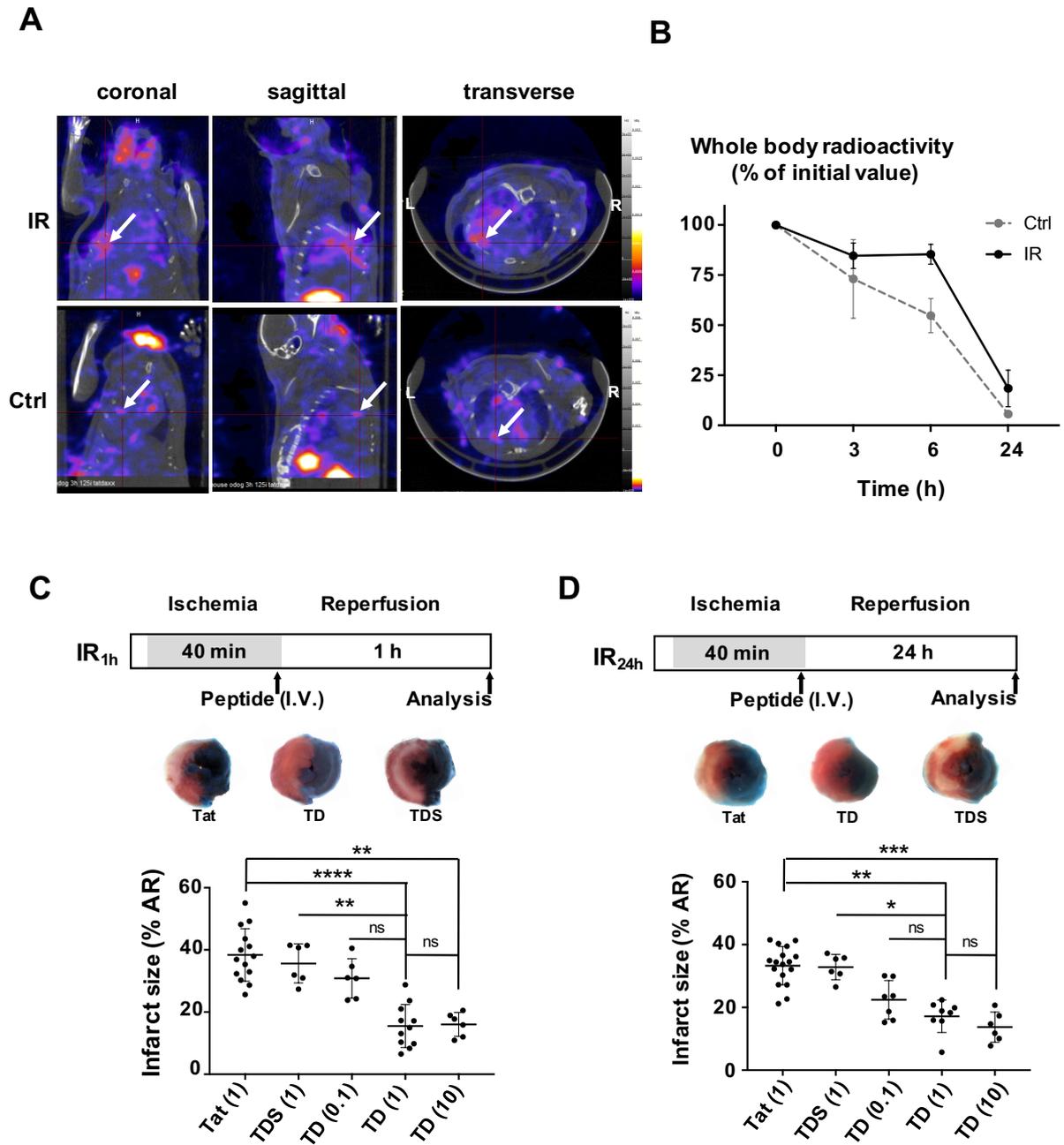
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Figure 1



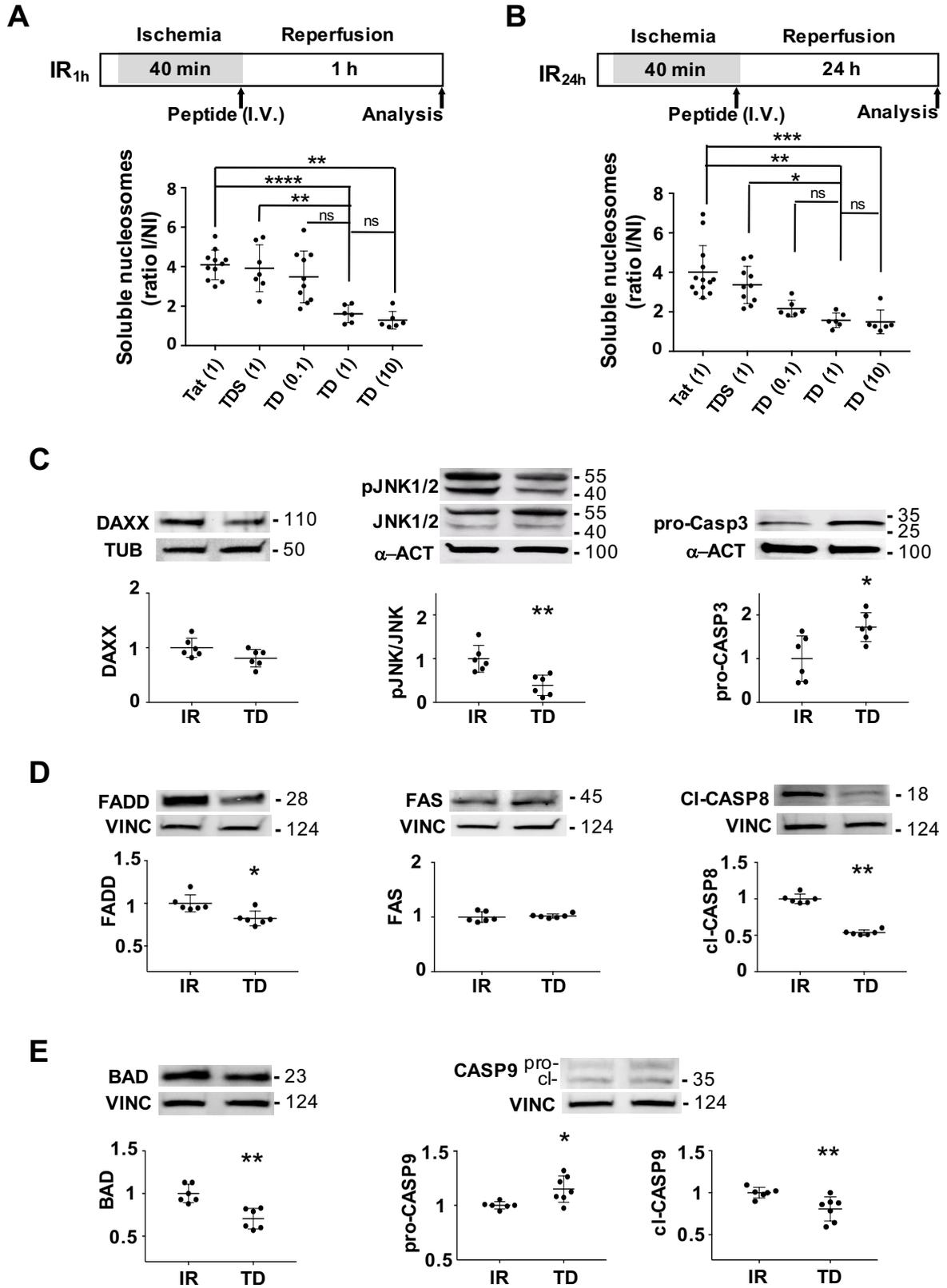
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Figure 2



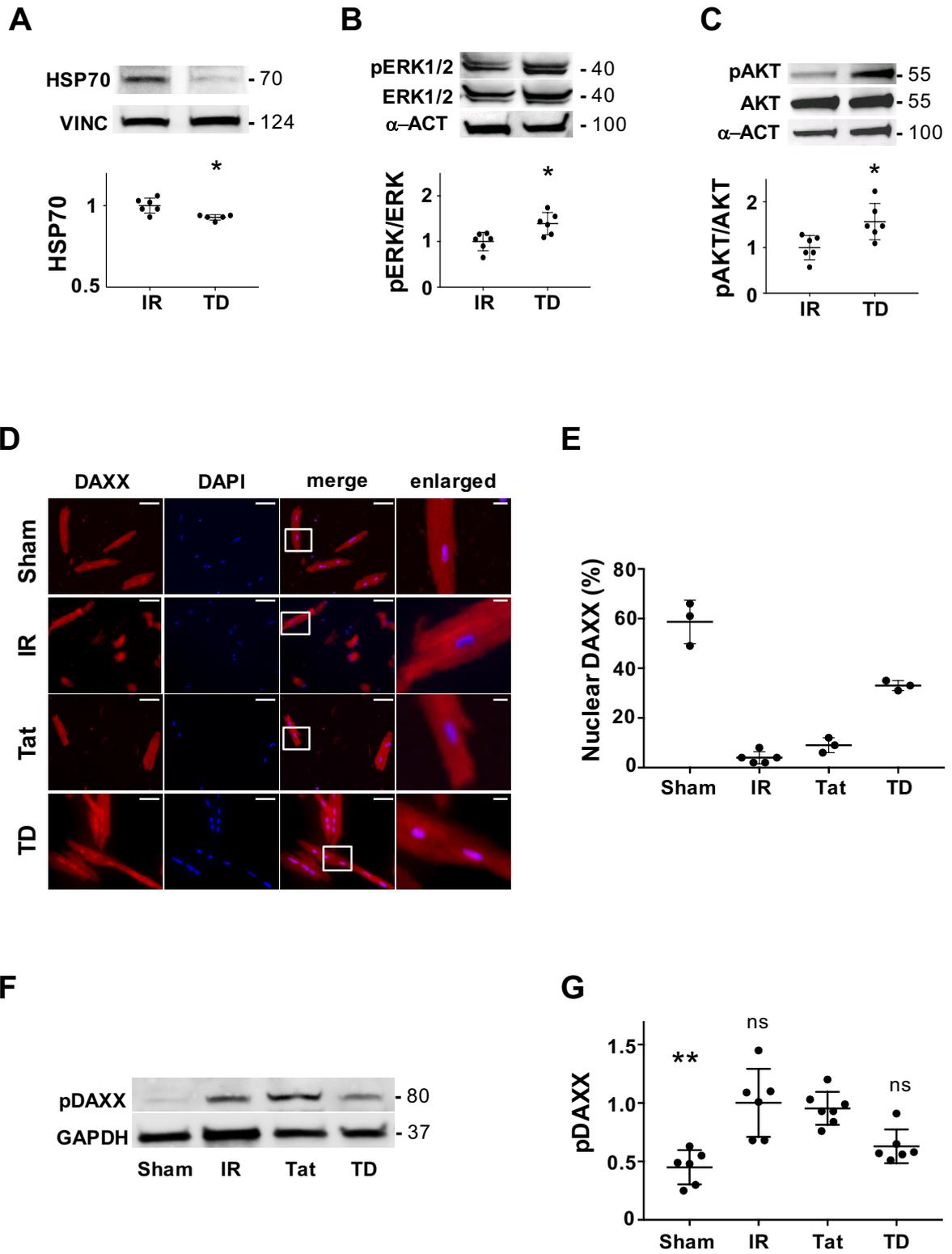
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Figure 3



