Lipocalin-2 regulates cardiomyocyte autophagy, apoptosis and insulin sensitivity: importance in heart failure

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Introduction

Lipocalin-2 (Lcn2; also termed neutrophil gelatinase-associated lipocalin (NGAL)) is a small, secreted adipokine that belongs to a diverse family of lipocalins. Lcn2 is a proinflammatory marker associated with insulin resistance and obesity-related metabolic disorders, including heart failure. However, the precise mechanisms via which Lcn2 alters cardiac function are not fully known. Here we aimed to elucidate the effects of Lcn2 on cardiomyocyte insulin sensitivity with a focus on autophagy and apoptosis as regulatory cellular mechanisms. We used H9c2 cells derived from rat ventricle treated with recombinant Lcn2 and translated these studies to animal models by performing coronary artery ligation surgery to induce ischemia in wildtype and lipocalin-2 knockout mice as follows:

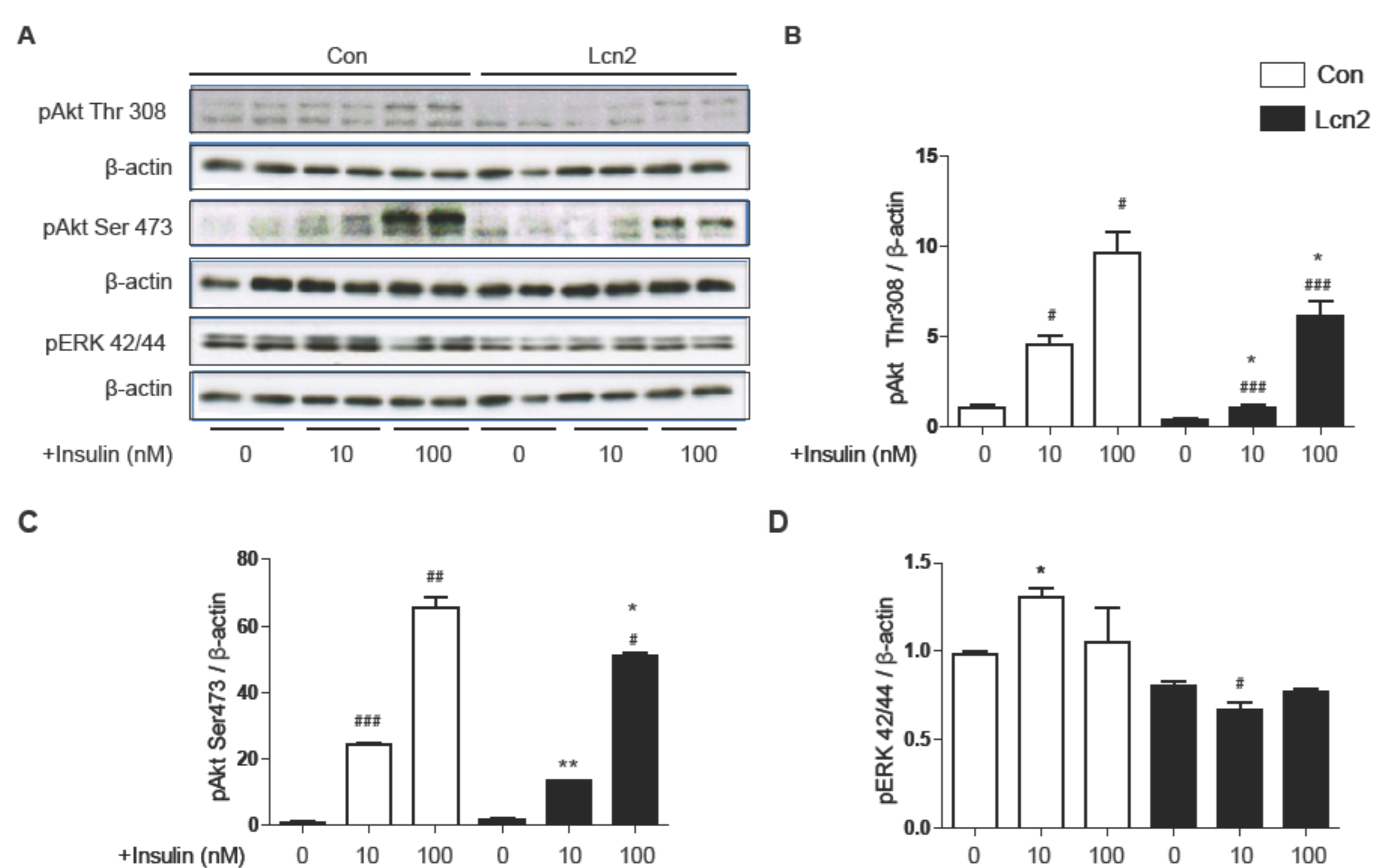### 1) Lcn2 blunted dose dependent insulin signaling in cardiomyocytes

Figure 1. Insulin signaling in cardiomyocytes was decreased by Lcn2, as indicated by decreased phosphorylation of Akt Thr308 , Akt Ser473 and ERK 42/44 in an insulin dose dependent manner.

