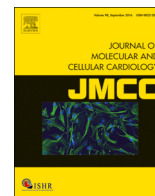




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Original article

## Polycystin-2-dependent control of cardiomyocyte autophagy

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## ABSTRACT

**Aims:** Considerable evidence points to critical roles of intracellular Ca<sup>2+</sup> homeostasis in the modulation and control of autophagic activity. Yet, underlying molecular mechanisms remain unknown. Mutations in the gene (*pkd2*) encoding polycystin-2 (PC2) are associated with autosomal dominant polycystic kidney disease (ADPKD), the most common inherited nephropathy. PC2 has been associated with impaired Ca<sup>2+</sup> handling in cardiomyocytes and indirect evidence suggests that this protein may be involved in autophagic control. Here, we investigated the role for PC2 as an essential regulator of Ca<sup>2+</sup> homeostasis and autophagy.

**Methods and results:** Activation of autophagic flux triggered by mTOR inhibition either pharmacologically (rapamycin) or by means of nutrient depletion was suppressed in cells depleted of PC2. Moreover, cardiomyocyte-specific PC2 knockout mice (*aMhc-cre;Pkd2<sup>F/F</sup>* mice) manifested impaired autophagic flux in the setting of nutrient deprivation. Stress-induced autophagy was blunted by intracellular Ca<sup>2+</sup> chelation using BAPTA-AM, whereas removal of extracellular Ca<sup>2+</sup> had no effect, pointing to a role of intracellular Ca<sup>2+</sup> homeostasis in stress-induced cardiomyocyte autophagy. To determine the link between stress-induced autophagy and PC2-induced Ca<sup>2+</sup> mobilization, we over-expressed either wild-type PC2 (WT) or a Ca<sup>2+</sup>-channel deficient PC2 mutant (PC2-D509V). PC2 over-expression increased autophagic flux, whereas PC2-D509V expression did not. Importantly, autophagy induction triggered by PC2 over-expression was attenuated by BAPTA-AM, supporting a model of PC2-dependent control of autophagy through intracellular Ca<sup>2+</sup>. Furthermore, PC2 ablation was associated with impaired Ca<sup>2+</sup> handling in cardiomyocytes marked by partial depletion of sarcoplasmic reticulum Ca<sup>2+</sup> stores. Finally, we provide evidence that Ca<sup>2+</sup>-mediated autophagy elicited by PC2 is a mechanism conserved across multiple cell types.

**Conclusion:** Together, this study unveils PC2 as a novel regulator of autophagy acting through control of intracellular Ca<sup>2+</sup> homeostasis.

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## 1. Introduction

Macroautophagy, hereafter referred to as “autophagy”, is a highly conserved catabolic process whereby intracellular components are degraded and recycled. In eukaryotic cells, basal autophagic flux is necessary to maintain cellular homeostasis [1]. Further, autophagic activity is altered – either up or down – in a wide range of circumstances, including disease [2,3]. As a result, there is great interest in deciphering mechanisms of autophagic control.

A key autophagic regulator is the serine/threonine protein kinase complex mTORC1 (mechanistic target of rapamycin complex 1) [4]. Active mTORC1 maintains autophagic flux at basal levels, whereas mTORC1 suppression, such as that elicited by nutrient withdrawal, triggers massive autophagy [1,5]. