SUPPLEMENTAL INFORMATION

Degradation of GRK2 and AKT is an early and detrimental event in myocardial ischemia/reperfusion

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SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Parallel changes in GRK2 protein levels during I/R with or without preconditioning in both nuclear and cytoplasmic fractions. Protein levels of GRK2 in post-nuclear clarified extracts (cytoplasmic fraction, SB1) and detergent-extracted nuclear fractions (SB2) obtained as described in Material and Methods in the different groups of treatments. GAPDH and nucleolin served as loading controls and markers of cytoplasmic and nuclear fractions, respectively, after subcellular fractionation. Representative blots are shown. No overt subcellular re-distribution of GRK2 proteins between cytosolic and nuclear cellular fractions is observed in the experimental conditions tested, suggesting the occurrence of protein degradation processes. Data are mean ± SEM, n=3 rats per condition. *p< 0.05 vs cytoplasmic extracts in the normoxia control group, ††p< 0.01 compared to normoxic nuclear fractions, Student's t test

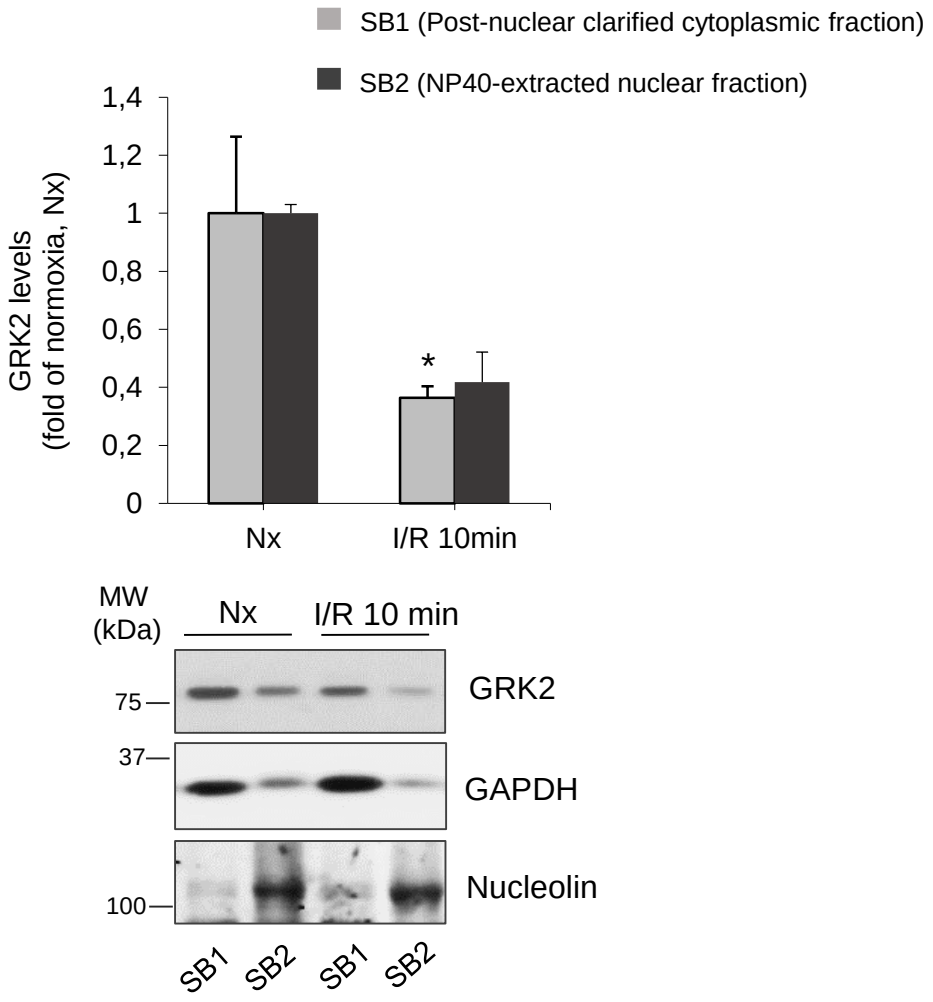
Supplementary Figure 2. Stimulation of PKA activity promotes the phosphorylation of GRK2 at Serine 685 and protein degradation. **(A)** HEK-293 cells were exposed to the cAMP-elevating agent forskolin (FO, 20 μM) for 10 min and levels of PKA-mediated phosphorylation of GRK2 were determined with a phospho-specific anti-pS685 GRK2 antibody. Blots were then stripped and probed with a polyclonal anti-GRK2 antibody. Actin was used as loading control. A gel representative of two independent experiments is shown. **(B)** Inhibition of PKA activity by addition of the PKA inhibitor peptide (PKI, 2.5μM) abrogates FO-induced GRK2 degradation. Pulse–chase ^{35}S-labeled proteins were immunoprecipitated with a specific anti-GRK2 antibody and resolved by SDS–PAGE followed by fluorography and densitometry. Data are the mean ± SEM of 2-4 experiments. **(C**) PKA-triggered GRK2 degradation is abrogated upon addition of the selective calpain protease inhibitor PD150606. GRK2 degradation was investigated by pulse-chase experiments as above in the presence of the indicated combinations of Forskolin, the proteasome inhibitor lacatcystin and the