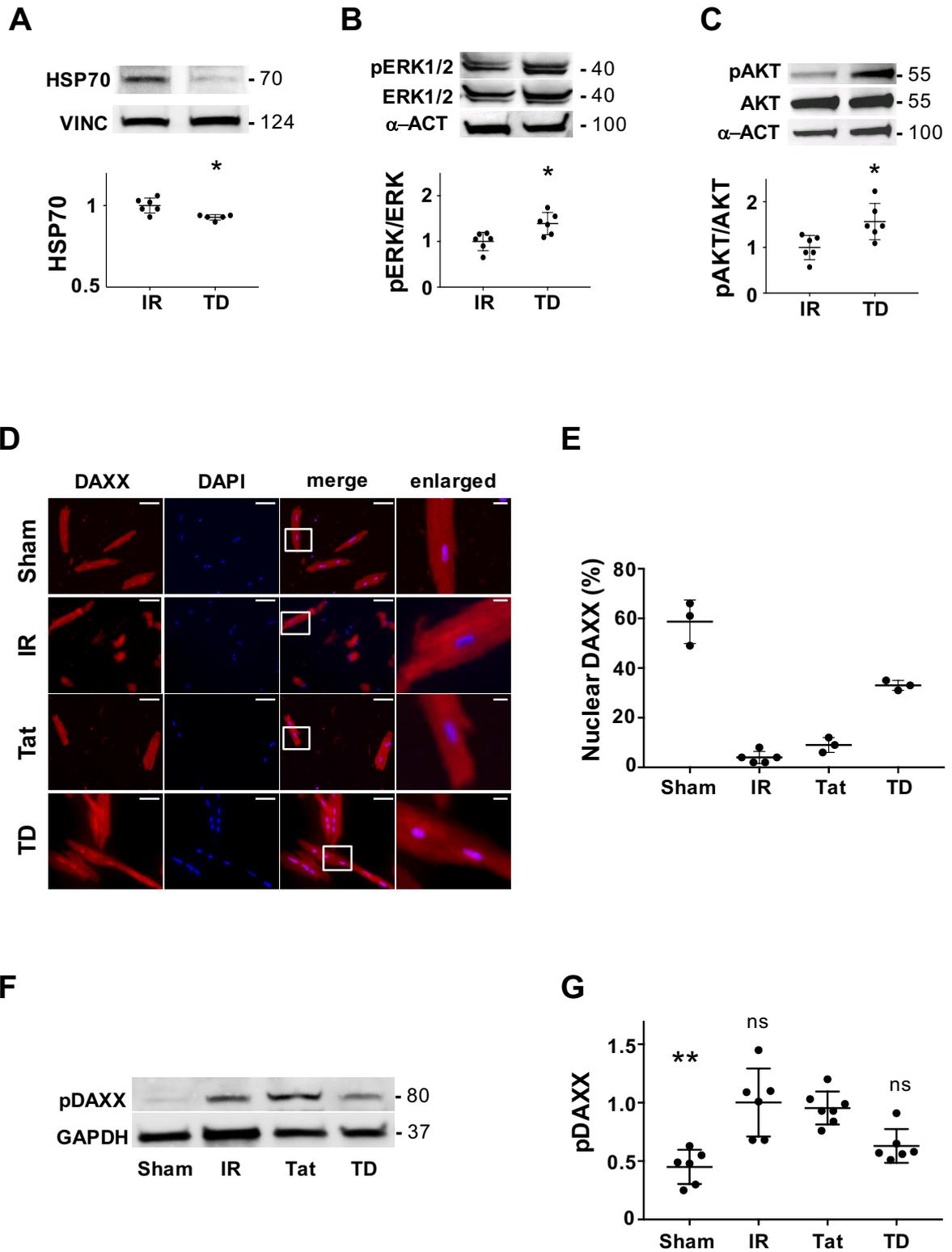
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Figure 4



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Figure 5



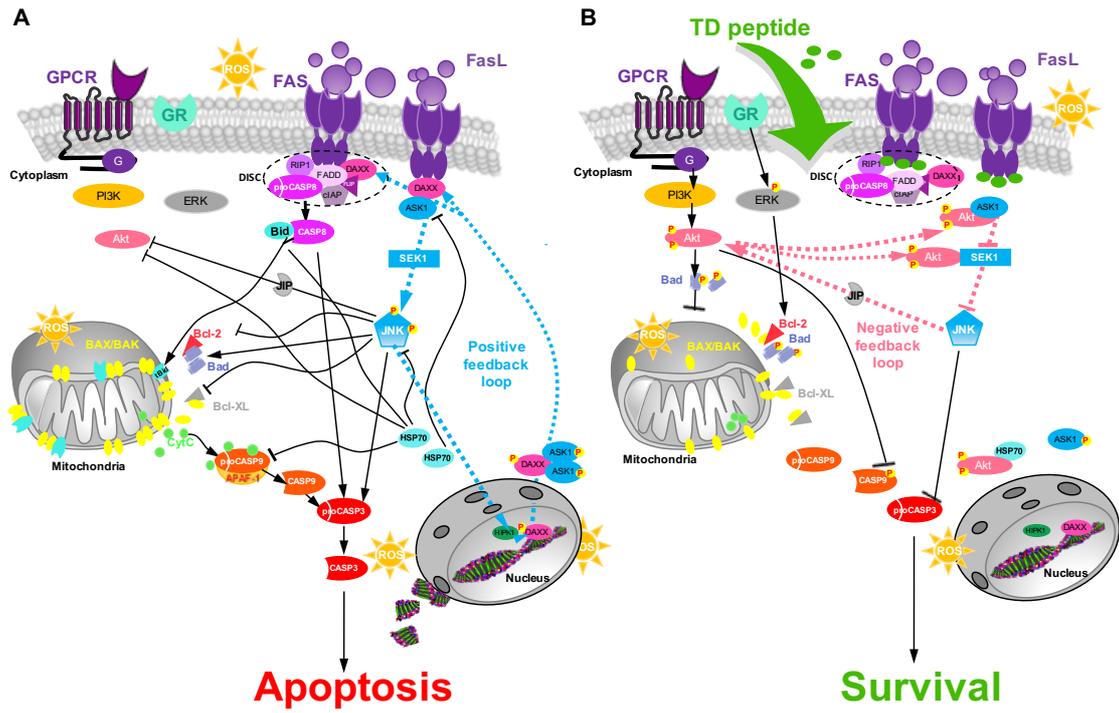
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Figure 6



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Figure 7

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A novel therapeutic peptide targeting myocardial reperfusion injury.

SUPPLEMENTARY MATERIAL

Boisguerin et al.

1 **1. Supplemental methods**

2

3 ***1.1. Peptide-array binding studies***

4 Peptide libraries were generated by a MultiPep SPOTrobot (*INTAVIS Bioanalytical Instruments*
5 *AG*) on N-modified CAPE-membranes¹ according to the standard SPOT synthesis.² The peptide
6 array was prewashed with EtOH (1x10 min), washed with Tris-buffered saline (TBS, pH 8.0,
7 3x10 min), and blocked for 4 h with blocking buffer (blocking reagent; *Sigma-Genosys*) in TBS
8 buffer (pH 8.0) containing 5% sucrose. Membranes were incubated with the polyhistidine-
9 tagged intracellular region of the FAS receptor (10 µg/ml of iFasR; *Sigma-Aldrich*) in blocking
10 buffer overnight at 4°C, and then washed with TBS (pH 8.0, 3x10 min). Bound intracellular
11 FAS receptor was detected using a mouse anti-polyHis antibody (*Sigma-Aldrich*; 1:3,000 in
12 blocking buffer, 2 h at room temperature) followed by horseradish-peroxidase conjugated anti-
13 mouse antibody (*Calbiochem*; 1:2,000 in blocking buffer, 1 h at room temperature). Detection
14 was carried out with Uptilight HRP blot chemiluminescent substrate (*Uptima*) with a 1 min
15 exposure time. The signal intensities with background subtraction were recorded as
16 biochemical light units (BLU) with a LumiImager (*Boehringer*) and analyzed using the
17 LumiAnalyst software (*Boehringer*).

18

19 ***1.2. Peptide synthesis***

20 Tat (13-mer), DAXXp (16-mer), Tat-DAXXp (TD; 29-mer), Tat-scrDAXXp (TDS; 29-mer
21 corresponding to the scrambled version DAXXp coupled to Tat) as well as the other deriving
22 sequences (see **Table S1**) were synthesized on resin by conventional Fmoc-chemistry [by the
23 group of Dr. Volkmer (Charité, Berlin) or by Intavis AG].
24 For microscopy experiments, the peptides were labeled with 3,5-carboxyfluorescein (CF) as
25 reported by Fischer *et al.*³ or with Cy5.