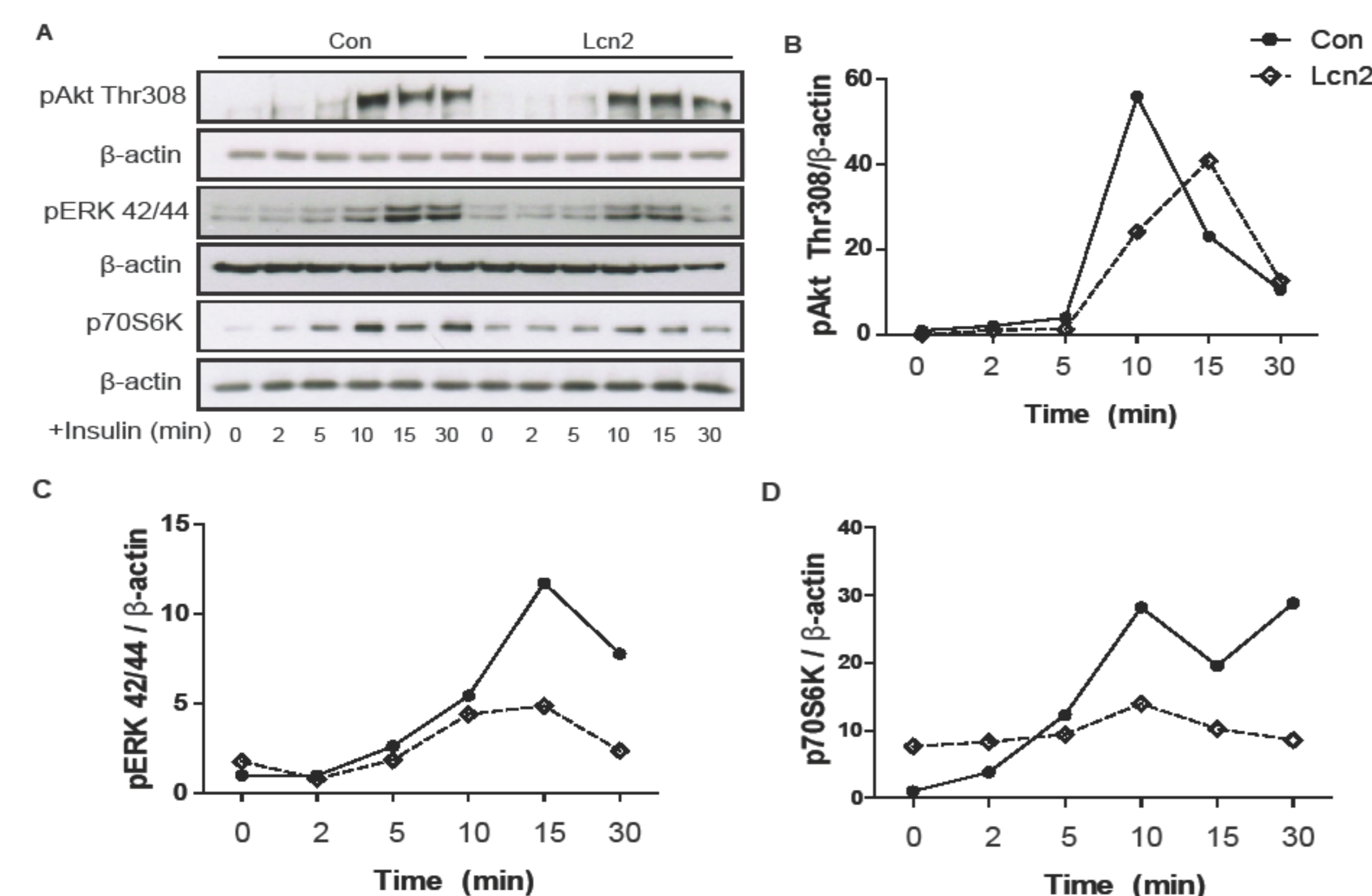### 2) Lcn2 blunted insulin signaling time dependently in cardiomyocytes