calpain inhibitors ALLN and PD150606. A gel fluorography representative of two experiments is shown. **p< 0.01; ***p< 0.001 compared to indicated conditions and ##p< 0.01, ### p< 0.001 compared to 0h [one-way ANOVA, Tukey post hoc test].

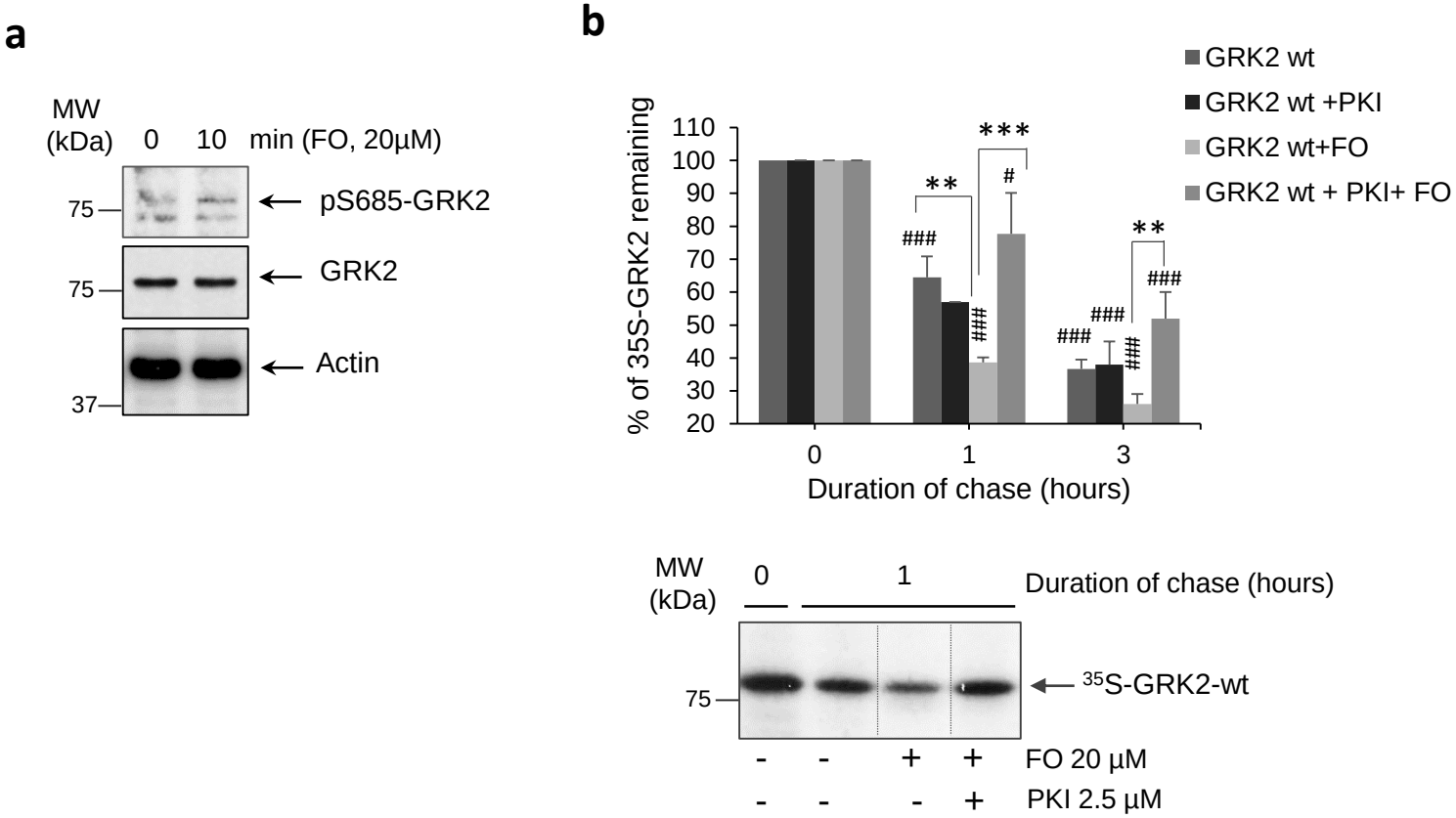
Supplementary Figure 3. (A) Cardiac ERK1/2 proteins levels remain unaltered upon ischemia, reperfusion and conditioning treatments. Rat heart lysates obtained after the different treatments detailed in Fig.1 were analyzed by western-blot using specific total ERK1/ERK2 antibodies. GAPDH was used as protein loading control and representative blots are shown. **(B, C)** Protein levels of AKT (B) and Pin1 (C) are similarly decreased in both post-nuclear clarified extracts (cytoplasmic fraction, SB1) and detergent-extracted nuclear fractions (SB2) obtained as in Suppl Fig. 1 in the different groups of treatments. Data are the mean ± SEM, n=3-4 rats per condition. **p< 0.01; ***p< 0.001 vs cytoplasmic extracts in the normoxia control group, †p< 0.05; ††p< 0.01 compared to normoxic nuclear fractions, Student's t test. GAPDH and Lamin B1 were used as protein loading controls and markers of cytoplasmic and nuclear fractions after subcellular fractionation. Representative blots are shown.

Supplementary Figure 4. Ischemia/reperfusion induces activation of PKA in porcine hearts. Global PKA activity was assessed in pig cardiac lysates from the indicated conditions by dot-blot, using a pan-specific phospho-substrate antibody that broadly detects phosphorylated proteins by PKA as indicated in Methods. Total GAPDH expression was used as loading control. Data were represented as fold-change with respect to control situation and are mean ± SEM, n=3-5 pigs per condition.*p<0.05; compared with the conditions indicated with the lines. Representative dot-blots are shown.