1 All peptides were purified by quantitative RP-HPLC and characterized by analytical RP-HPLC
2 and mass spectrometry to ensure a purity of >95%.
3 For SPECT-CT, TD sequence with an additional tyrosine residue (Y-TD) was labeled with ¹²⁵I
4 (*Perkin-Elmer*; specific activity of 370 MBq/mg) using the IODO-GEN (*Pierce Chemical*)
5 method and previously described protocols.⁴

6

7 **1.3. Tat-DAXXp and DAXXp stability assays**

8 The peptides were dissolved in 20% mouse serum (in house preparation) in water at 1 mg/mL
9 concentration. The peptides were incubated at 37°C and at various times (0 h, 1 h, 2 h, 4 h, 8
10 h). 50 µmol/L of the solution was precipitated in 100 µmol/L 10% dichloroacetic acid (DCA)
11 in H₂O/CH₃CN (50/50). For a better precipitation, the samples were kept overnight at -20°C.
12 After a centrifugation (5 min, at 14,000 rpm), 50 µmol/L of the samples were injected for HPLC
13 analysis (*Waters*). The peak areas (A [µV*sec]) of the different incubation times were
14 determined and compared.

15

16 **1.4. Cell cultures**

17 Mouse myoblast cells (C2C12 – ATCC: CRL-1772™) were cultured in Dulbecco's Modified
18 Eagle's Medium (DMEM, Gibco®, *Life Technologies*) supplemented with 10% (v/v) fetal
19 bovine serum (FBS; *BioWest*), 1 mM Na pyruvate Gibco®, (*Life Technologies*), 1% (v/v) non-
20 essential amino acids (100X, Gibco®; *Life Technologies*) and 1% (v/v) penicillin-streptomycin-
21 neomycin (PSN, Gibco®; *Life Technologies*).

22 Primary neonate cardiomyocytes were isolated from 1- to 2-day-old C57BL/6J mice (*Charles*
23 *River Laboratories*) ventricles by enzymatic digestion (type-4 collagenase; *Serlabo*
24 *Technologies* and pancreatin, Gibco®; ***Life Technologies***) as described previously.⁵ Freshly
25 isolated cells were seeded in 90-mm Petri dishes to allow selective adhesion of cardiac

1 fibroblasts⁶ in plating medium: 250 mL DMEM (Gibco®, *Life Technologies*), 250 mL M199
2 (*Sigma-Aldrich*), 5 mL glutamine-PS (100X, Gibco®, *Life Technologies*), 50 mL HS (Horse
3 serum; *Sigma-Aldrich*), 25 mL FBS (Fetal bovine serum; *PAA Laboratories*). Cardiomyocytes
4 remaining in the supernatant were seeded in 12- or 24-well plates coated with 0.1% gelatin
5 (*Sigma-Aldrich*) or in glass bottom Petri dishes coated with laminin (*Life Technologies*).
6 Medium was changed daily until the cells were used.

7 Isolated adult murine cardiomyocytes were obtained from mouse hearts enzymatically
8 dissociated. C57BL/6J mice were injected intraperitoneally with a single dose of heparine (250
9 U; Héparine Choay®, 25000 U.I. /5 mL; *Sanofi-Aventis*). Anesthesia was induced 10 min later
10 by injecting a mixture of ketamine (14 mg/kg; Imalgène® 500; *Merial*), xylazine (14 mg/kg;
11 Rompun® 2%; *Bayer*) and pentobarbital (76.6 mg/kg; *Ceva Santé Animale*). The chest was
12 opened and the heart was harvested (leading to the sacrifice of the animal).

13 Hearts were mounted on a Langendorff apparatus (homemade) and perfused for 15 min with
14 normal Tyrode buffer [140 mM NaCl (*Sigma-Aldrich*), 5.4 mM KCl (*Sigma-Aldrich*), 1 mM
15 MgCl₂ (*Sigma-Aldrich*), 5 mM HEPES (*Sigma-Aldrich*), 5.5 mM Glucose (*Sigma-Aldrich*), 147
16 mM CaCl₂ (*Sigma-Aldrich*)]. After stabilization, hearts were subjected to a 30 min global
17 ischemia followed by 60 min of reperfusion (IR protocol) with Tyrode buffer. The peptide
18 treatment (1 µmol/L in Tyrode buffer) was applied at the onset of reperfusion during 1 min.
19 At the end of reperfusion (IR), the heart was perfused with a Ca²⁺-free Tyrode buffer for 4
20 min followed by an enzymatic solution of LiberaseTM (0.2 mg/mL; *Roche*) for 2-4 min as
21 previously described.⁷ Hearts were removed from the apparatus and stored in a "Stop
22 solution" (10 mM 2-3 butanedione monoxime (*Sigma-Aldrich*), 5.5 mM glucose (*Sigma-*
23 *Aldrich*), 12.5 µM CaCl₂ (*Sigma-Aldrich*) and 5% FBS) to block enzymatic digestion. Atria
24 were quickly removed. Single rod-shape myocytes were collected after mechanical
25 dissociation of the ventricles and preplate to remove fibroblasts. Cardiomyocytes were then

1 plated on coverslips in culture medium contained DMEM (Dulbecco Modified Essential
2 Medium; *Invitrogen*), 20% FBS (*Invitrogen*), 1%-non essential amino acids (*Sigma-Aldrich*),
3 1%-Insulin-Transferrin-Selenium and 1% Penicillin-Streptomycin solution (*Invitrogen*) at
4 least 2 h before immunohistochemistry.

5

6 **1.5. Tat-DAXXp treatment on cell cultures**

7 Cellular localization: C2C12 cells or primary neonatal cardiomyocytes were seeded on glass
8 bottom dish (FluoroDish, *World Precision*) in the appropriate medium (see above). After 24
9 h, the cells were incubated in OptiMEM (Gibco®; *Life Technologies*) with CF-labeled peptides
10 (1 μ M, 1 h or 4 h). Thereafter, they were washed with PBS and co-incubated with Hoechst
11 33342 dye (blue fluorescence) for 10 min in order to stain nuclei. The distribution of
12 fluorescence in live unfixed cells was analyzed on a Zeiss Axiovert 200 M fluorescence
13 microscope.

14 Internalization mechanism: C2C12 cells (20,000 cells/well) were seeded in a 24-well plate and
15 grown overnight. The next day, the cells were pre-incubated for 30 min at 4°C or with the
16 different endocytosis inhibitors at the indicated concentrations (in OptiMEM) at 37°C.
17 Afterwards, CF-peptides [1 μ mol/L final concentration] were added for an additional 1 h
18 incubation using the same incubation temperature as applied in the pre-incubation. Then, cells
19 were washed 2x in PBS and extracellular membrane-bound peptide were removed by a 10 min
20 incubation with 100 μ L 0.05% trypsin (Gibco®; *Life Technologies*) at 37°C, 5% CO₂. Detached
21 cells were transferred in 1.5 mL tubes after addition of 400 μ L D-PBS (Gibco®; *Life*
22 *Technologies*) complemented by 5% FBS (*PAA Laboratories*), followed by centrifugation (10
23 min, 4°C, 1,500 rpm). Pellets were resuspended in 500 μ L D-PBS complemented by 0.5% FBS
24 and 0.1% propidium iodide (PI) (*Molecular Probes*) and cell suspensions were transferred to
25 polystyrene round-bottom tubes (*Falcon*). Fluorescence analysis was performed with a BD

1 FacsCanto flow cytometer (*BD Biosciences*). Cells stained with PI were excluded from further
2 analysis. A minimum of 10,000 events per sample was analyzed.

3 The following endocytosis inhibitors were used: nystatin (NYS, 50 $\mu\text{mol/L}$), chlorpromazine
4 (CPZ, 7.5 $\mu\text{mol/L}$), 5-(N-ethyl-N-isopropyl)-amiloride (EIPA, 10 $\mu\text{mol/L}$), sodium azide
5 (NaN_3 , 10 mmol/L) and 2-deoxy-glucose (DG, 6 mmol/L) (for ATP depletion) (all from *Sigma-*
6 *Aldrich*).

7

8 **1.6. Animal experiments**

9 All experiments were carried out with C57BL/6J mice (*Charles River laboratories*) in
10 accordance with the European Communities Council directive of November 1986 and
11 conformed to the "Guide for the Care and Use of Laboratory Animals" published by the US
12 National Institutes of Health (NIH publication 8th Edition, 2011). The surgical protocols were
13 approved by the "Comité d'Ethique pour l'Expérimentation Animale Languedoc-Roussillon"
14 (CEEA-LR) with the authorization number CE-LR-0814.

15

16 **1.7. Langendorff ex vivo studies**

17 For *ex vivo* experiments, C57BL/6J male mice were anesthetized with a first intramuscular
18 injection of an anesthetic cocktail comprising ketamine (14 mg/kg , Imalgène®; *Merial*) and
19 xylazine (14 mg/kg , Rompun®; *Bayer*), and by a second injection of pentobarbital (76.6 mg/kg ;
20 *Sanofi-Aventis*). Mice were treated (IP injection) with heparin (250U, Héparine Choay®; *Sanofi*
21 *Aventis*) in order to prevent the blood clot formation. Hearts were excised after sternotomy and
22 cannulated through the ascending aorta. Then, the heart was quickly mounted on the
23 Langendorff system and retrogradely perfused with a pre-warmed Krebs buffer [116 mM NaCl
24 (*Sigma-Aldrich*), 5 mM KCl (*Sigma-Aldrich*), 1.1 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (*Sigma-Aldrich*), 0.35 mM
25 NaH_2PO_4 (*Sigma-Aldrich*), 27 mM NaHCO_3 (*Sigma-Aldrich*), 10 mM Glucose (*Sigma-*

1 Aldrich), 1.8 mM CaCl₂ (Sigma-Aldrich), pH 7.4, oxygenated with carbogen (95% CO₂, 5%
2 O₂; Linde)] at constant pressure and temperature (37°C).

3 Ischemia-reperfusion protocols

4 IR was achieved *ex vivo* by either using global (I₃₀R) or regional ischemia (IR) models.

5 - Global ischemia was induced on a homemade Langendorff system by stopping the
6 perfusion during 30 min (no-flow). Reperfusion was achieved by restoring the liquid perfusion
7 during 60 min with the Krebs solution (control) or with peptide solutions administered during
8 the first minute of reperfusion.