Figure 2. Insulin signaling in cardiomyocytes was decreased by Lcn2 indicated by decreased phosphorylation of Akt Thr308 and Akt Ser473 and p70S6K in a time dependent manner.
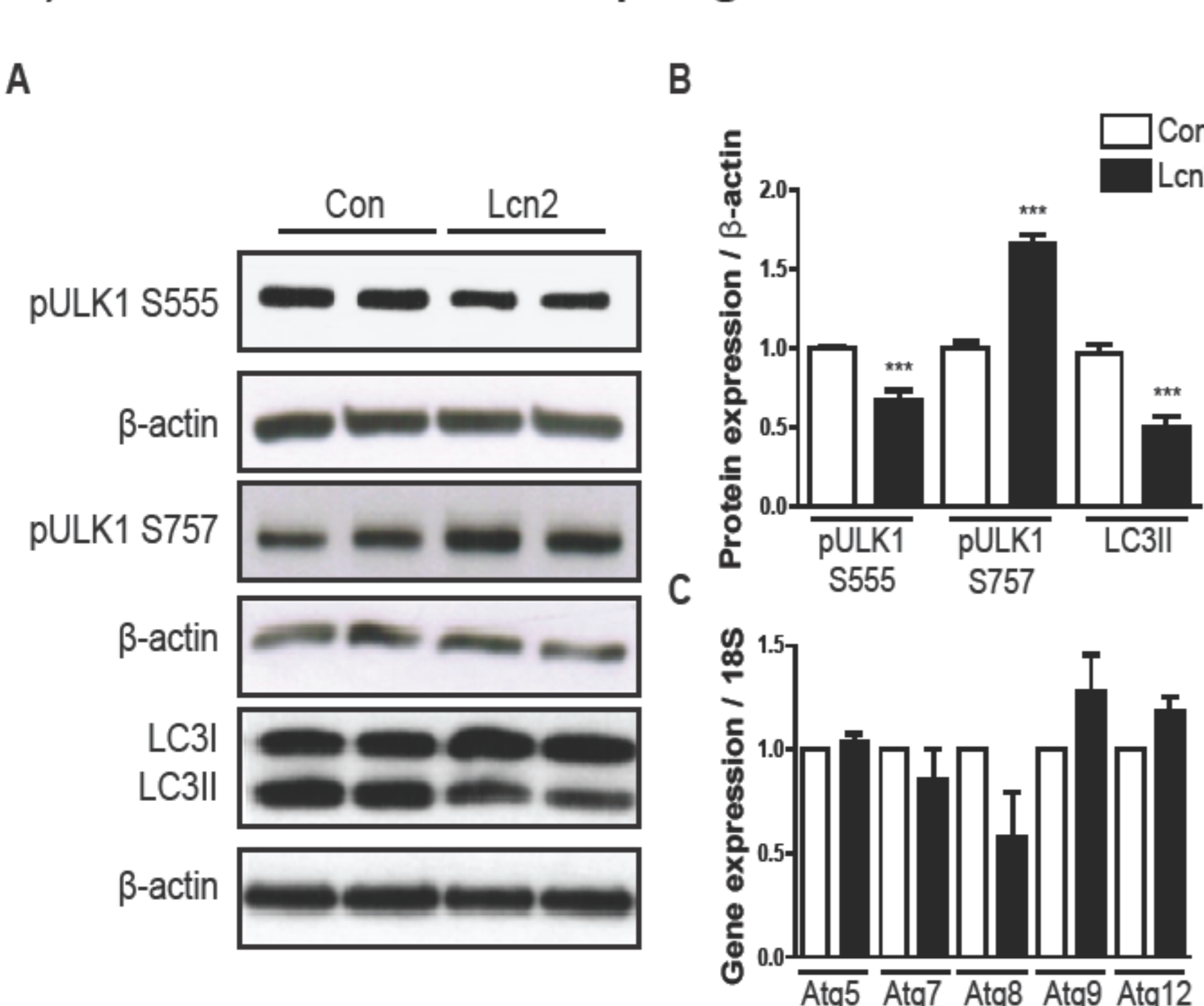
3) Lcn2 decreased autophagosomes formation

Figure 3. Initiation of autophagy was inhibited by Lcn2 in cardiomyocytes. Western blots showing protein expression of pULK1 S555, pULK1 S757, LC3II and the reference protein β-actin (A) and quantifications (B). Gene expression of autophagy genes including Atg5, Atg7, Atg8, Atg9 and Atg12 by qPCR was quantified with 18S as the reference gene using the delta delta CT method (C). Autophagosomes were visualized as endogenous LC3 puncta (D) and quantified (E).