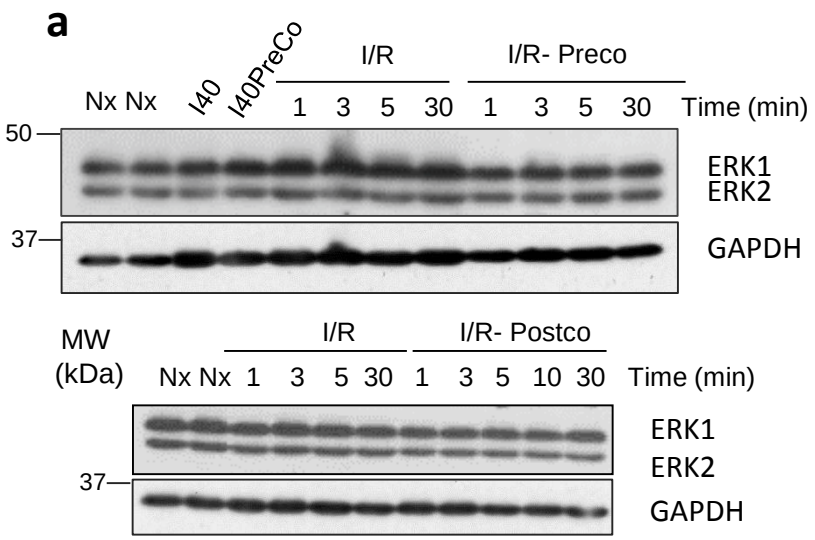
a

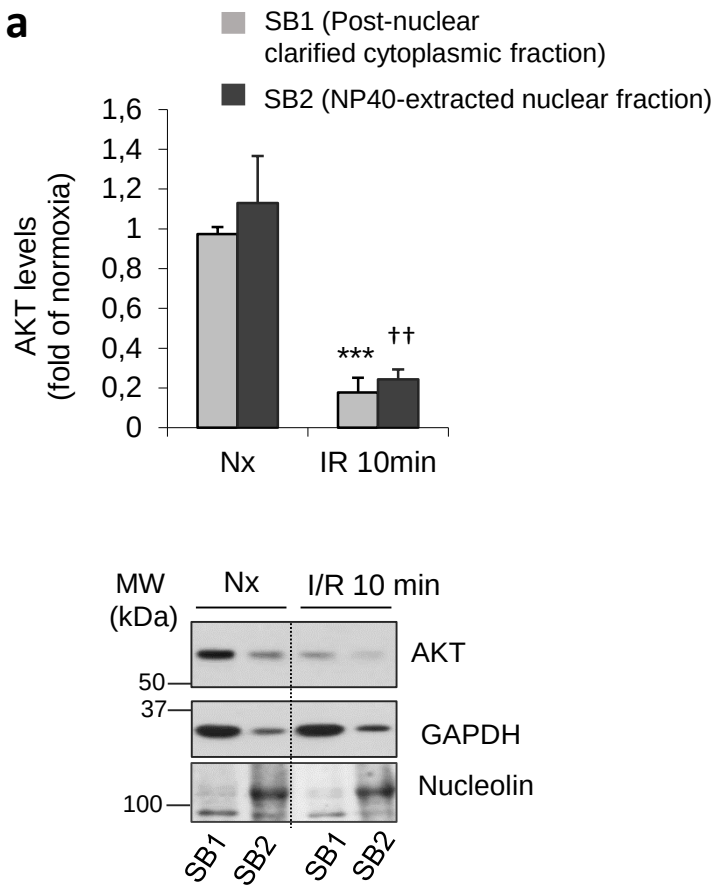


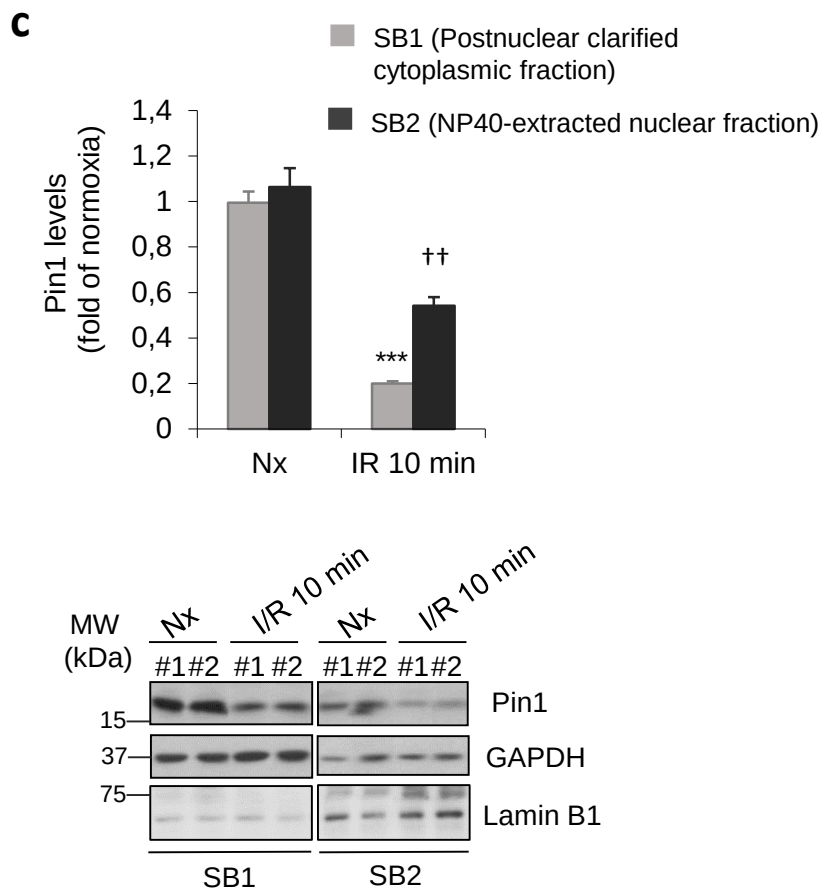