9 - Regional IR was obtained on an EMKA system (EMKA Technologies) allowing
10 coronary blood flow and ECG recordings. The coronary circulation was perfused at 80 mmHg
11 over a 2 to 4 mL/min flow range with an oxygenated Krebs buffer. During the stabilization
12 phase, a 7.0 prolene ligature (Ethicon, Johnson & Johnson) was placed around the left coronary
13 artery 1-2 mm distal from where it emerges beneath the left atrium and the ends of the ligature
14 were passed through an occluder tube, initially without tension. Heart temperature was
15 continuously assessed by a probe positioned on the base of the heart and recorded by using a
16 TH-5 monitoring thermometer (Phymep). Perfused hearts were then immersed in a water-
17 jacketed bath and continuously maintained at 37°C. After a stabilization phase of 20 min
18 (baseline), regional normothermic ischemia was induced by tightening the suture with the
19 occluder tube for 40 min. After ischemia, reperfusion was allowed for 1 h by loosening the
20 ligature. Peptides were perfused during the first minute after the onset of reperfusion, following
21 by a perfusion of 59 min with Krebs buffer. At the end of reperfusion, the artery was reoccluded,
22 the hearts were removed from the perfusion system. For infarct size analysis, hearts were
23 injected with phthalocyanine blue through the aortic cannula and allowed to perfuse the non-
24 ischemic portions of the myocardium, making possible a delineation of the area at risk. For
25 *Western blots* analysis, the hearts were removed from the Langendorff without any blue dye

1 injection and stored at -80°C until protein extraction. Sham hearts were perfused the same time
2 as those subjected to IR without any coronary ligation.

3 Recording of the developed tension:

4 Developed tension was measured on a homemade Langendorff system comprising a transducer
5 connected to the DMT amplifier (*DMT 920 CS*). The transducer position was adjusted using a
6 micromanipulator to give a diastolic tension of 1.0 g. Apicobasal displacement was measured
7 through a small hook attached to the apex of the heart. The recordings were obtained on a PC
8 computer through a digidata and PClamp 9.2. All data were analyzed using Clampfit 9.2
9 software (*Axon Instruments*).

10 Recording of coronary blood flow:

11 During regional *ex vivo* IR protocol, the coronary flow was continuously digitized at 0.1 Hz
12 and recorded using an IOX data acquisition system (*EMKA Technologies*). Excel software
13 (*Microsoft*) was used to perform offline analysis of the recorded data. For each mouse, a mean
14 value of maximal coronary flow was calculated each 10 min. Values during ischemia and
15 reperfusion were normalized with respect to the pre-ischemia phase (baseline) and were
16 expressed in percentages.

17 Electrocardiographic recordings and analysis:

18 During regional *ex vivo* IR protocol, ECG was continuously recorded by Ag/AgCl electrodes
19 positioned on the right atrium and near the apex. Signals were digitized continuously at 1 kHz
20 and recorded using an IOX data acquisition system (*EMKA Technologies*). The software
21 ecgAUTO (*EMKA Technologies*) was used to perform offline analysis of the recorded data. For
22 each mouse the mean heart rate (HR) value during baseline, ischemia and reperfusion was
23 calculated.

24

25

1 **1.8. Surgical protocol of myocardial ischemia-reperfusion in vivo**

2 Surgical preparation: Acute myocardial ischemia and reperfusion were performed in
3 C57BL/6J mice as previously described.⁸ Male mice (22-30 g) were anaesthetized with an
4 intramuscular (IM) injection of an anaesthetic mixture comprising ketamine (50 mg/kg;
5 Imalgène[®] 500; Merial), xylazine (10 mg/kg; Rompun[®] 2%; Bayer) and chlorpromazine (1.25
6 mg/kg; Largactil[®] 5 mg/ml; Sanofi-Aventis). Mice were ventilated via a tracheal intubation on
7 a Harvard rodent respirator (tidal volume 7.2 μ L/g body mass; respiratory rate 200 breaths per
8 min). The body temperature was maintained constant between 36.8°C and 37.0°C owing to the
9 thermo-regulated surgical table connected to a rectal probe. After a second injection of
10 ketamine (50 mg/kg) and xylazine (10 mg/kg), the chest was opened by a left lateral
11 thoracotomy and a reversible coronary artery snare occluder was placed around the left
12 coronary artery. All animals underwent 40 min of ischemia followed by reperfusion by
13 loosening the knot. When reperfusion lasted 60 min, a third administration of ketamine/xylazine
14 was performed (same dose as in the previous injection, IM). The heart was removed at the end
15 of the surgical protocol.

16 When reperfusion lasted 24 h, mice underwent a recovery protocol comprising subcutaneous
17 administration of lidocaine (1.5 mg/kg; Xylocain[®] 10 mg/mL; AstraZeneca), and a post-
18 operative awakening in an emergency care unit maintained at 28°C, with oxygen supply and
19 controlled humidity. After 2 h of stabilization, mice were allowed to recover in a ventilated
20 cabinet during 20 h. Mice subjected to 24 h reperfusion were anesthetized again according to
21 the same protocol described for a short period of reperfusion and the heart was removed for
22 infarct size and apoptosis analysis. In SHAM-operated mice (n=3), the suture was passed but
23 not tied and the heart was removed as described before.

24 In vivo experimental design: Mice were randomly allocated to two different surgical protocols
25 of myocardial IR. **IR 1h**: 40 min ischemia, 1 h reperfusion and **IR 24h**: 40 min ischemia and

1 24 h reperfusion. All peptides were diluted in physiological saline serum and administered
2 intravenously 5 min before reperfusion or 15, 30 or 45 min after the onset of reperfusion. The
3 control groups were treated with the free Tat peptide (CPP alone), the scrambled version of the
4 peptide (TDS) or with physiological saline serum alone. All peptides were used at the indicated
5 concentrations indicated in the figures legends.

6 At the end of reperfusion, the artery was re-occluded. The phthalocyanine blue dye (TTC; *Sigma-*
7 *Aldrich*) was injected into the left ventricle (LV) cavity and allowed to perfuse the non-ischemic
8 portions of the myocardium. Hearts were harvested, atria and right ventricle removed and
9 dedicated to infarct size or DNA fragmentation measurements.

10

11 **1.9. DNA fragmentation**

12 *In vitro assay*: Cells were seeded in 24-well plates and grown for 24 h to 48 h (37°C, 5% CO₂).
13 Cells were incubated with staurosporin (STS, 0.1 µmol/L for C2C12 and 1 µmol/L for
14 cardiomyocytes) with or without peptide (in OptiMEM, Gibco®; *Life Technologies*) as
15 described in **Figure 1D** and **Figure S2**. After apoptosis induction, the solution was replaced
16 by plating medium (250 ml DMEM (Gibco®; *Life Technologies*), 250 mL M199 (*Sigma-*
17 *Aldrich*), 5 ml glutamine-PS (100X, Gibco®; *Life Technologies*), 50 mL HS (Horse serum;
18 *Sigma-Aldrich*), 25 mL FBS (Fetal bovine serum; *PAA Laboratories*) and plates were further
19 incubated for 40 h. DNA fragmentation was quantified on cell lysates with an enzyme-linked
20 immunosorbent assay kit (Cell Death ELISA; *Roche Diagnostics*) designed to quantitate
21 histone-complexed DNA fragments (mono- and oligonucleosomes) in the cytoplasm of cells.
22 DNA fragmentation data were expressed as a “DNA enrichment factor” according to
23 manufacturer’s instructions. These values were first normalized to the negative control
24 (condition without STS and peptide) and thereafter related to the STS-condition (corresponding
25 to 100% DNA fragmentation).

1 *In vivo assay*: Lysates of transmural samples of non-ischemic (NI) or ischemic (I) areas of the
2 left ventricles were prepared as previously described.⁹ In both cases, DNA fragmentation was
3 quantified with an enzyme-linked immunosorbent assay kit as described in the previous section.
4 Transmural samples from 30 mg of non-ischemic areas of the left ventricle (central portion of
5 the septum) and ischemic areas (portion of the LV free wall directly under the silk ligation)
6 were harvested on mice submitted to the surgical protocol. Tissues samples were disintegrated
7 in a tissue grinder in 400 µL of the lysis buffer supplied with the kit. The homogenate was
8 centrifuged at 13,000 g for 10 min. The supernatant was used as antigen source in the sandwich
9 ELISA. Incubation buffer, instead of the sample solution, and DNA-histone complex were used
10 as negative and positive controls, respectively. Two values from the double absorbance
11 measurements (405 nm / 490 nm) of the samples were averaged, and the background (negative
12 control) was subtracted from each of these averages. The positive control was used as an
13 internal control for daily variability. DNA soluble nucleosomes were quantified both in blue-
14 colored region (non-ischemic region = NI) as well as in the non blue-colored areas (ischemic
15 region = I). This allows to normalize the rate of DNA fragmentation (internal control) and to
16 avoid the introduction of variability in the results due to differences in temperature, timing of
17 the measurement.

18

19 ***1.10. Infarct size assessment***

20 LV were sliced transversally into 1-mm-thick sections and incubated in a 1% solution of 2,3,5-
21 triphenyltetrazolium chloride (TTC, *Sigma-Aldrich*) for 15 min at 37°C. After fixation in a 4%
22 paraformaldehyde–PBS solution, the slices were weighted and each side was photographed
23 (*Olympus* camera). The ischemic risk area and the infarcted area were measured by planimetry
24 with ImageJ software (*U. S. National Institutes of Health*). Infarct size was expressed as a
25 percentage of the ischemic risk area.