4) Lcn2 decreased autophagic flux: analyzed using tandem fluorescent LC3 (tf-RFP/GFP-LC3)
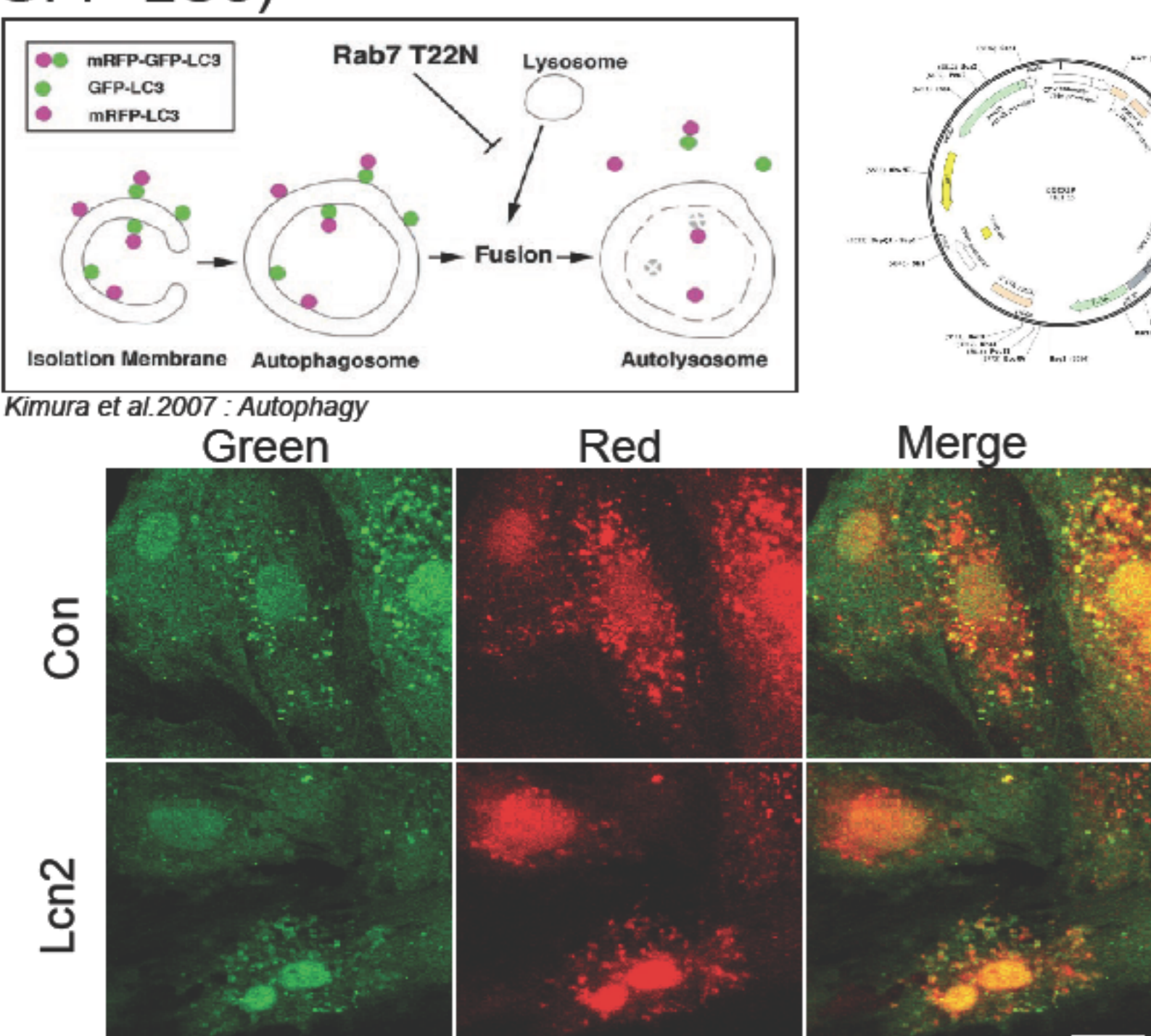
We used retroviral vector containing antibiotic resistance cassette for selection to make stable pools of cells expressing tf-RFP/GFP-LC3. This construct enables analysis of autophagosome content (orange puncta) and since GFP is sensitive to lysosomal degradation whereas RFP is not, the completion of autophagic flux can be inferred from disappearance of green signal.

Figure 4. Representative images from cells treated ± Lcn2 for 24h and showing less RFP/GFP ratio of the LC3 puncta in the tf-GFP/RFP-expressing H9c2 cells.