Suppl figure 1



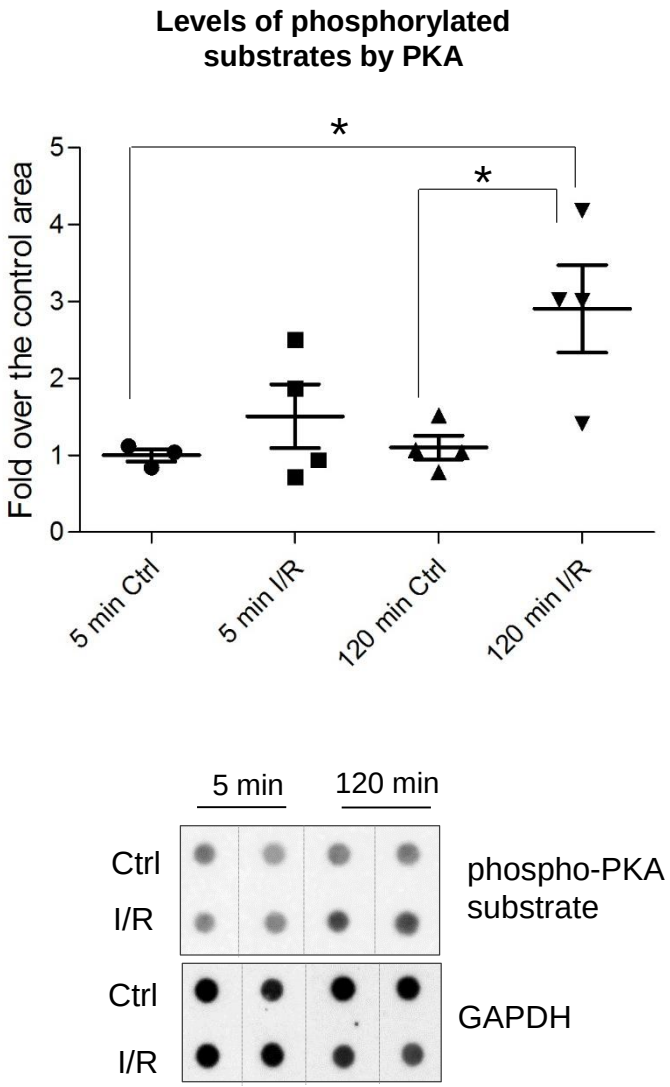
Suppl figure 2







Suppl. Figure 3



Suppl. Figure 4