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1.11. Immunoblotting

Tissue samples (left ventricle) were rapidly frozen in liquid nitrogen after the end of *ex vivo* IR protocols. Samples were homogenized with a grinder in RIPA buffer [50 mM Tris (*Sigma-Aldrich*), 150 mM NaCl (*Sigma-Aldrich*), 1% Triton X-100 (*Sigma-Aldrich*), 0.1% sodium dodecyl sulfate (*Sigma-Aldrich*) pH 8.0] supplemented with EDTA-free protease and phosphatase inhibitor (Halt™ Protease and phosphatase single-use inhibitor Cocktail-100X, *Thermoscientific*). After centrifugation, pellets were resuspended in RIPA buffer for protein purification and incubated for 1 h at 4°C. Protein concentrations were determined with the bicinchoninic acid (BCA) protein assay kit (*Pierce*). Samples (25 µg) of protein were resolved by SDS polyacrylamide gel electrophoresis (4-20% mini-Protean®TGX™ precast gels; *Biorad*) and transferred to nitrocellulose (Trans-Blot®Turbo™; *Biorad*). The following antibodies and suppliers were used: 1) EMD Millipore: anti-caspase 3 (Upstate 06-735), 2) Cell Signaling Technology: anti-phospho ERK1,2; anti-ERK1,2; anti-phospho JNK1,2; anti-JNK1/2; anti-phospho AKT; anti-AKT; cleaved caspase-8 (ASP387)(D5B2); RIP1 (D94C12); RIP3 (D8J3L); caspase 9; Beclin-1 (D40C5); LC3A/B (D3U4C); β-Tubulin; anti-α-actinin; vinculin (E1E9V), GAPDH, 3) Abcam: FAS, DAXX (M-112), anti-phosphoMLKL (phosphoS345), FADD (EPR5030), HSP70, BAD (Y208), 4) Biorbyt Ltd: phosphoDAXX and 5) Jackson ImmunoResearch: horseradish peroxidase-conjugated anti-rabbit. Protein bands were visualized by enhanced chemiluminescence method using an ECL kit (SuperSignal™ West Pico chemiluminescent Substrate; *Thermo Scientific*™). Densitometry analysis was performed using Chemidoc™ (*Biorad*) and ImageJ software (*U. S. National Institutes of Health*). Signal intensities of each protein band were corrected with the corresponding value obtained for the loading controls (tubulin, vinculin, GAPDH or α-actinin) and then normalized with the mean value obtained for the IR control conditions.

1 **1.12. Immunostaining**

2 Cultured ventricular myocytes were fixed in 4%-paraformaldehyde (in PBS solution) and co-
3 incubated 2 h at room temperature using anti-DAXX primary antibody (sc-7152; *Santa Cruz*
4 *Biotechnologies*).

5 After primary antibody incubation, cells were washed in PBS, and then incubated (1 h at room
6 temperature) with the secondary antibody (1:2000; *Jackson ImmunoResearch*). Cell nuclei
7 were stained with DAPI (*Sigma-Aldrich*). Coverslips were mounted in Citifluor™ (*Biovalley*)
8 and slides were imaged with a microscope (40x oil objective).

9

10 **1.13. SPECT-CT imaging**

11 C57BL/6J (male, 6–8 weeks old) were force-fed with Lugol solution one day before imaging,
12 and stable iodine was added to their drinking water for the entire experimental period.

13 Mice were anesthetized with 2% isoflurane and positioned on the head of 4-head multiplexing
14 multipinhole NanoSPECT camera (*Bioscan Inc.*). Whole-body SPECT/CT images were
15 acquired at various times (0, 3, 6, and 24 h) after tail vein injection of 10 MBq radiolabeled
16 ¹²⁵I-TD in control mice or at the time of reperfusion in IR mice.

17 Energy window was centered at 28 keV with ±20% width. Acquisition times were defined to
18 obtain 30,000 counts for each projection with 24 projections. Images and maximum intensity
19 projections (MIPs) were reconstructed using the dedicated Invivoscope® (*Bioscan Inc.*) and
20 Mediso InterViewXP® software (*Mediso Medical Imaging System*). Concurrent microCT
21 whole-body images were performed for anatomic co-registration with SPECT data.
22 Reconstructed data from SPECT and CT were visualized and co-registered using
23 Invivoscope®.

24

25

1 **1.14. Statistical analysis**

2 All values are expressed as mean \pm SD. Statistical analysis was performed only for $n \geq 5$
3 independent experiments. Data were analyzed with nonparametric Kruskal-Wallis test for
4 multiple comparison or Mann-Whitney when appropriate. For repeated measures, data were
5 analyzed with Two-way RM ANOVA and the Tukey's or Sidak's post-test. Concerning
6 **Figures 6B and C**, infarct size data (mean \pm SD) were fitted by a linear regression model using
7 the least squares method based on the minimization of the sum of squares of the vertical
8 distances of the points from the line. Values of $P < 0.05$ were accepted as statistically significant.
9 P values, indicated in the text, were noted in the figures as * for $p < 0.05$, ** for $p < 0.01$, *** for
10 $p < 0.001$ and **** for $P < 0.0001$. Data were analyzed with GraphPad Prism (*GraphPad*
11 *Software*).

12

1 2. Supplemental Results

2

3 **2.1. Screening of DAXXp interfering peptides**

4 A peptide library of 15-mer overlapping peptides (PepScan array) spanning the entire primary
5 sequence of the human DAXX was screened with the intracellular region of the FAS receptor
6 (FasR) as outlined in **Figure S1A**. The highest signal intensities were measured for peptides
7 209 to 212 on the array (**Figure S1B**). Since this set of peptides is included in the DAXX-DN
8 (dominant-negative) construct found to be cardioprotective in our previous studies,⁸ we focused
9 on this region to design a cardioprotective peptide.

10

11 **2.2. Determination of the optimal anti-apoptotic DAXXp peptide**

12 The interfering peptide (DAXXp) was coupled to the Tat cell penetrating peptide for the cellular
13 internalization resulting in the Tat-DAXXp (TD). First, we analyzed the cell viability on the
14 C2C12 model cell line by incubating them with TD in a dose-dependent manner (**Figure S2A**).
15 No toxic effect could be detected using concentrations between 0.5 $\mu\text{mol/L}$ and 2.5 $\mu\text{mol/L}$ of
16 TD after 24 h incubation, which is an important prerequisite in the development of a therapeutic
17 drug development (p=ns *versus* untreated).

18 More importantly, the anti-apoptotic activity of TD was then evaluated in C2C12 cells
19 submitted to a staurosporin (STS)-induced apoptotic stress (0.1 $\mu\text{mol/L}$; see protocol in **Figure**
20 **S2B**) followed by specific DNA fragmentation quantification (**Figure S2C**). A 23%-decrease
21 in DNA fragmentation was observed in cells treated with 1 $\mu\text{mol/L}$ TD (p<0.05 *versus* Tat). As
22 a control, we also evaluated the murine peptide sequence (Tat-mDAXXp) and determined
23 exactly the same 23%-reduction of DNA fragmentation (p<0.05 *versus* Tat) confirming the
24 homology of both DAXX sequences. Neither the Tat delivery vector alone nor a scrambled
25 version of the peptidic inhibitor (TDS) were able to inhibit apoptosis. As expected from its poor

1 *in vitro* uptake, the free DAXXp did not induce any anti-apoptotic activity even at a 10 $\mu\text{mol/L}$
2 concentration.

3

4 **2.3. Evaluation of Tat-DAXXp trafficking in vitro**

5 The sub-cellular localization of a carboxy-fluorescein (CF)-labelled TD revealed a punctuated
6 cytosolic pattern after 1 h incubation in C2C12 cells (**Figure S3A**, left panel). This indicates,
7 that TD is probably internalized via an endocytotic pathway. However, after a prolonged
8 incubation time (4 h), TD seems to escape from the endosomal vesicles as shown by a more
9 diffuse peptide pattern in the cytosol (**Figure S3A**, right panel).

10 To determine which endocytotic pathways are involved in the trafficking of TD in
11 C2C12 cells, we took advantage of pharmacological inhibitors of the major endocytic
12 pathways. C2C12 cells were treated with chlorpromazine (CPZ) for clathrin-mediated
13 endocytosis inhibition, with nystatin (Nys) for caveolae-mediated endocytosis inhibition or
14 with 5-(N-ethyl-N-isopropyl) amiloride (EIPA) for macropinocytosis inhibition. Furthermore,
15 cells were incubated at 4°C for membrane fluidity reduction or with NaN_3 and 2'-Deoxy-D-
16 Glucose (DDG) for ATP-depletion (-ATP). Fluorescence spectroscopy analysis showed that
17 the peptide is mainly internalized *via* an energy- and clathrin-dependent pathway as shown by
18 a significant uptake reduction using the 4°C (-74%) and the ATP depletion (-63%) conditions
19 as well as with chlorpromazin (-57%) (**Figure S3B**).

20

21 **2.4. Determination of the optimal DAXXp length**

22 The minimal active sequence (MAS) of the interfering peptide was investigated first by
23 synthesizing the peptides corresponding to sequences 209 to 212 issued from PepScan
24 screening (**Figure S1B**) to confirm that the TD sequence has the highest anti-apoptotic effect
25 as revealed by the DNA fragmentation assay (**Figure S4A**). Thereafter, length analyses were

1 performed based on the 18-mer variants 209-210 and 211-212 by cutting the respective
2 sequences from the N-terminus or the C-terminus (**Figure S4B**). The highest signal intensities
3 were observed with spot #6 (= sequence 211) and spot #27 (= sequence 209), showing that the
4 peptide could not be elongated or shortened. This was further confirmed with several shorter
5 TD variants (15-, 14-, 13 and 9-mer) having no or significant less anti-apoptotic effect
6 compared to TD as observed by DNA fragmentation quantification (**Figure S4C**).

7 Altogether, we could define the minimal active sequence issued from the human DAXX
8 protein as the 16-mer DAXXp sequences, which was therefore used coupled to the Tat vector
9 in all further experiments.

10

11 **2.5. Time-dependent level of phospho-DAXX during myocardial IR.**

12 As reported in our previous work, the time window of cardioprotection offered by
13 postconditioning in mice subjected to IR injury may be larger than initially reported.¹⁰ In this
14 study, we show that postconditioning when applied at 10 seconds or 1, 5, 10, 15, or 30 minutes
15 after the onset of reperfusion reduced infarct size and DNA fragmentation.