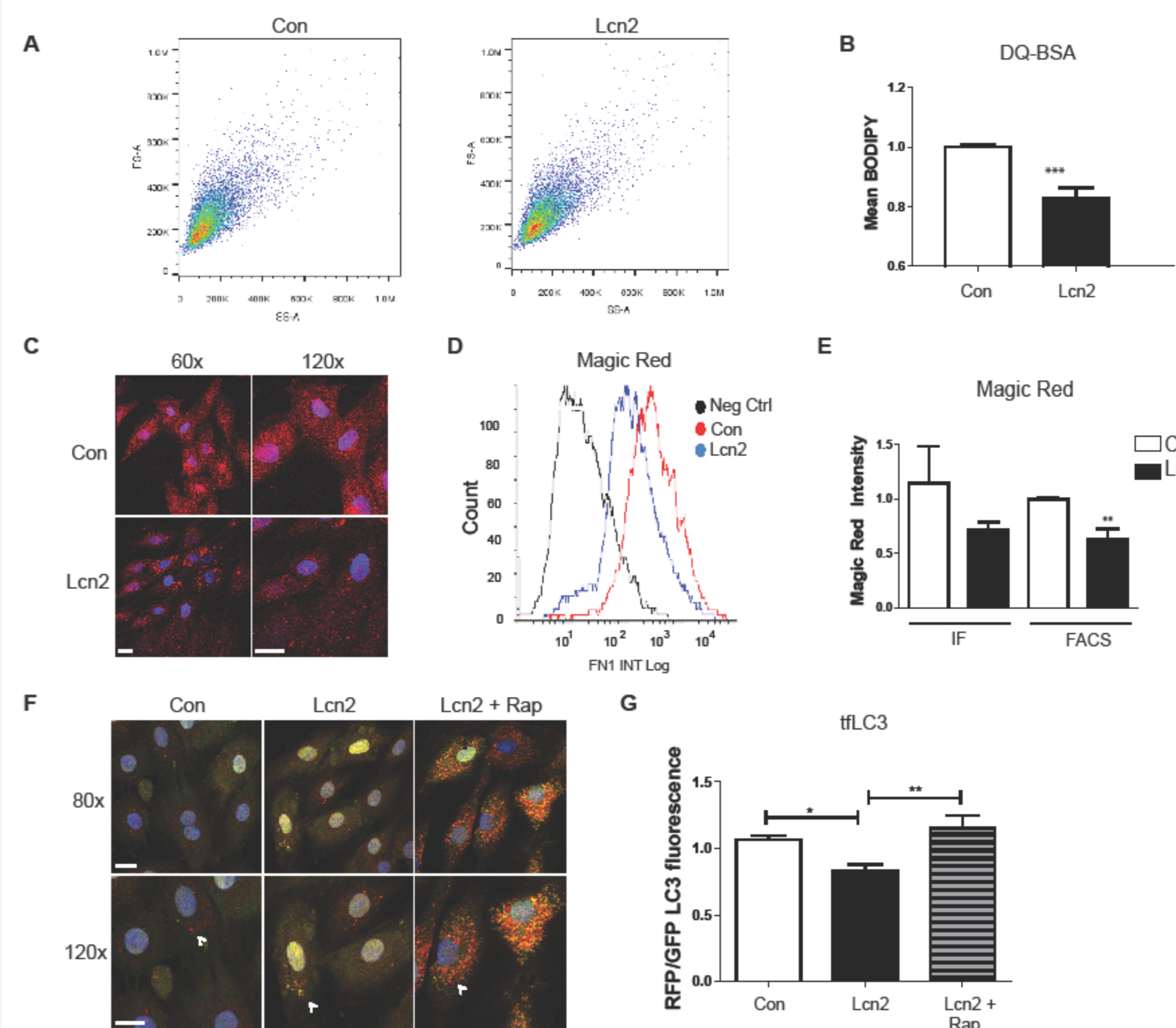### 5) Lcn2 decreased lysosomal activities and autophagic flux

Figure 5. Lcn2 decreased lysosomal cathepsin B activity and autophagic flux. The overall proteolysosomal activity was examined by quantifying fluorophore released by DQ-BSA using flow cytometry (A, B). The lysosomal cathepsin B activity was examined using Magic Red dye with immunofluorescence microscopy (C); FACS (D) and their quantifications (E). Autophagic flux was evaluated by looking at the RFP/GFP ratio of the LC3 puncta in the tf-GFP/RFP-H9c2 cell line, with rapamycin, an inducer of autophagy as a positive control (F).

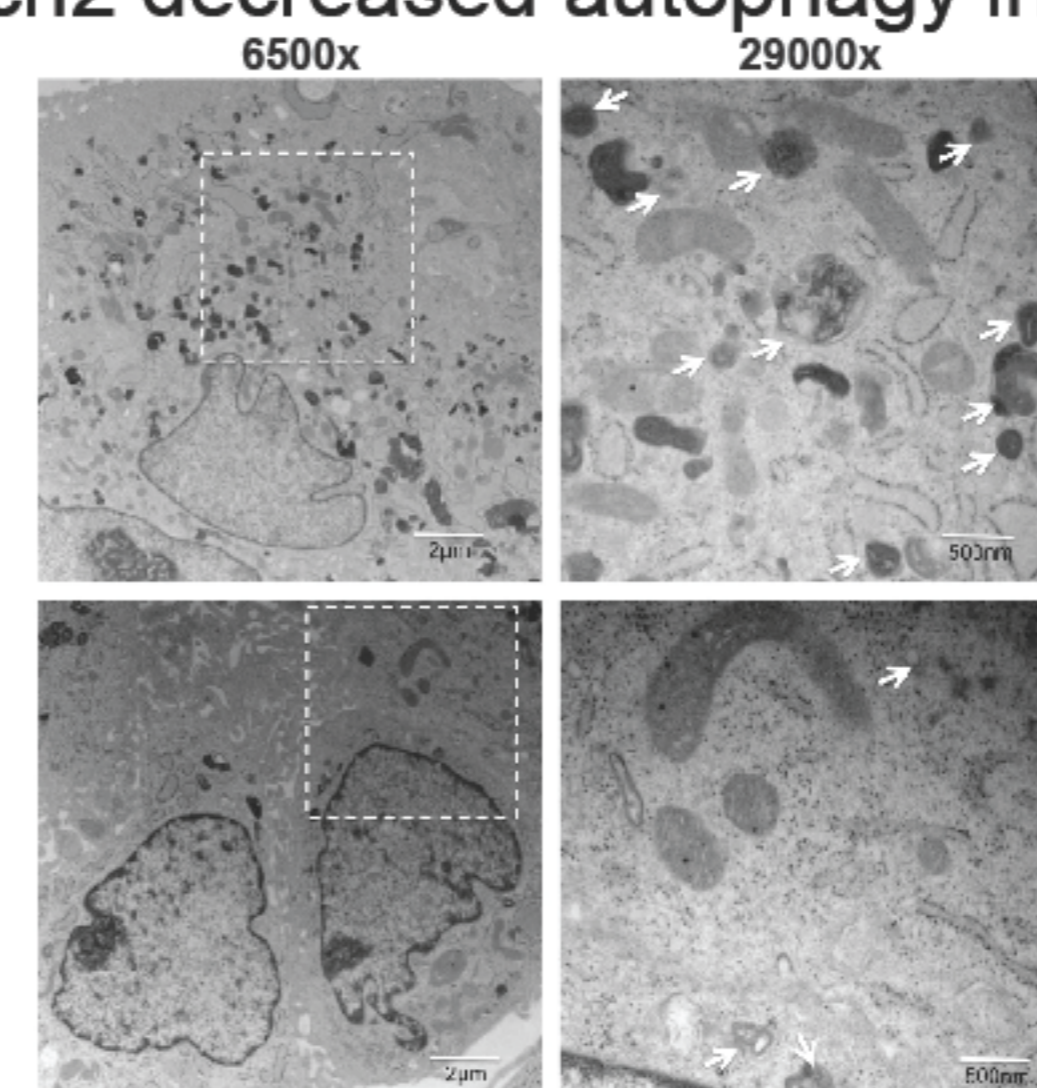### 6) Lcn2 decreased autophagy initiation and resulted in minimal of autolysosomes