16 This delayed time window of 15 to 30 min was also observed for the TD administration
17 after the onset of reperfusion (**Figure 6**). TD cardioprotection is effective even if the DAXX
18 protein is phosphorylated 15 min after reperfusion (**Figure S9**). The amount of phosphorylation
19 reached 63% of that obtained in the non-treated IR₆₀ condition. If we consider the corresponding
20 apoptosis rates evaluated *in vivo* in the hearts subjected to the same injury (40 min ischemia-15
21 min reperfusion), we evidenced that in the IR₁₅ group, specific DNA fragmentation level
22 represents at this time only 43% of the value obtained for IR (maximal level; for details, see¹⁰).
23 These results confirm that DAXX phosphorylation is an early mechanism upstream the rise in
24 apoptosis that occurs later reaching a plateau at 60 min. In addition, we evidenced an efficient
25 cardioprotection for TD_{Δ15} *versus* Tat when the peptide was administered 15 minutes after the

1 onset of reperfusion (see **Figures 6B and C**) with a 42%-decrease in both infarct size and the
2 release of soluble nucleosomes (specific DNA fragmentation) (TD_{Δ15} *versus* Tat, p*; see
3 **Figures 6B and D**). This suggests that DAXX nucleo-cytoplasmic export, as a consequence of
4 DAXX phosphorylation, happens during the first minutes of the wave front of IR injury and
5 that the strong inhibition of apoptosis due to the TD administration happens even if DAXX is
6 exported from the nucleus. In sum, TD cardioprotection occurs even if DAXX protein is
7 phosphorylated and probably exported to the cytoplasm.

8

9 **2.6. Indirect role of Tat-DAXX on the DAXX nuclear export.**

10 Previous studies have shown that DAXX protein, which contains two nuclear localization
11 signals, is mainly located in the nucleus of unstressed cells¹¹. DAXX nucleocytoplasmic export
12 is triggered by oxidative stress in cardiac cells^{12, 13} or by glucose deprivation¹⁴. Upon stress,
13 DAXX phosphorylation (Serine 667) results in conformational changes exposing the nuclear
14 export signal (NES), which is recognized by the chromosomal region maintenance 1 receptor
15 (CRM1 or exportin 1).^{15, 16} The CRM1 is a carrier protein and a receptor recognizing the NES
16 of DAXX protein having the following amino acid sequence: LFELEIEALPL (565–575 AA
17 sequence of the DAXX protein). TD peptide (625-637 AA sequence of the DAXX protein) is
18 not issued from the NES region, is not homologue to this NES region and more importantly did
19 not contain the serine 667. For all mentioned reasons, we estimate that TD peptide could not
20 act as a bait on CRM1 receptor in order to block DAXX nuclear export.

21 ASK1 (Apoptosis signal-regulating kinase 1) is also a DAXX-interacting protein that
22 insures shuttle DAXX-shuttling during nucleo-cytoplasmic transport into the cytoplasm.¹⁷
23 Because ASK1 binds to the DAXX protein by interaction with the region spanning sequence
24 from amino acid 501 to 625¹⁴, we exclude the possibility that TD interacts with ASK1 (so no
25 change in the feedback loop controlling DAXX nucleo-cytoplasmic export).

1 In conclusion, if DAXX nucleo-cytoplasmic transport depends on specific interactions
2 between first DAXX and CRM1 and second DAXX and ASK1 after DAXX nuclear export,
3 there is no chance for the TD peptide to interact with these two proteins instead of DAXX,
4 leading to a direct impact of TD on the nucleo-cytoplasmic export.

5 Altogether these data suggest that DAXX nuclear trafficking may be indirectly
6 influenced by the TD treatment as a result of its impact on both apoptotic and survival pathways
7 as well as on the feedback loops controlling DAXX nucleo-cytoplasmic ratio (**Figure 7**).

8

9

1 **3. Supplemental Tables**2 **Table S1: Peptide sequences.**

Peptide	Sequence	AA
Tat	GRKKRRQRRRPPQ	13
Tat-DAXXp (TD)	GRKKRRQRRRPPQ-KKSRKEKKQTGSGPLG	29
Tat-scrDAXXp (TDS)	GRKKRRQRRRPPQ-KKGRKQSGESLGTPEK	29
Tat-DAXXp-209	GRKKRRQRRRPPQ-SGPPCKKSRKEKKQT	28
Tat-DAXXp-210	GRKKRRQRRRPPQ-PCKKSRKEKKQTGSG	28
Tat-DAXXp-211	GRKKRRQRRRPPQ-KSRKEKKQTGSGPLG	28
Tat-DAXXp-212	GRKKRRQRRRPPQ-KEKKQTGSGPLG	28
Tat-DAXXp-14	GRKKRRQRRRPPQ-SRKEKKQTGSGPLG	27
Tat-DAXXp-13	GRKKRRQRRRPPQ-RKEKKQTGSGPLG	26
Tat-DAXXp-9	GRKKRRQRRRPPQ-KSRKEKKQT	22

3 **Footnote:** Tat-DAXXp-211 correspond to Tat-DAXXp-15 in Figure S4C

4

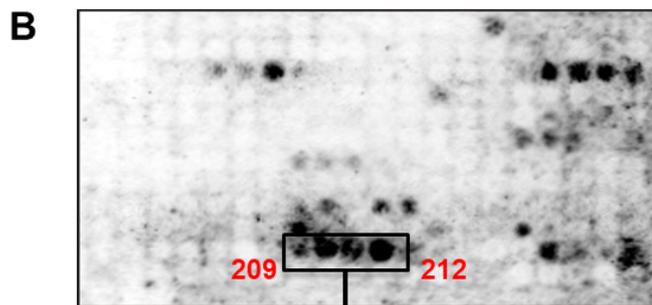
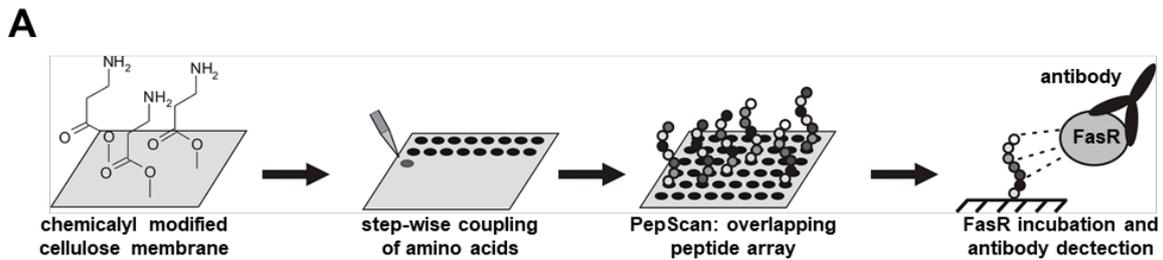
5 **Table S2: Summary of the *in vivo* results.**

Conditions	Peptides	Dose (mg/kg)	Infarct size (% of AR) [n]	Area at risk (% of LV) [n]	Soluble nucleosomes (ratio I/NI) [n]
40 min I / 1 h R	Tat	1	38.37 ± 8.46 [14]	43.42 ± 5.74 [14]	4.09 ± 0.75 [11]
	TD	0.1	30.85 ± 6.34 [6]	46.19 ± 3.65 [6]	3.30 ± 1.37 [11]
	TD	1	15.54 ± 6.90 [11]	45.47 ± 5.18 [11]	1.61 ± 0.44 [6]
	TD	10	16.02 ± 3.84 [6]	43.06 ± 5.84 [6]	1.29 ± 0.45 [6]
	TDS	1	35.64 ± 6.28 [6]	46.66 ± 4.76 [6]	3.92 ± 1.19 [7]
	TD _{Δ15}	1	21.97 ± 0.95 [6]	46.12 ± 4.90 [6]	2.38 ± 0.27 [6]
	TD _{Δ30}	1	25.12 ± 4.95 [10]	44.92 ± 5.05 [10]	2.58 ± 0.51 [11]
	TD _{Δ45}	1	36.80 ± 7.15 [9]	43.95 ± 5.06 [9]	4.17 ± 0.35 [8]
	40 min I / 24 h R	Tat	1	33.29 ± 6.07 [17]	44.41 ± 4.99 [17]
TD		0.1	22.41 ± 6.15 [7]	46.48 ± 5.20 [7]	2.17 ± 0.43 [6]
TD		1	17.10 ± 5.16 [8]	41.99 ± 4.57 [8]	1.57 ± 0.37 [6]
TD		10	13.73 ± 4.79 [6]	46.07 ± 3.41 [6]	1.49 ± 0.60 [6]
TDS		1	32.84 ± 4.01 [6]	45.18 ± 5.20 [6]	3.37 ± 0.94 [10]
TD _{Δ15}			19.70 ± 3.87 [6]	45.34 ± 3.49 [6]	1.59 ± 0.29 [6]
TD _{Δ30}		1	23.62 ± 4.69 [14]	47.13 ± 3.82 [14]	2.34 ± 0.72 [10]
TD _{Δ45}		1	36.26 ± 6.03 [9]	44.06 ± 7.23 [9]	3.52 ± 0.63 [8]

6 **Footnote:** numbers in square brackets represent the number of treated animals.

1 **4. Supplemental Figures**

2



Selected DAXX epitope

3

4 **Supplemental Figure S1 : Screening of the DAXX epitope by SPOT synthesis.**

5 (A) Principle of the SPOT synthesis: after the amino functionalization of the cellulose
6 membrane, the amino acids were spotted step-wise according to the standard SPOT synthesis.²
7 The peptide library was then incubated with the intracellular region of the Fas receptor (FasR)
8 and the interactions were revealed by antibody detection.

9 (B) Peptide library screening: The primary amino acid sequence of the DAXX protein was
10 dissected in overlapping peptides, which were synthesized as an array (PepScan; 15-mer
11 peptides with a 3 amino acids shift). The peptide array was probed for FAS interaction and
12 analyzed in enzyme-linked blot. The black rectangle shows the brightest spots with the
13 corresponding epitope sequence.

14

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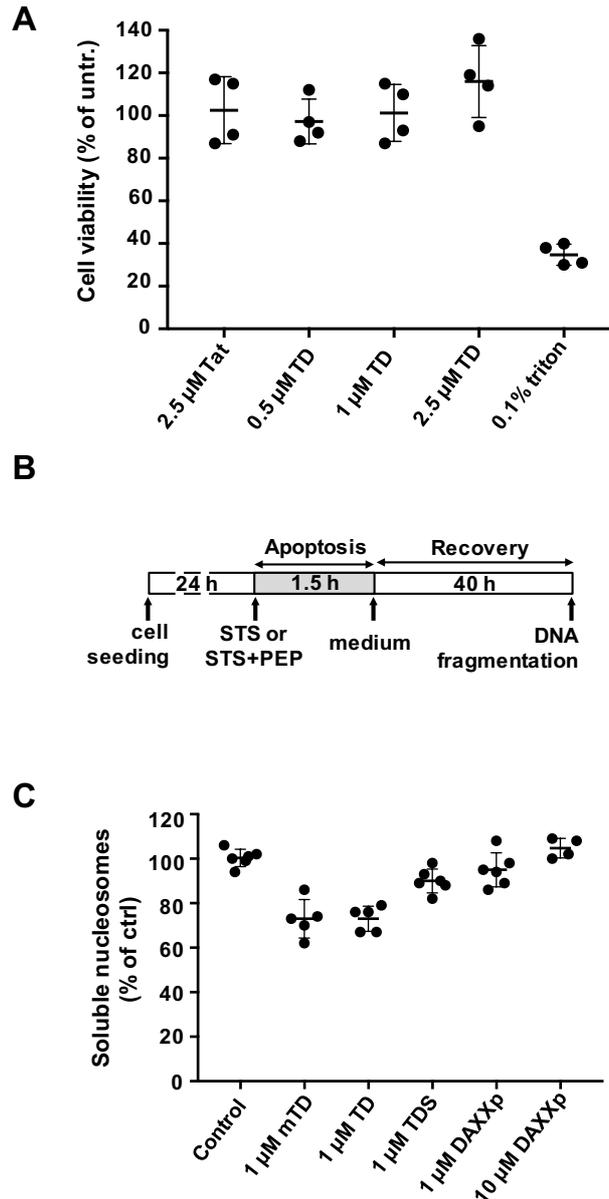
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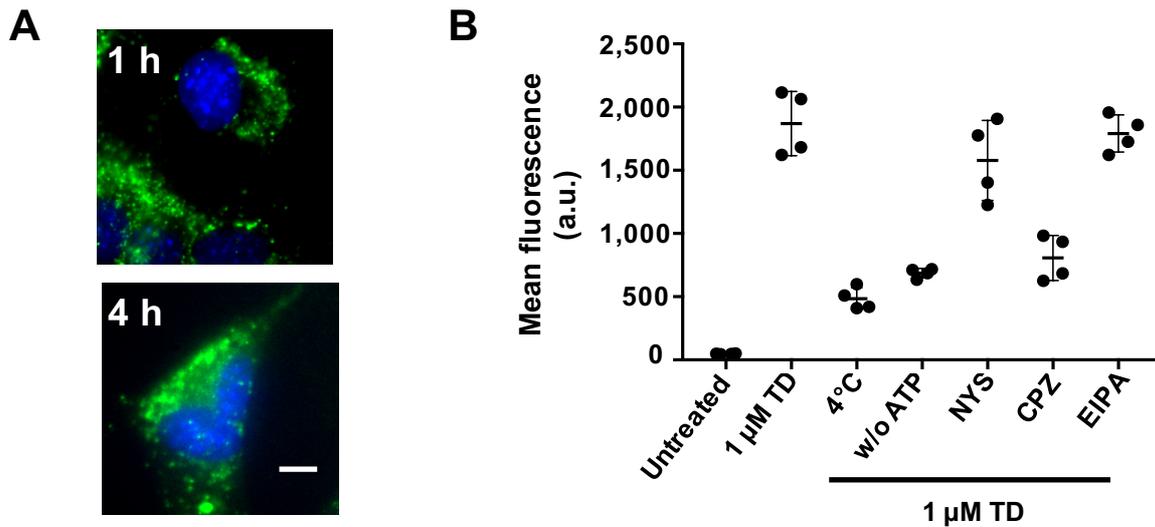
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23 **Supplemental Figure S2: *In vitro* evaluation of Tat-DAXXp in C2C12 cells.**

4 (A) Cell viability: C2C12 were incubated with increasing concentrations of TD for colorimetric
 5 assessment of the cell viability. Data shown are the means \pm SD, with n=4 independent
 6 experiments. (B) Treatment: C2C12 cells were subjected to 0.1 μ mol/L staurosporine (STS) for
 7 1.5 h, with or without additional peptide treatment, and followed by 40 h recovery after medium
 8 replacement. (C) Anti-apoptotic effect: Quantification of DNA fragmentation in C2C12 cells
 9 treated by STS and anti-apoptotic peptides (1 μ mol/L). Note a 22%-decrease in DNA
 10 fragmentation with the murine mTD (for Tat-mDAXXp) as well as with the human TD
 11 sequence (1 μ mol/L each). Data were normalized relative to control value (STS alone). Data
 12 shown are the scatter dot blots and the means \pm SD, with $n \geq 4$ independent cultures.

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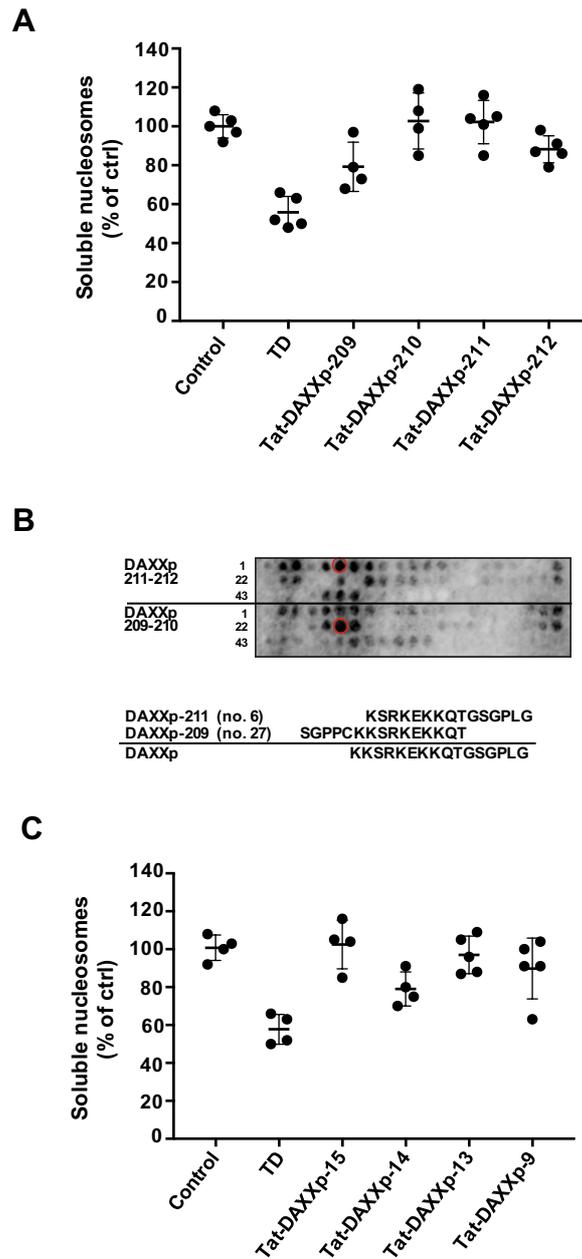
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4 **Supplemental Figure S3: Mechanism of cellular internalization of Tat-DAXXp in C2C12**
 5 **cells.**

6 (A) Cellular localization: Representative images of C2C12 cells, which were incubated with
 7 1 μ mol/L CF-labeled TD incubated for 1 h or 4 h. Cell nuclei were stained with Hoechst-dye
 8 (blue). Bar scale = 10 μ m. Original magnification: x63 oil immersion.

9 (B) Trafficking mechanism: C2C12 were incubated with carboxyfluorescence (CF)-labeled TD
 10 (1 μ mol/L) for 1 h and uptake analyzed by flow cytometry. Untreated cells represent the
 11 negative control. To evaluate the internalization pathway, cells were pre-treated 30 min in the
 12 following conditions: 4 $^{\circ}$ C, ATP-depletion (w/o ATP: 10 mM NaN₃ + 6 mM DDG),
 13 chlorpromazine (CPZ: 7.5 μ mol/L), nystatin (NYS: 50 μ mol/L), 5-(N-ethyl-N-isopropyl)
 14 amilorid (EIPA: 10 μ mol/L). Data shown are scatter dot blots and mean \pm SD, with n=4
 15 independent experiments.

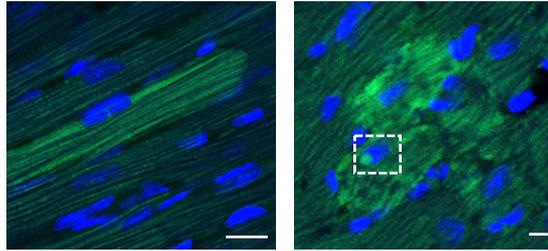
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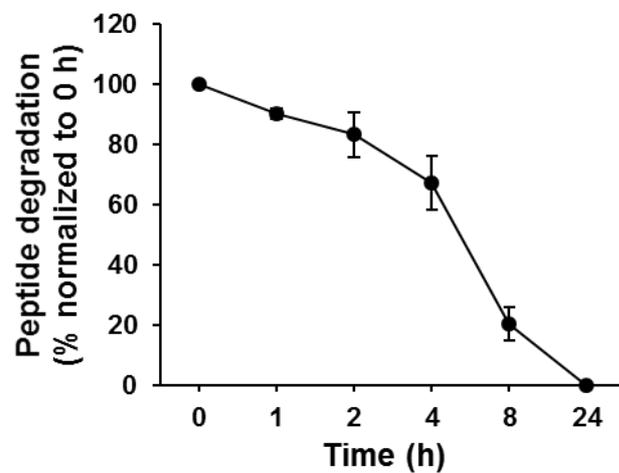
Supplemental Figure S4: Determination of the optimal Tat-DAXXp sequence.