Figure 6. Lcn2 decreased number of autophagosomes and autolysosomes in cardiomyocytes. H9c2 cells were treated with Lcn2 (1μg/ml) (Lcn2) or without (Con) for 1 hour. Autophagy was evaluated using transmission electron microscopy. Selected areas were magnified with white arrows indicating the presence of any autophagic vacuole in cardiomyocytes.

7) Lcn2 reduced insulin signaling due to its inhibition of autophagy
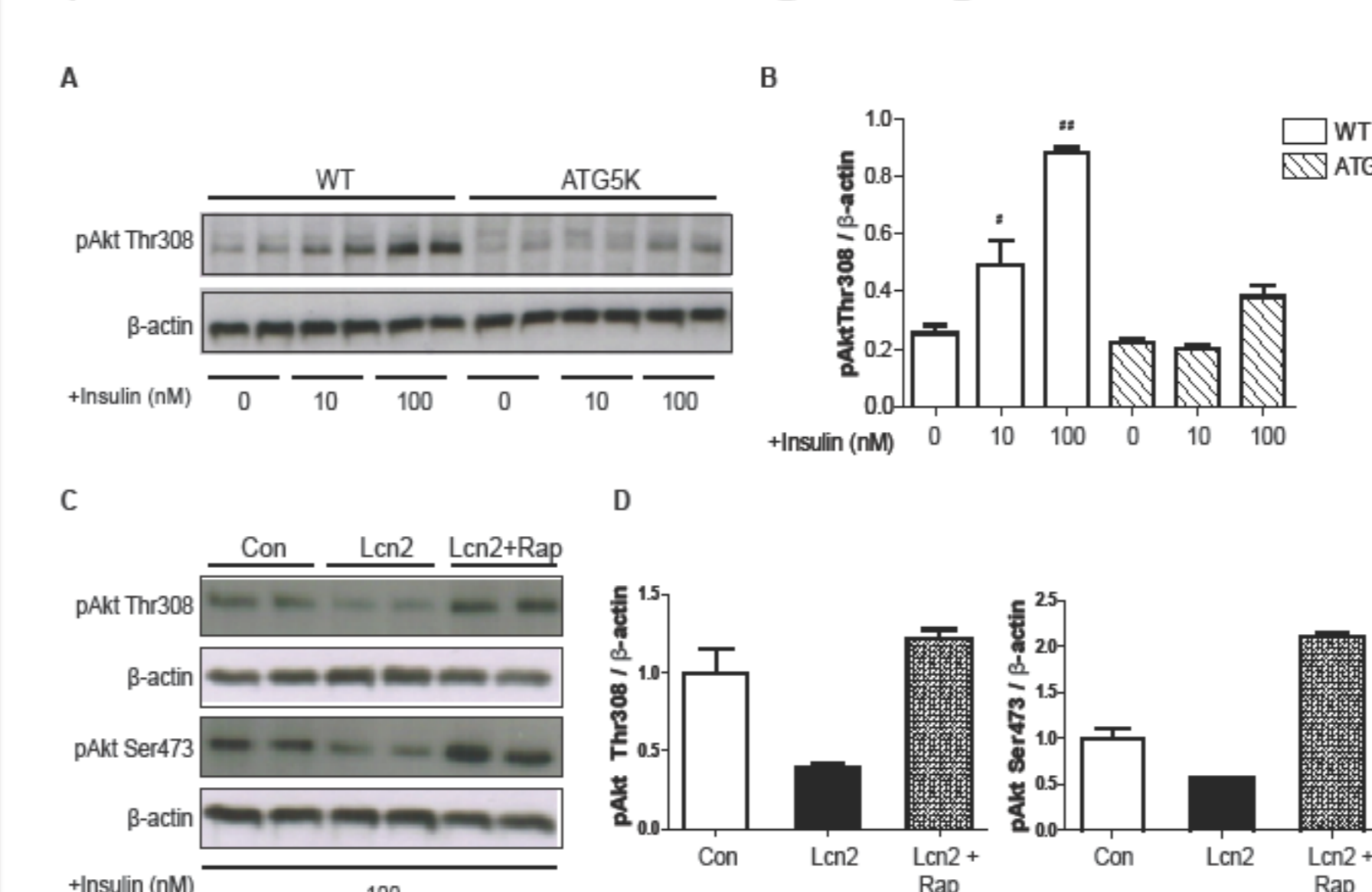