(A) Evaluation of the anti-apoptotic activity of TD variants in primary cardiomyocytes using DNA fragmentation. Data are normalized to 100% STS. Data shown are scatter dot blots and means \pm SD, with $n \geq 4$ independent experiments. (B) The DAXXp-211 and DAXXp-209 peptides were successively shortened by one amino acid residue at the N-terminus, at the C-terminus or both at the N- and the C-termini, and analyzed using enzyme-linked blot. The spots indicated by a red circle exhibited the highest signal intensities (BLU). (C) Evaluation of the anti-apoptotic activity of shorter TD variants in primary cardiomyocytes using DNA fragmentation. Data are normalized to 100% STS. Data shown are scatter dot blots and the means \pm SD, with $n \geq 4$ independent experiments.

A



B



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3 **Supplemental Figure S5: Behavior of Tat-DAXXp *in vivo* and *in vitro*.**

4 (A): Representative confocal images from LV slices of mice hearts subjected *in vivo* to

5 myocardial IR and injected with CF-TD showing cytoplasmic localization of the peptide

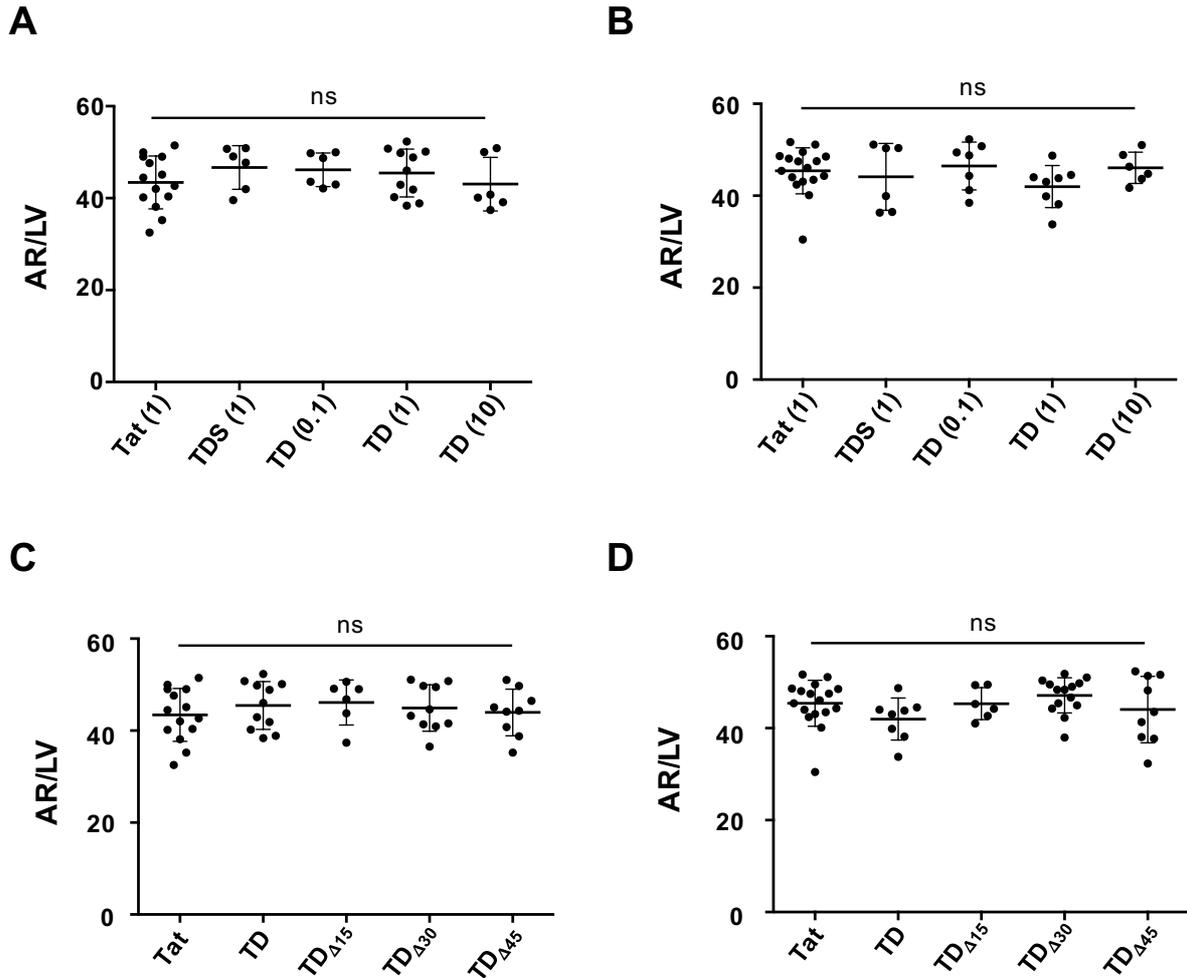
6 (green) and few nuclear localization (DAPI in blue). Bar scales = 50 μm (left), 10 μm (right);

7 (B) Stability of TD was measured in 20% mouse adult serum (37°C) and peptide degradation

8 was analyzed by reverse-phase HPLC (measurement of the peak area in $\mu\text{V}\cdot\text{sec}$) after 0 h, 1 h,

9 2 h, 4 h, 8 h and 24 h incubation. n = 3.

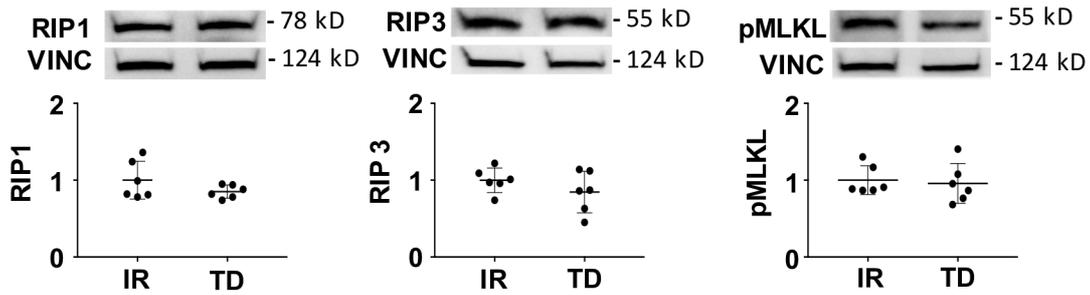
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 2 **Supplemental Figure S6: Area at risk measured in the study groups of mice subjected to**
 3 **IR.**

4 Area at risk expressed in % of LV mass (AR/LV) was quantified in mice subjected to 40 min-
 5 ischemia followed by 1 h (A, C) or 24 h of reperfusion (B, D). (A, B): Scatter dot blots and
 6 means ± S.D were plotted for Tat (1 mg/kg), TD (0.1, 1 and 10 mg/kg) or TDS (1 mg/kg)
 7 injected intravenously 5 min before reperfusion. (C, D): Scatter dot blots and means ± S.D were
 8 plotted for all peptides (1 mg/kg) injected 5 min before reperfusion (TD) or for the delayed
 9 administration at 15 min (TD_{Δ15}), 30 min (TD_{Δ30}), and 45 min (TD_{Δ45}) after the onset of
 10 reperfusion. Statistical significance compared to Tat is noted ns for p>0.05. n values are
 11 reported in Table S2.

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2 **Supplemental Figure S7: Effects of Tat-DAXXp treatment on necroptosis.**

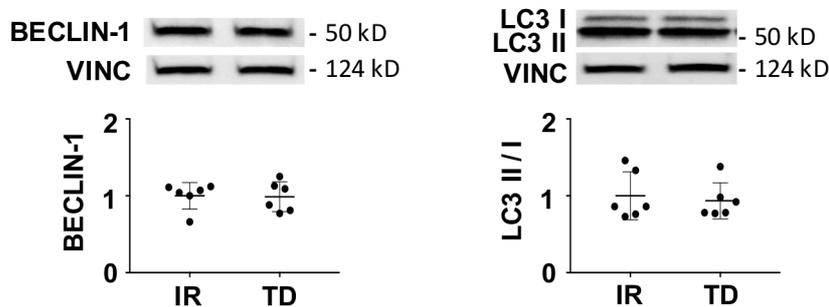
3 *Western blot* analysis was performed on LV protein extracts. Scatter dot blots and mean ± SD
 4 were plotted for RIP1 (p=0.3723), RIP3 (p=0.3939) and pMLKL (p=0.5887) tested in TD (n=6)
 5 *versus* IR (n=6) protein extracts. Representative gel blots are presented for each protein.
 6 Vinculin (VINC) was used as protein loading control. Statistical analysis was performed using
 7 non-parametric Mann-Whitney test revealing no significant difference between groups.

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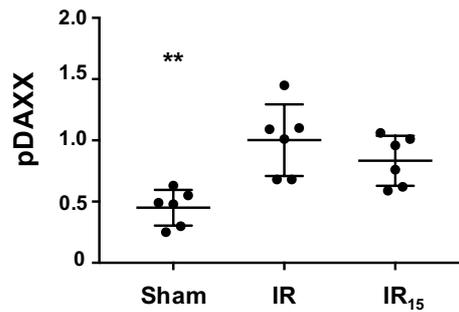
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16 **Supplemental Figure S8: Effects of Tat-DAXXp treatment on autophagy flux.**

17 *Western blot* analysis was performed on LV protein extracts. Scatter dot blots and mean ± SD
 18 were plotted for Beclin-1 (p=0.9372) and LC3 II/I ratio (p=0.8939) tested in protein extracts
 19 from TD (n=6) *versus* IR (n=6) hearts. Representative gel blots are presented for each protein.
 20 Vinculin (VINC) was used as protein loading control. Statistical analysis was performed using
 21 non-parametric Mann-Whitney test revealing no significant difference between groups.

22



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3 **Supplemental Figure S9: Effects of Tat-DAXXp treatment on DAXX phosphorylation.**

4 *Western blot* analysis was performed on LV protein extracts. Scatter dot blots and mean \pm SD
5 were plotted for pDAXX protein tested in Sham (n=6), IR (n=6) and IR _{Δ 15} (n=6) protein
6 extracts. Representative gel blots are presented for each protein. GAPDH was used as protein
7 loading control. Statistical analysis was performed using non-parametric Kruskal-Wallis
8 followed by the Dunn's post test. Statistical significance was noted p** = 0.0032 for Sham
9 *versus* IR.

10

11

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14

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