Figure 7. Autophagy was verified as being essential for insulin signaling as indicated by decreased phosphorylation of Akt Thr308 in autophagy deficient ATG5K cells (A) Western blots, (B) quantification. H9c2 cells were treated with Lcn2 (1μg/ml) (Lcn2) or without (Con) for 1 hour. Rapamycin (Rap) (250nM) was added 30 minutes after Lcn2 treatment to stimulate autophagy, and rescued the insulin signaling indicated by increased phosphorylation of Akt Thr308 and Akt Ser473 in Western blots (C) and its quantification (D).

8) Lcn2 increased intracellular iron levels

Figure 9. Intracellular free iron levels were monitored in cells treated ± Lcn2 for 24 h, using the cell-permeable fluorescent dye PG-SK, which is quenched upon binding iron. Treatment of FeSO$_4$ (100 μM) was used as positive control. A. representative segmented images and B. quantitation of fluorescent intensity per cell and C. distribution of values is shown here. Quantitative analysis of cell by cell data is shown for each condition with number of cells, median fluorescence, and number of outlying values shown. *Taken from J. Biol. Chem. (2012) 287(7):4808–4817.*
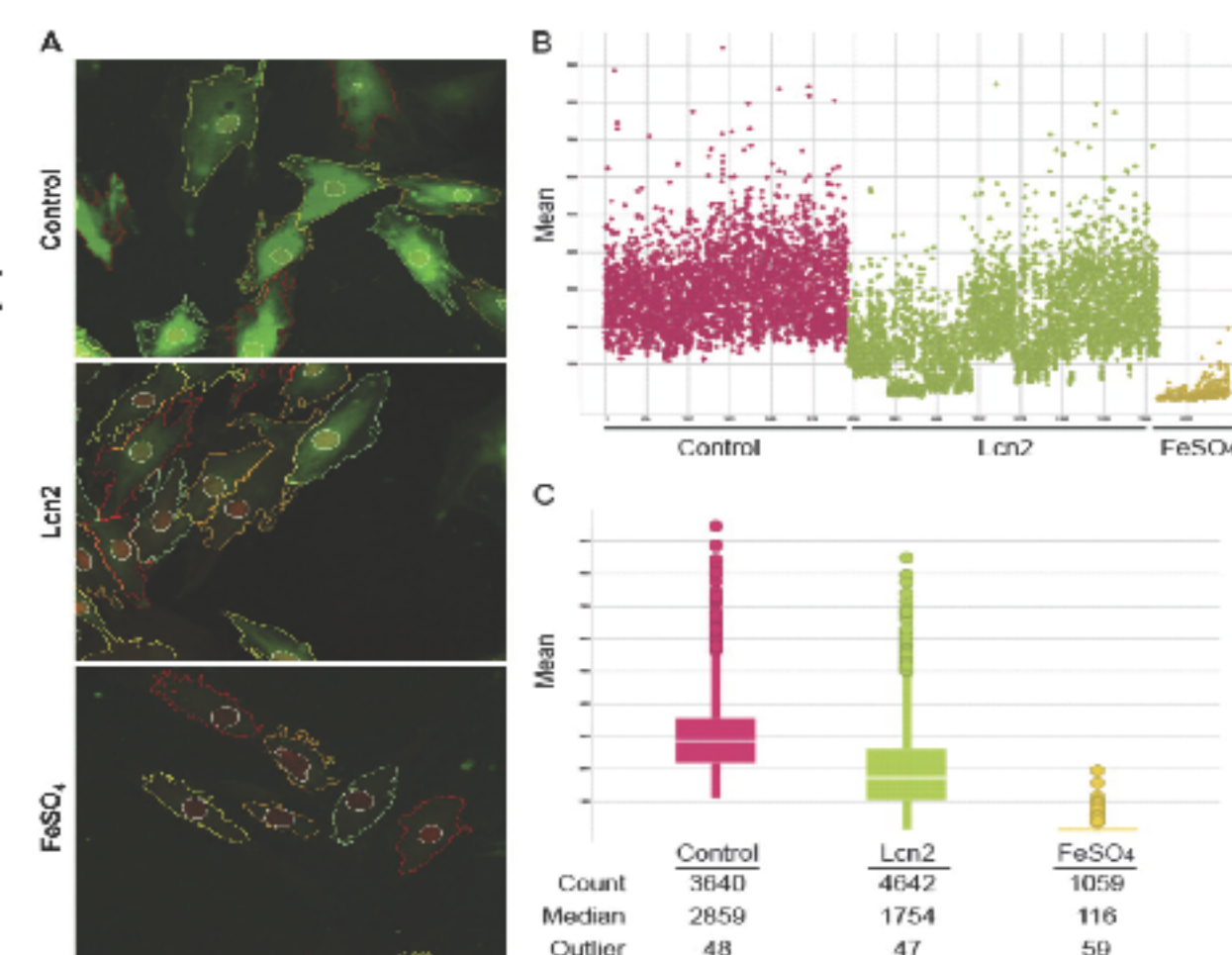
9) Caspase 3 and caspase12 activity in mouse hearts after 30min ischemia was attenuated in Lcn2-KO mice
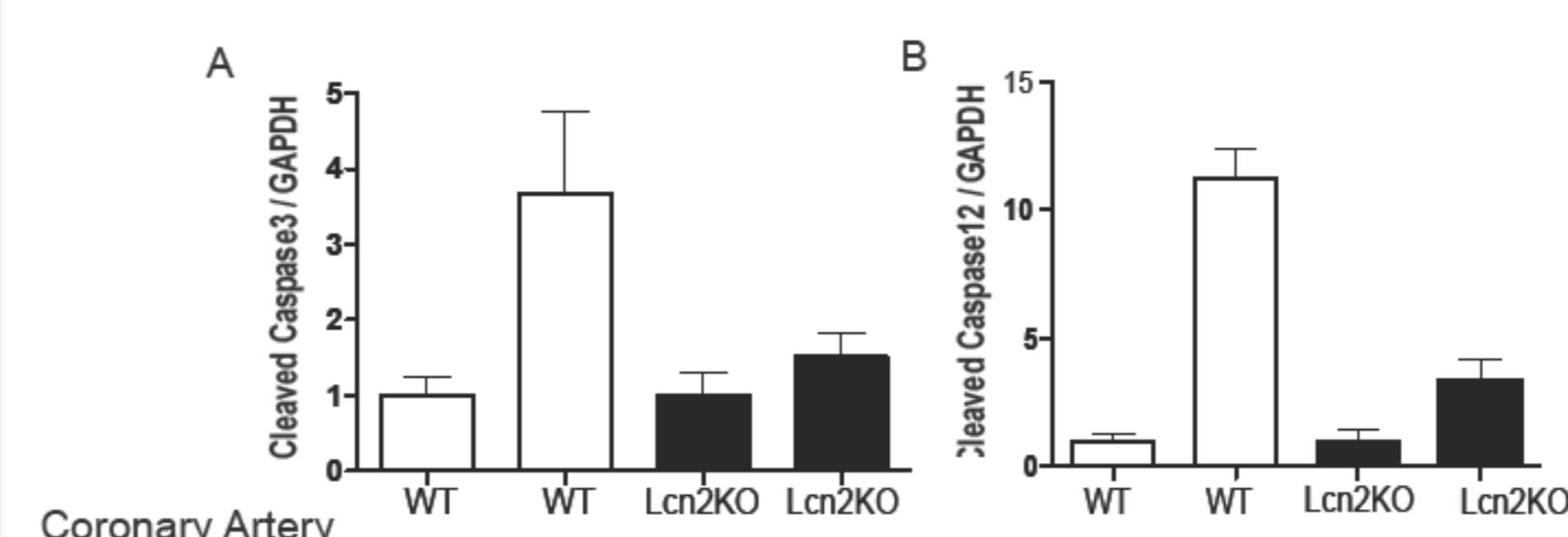

Figure 9. ischemia-induced caspase-3 and -12 activity was attenuated in Lcn2 KO versus wildtype. C57 wild type mice (WT) or Lcn2KO received sham or coronary artery ligation (CAL) surgery to induce 30 minutes ischemia before sacrifice. Western blot quantifications showing levels of (A) Caspase 3 and (B) Caspase 12.

Conclusions

- Lcn2 induced insulin resistance in H9c2 cardiomyocytes.
- Lcn2 reduced autophagy and this contributed to development of insulin resistance.
- Lcn2 mediated changes in apoptosis by increasing intracellular iron levels.
- Lcn2 knockout mice hearts had reduced levels of apoptosis in response to 30min ischemia.

In summary, lipocalin-2 regulated cardiomyocyte autophagy, apoptosis and insulin sensitivity and the exact physiological significance of these cardiac remodeling effects must now be determined.

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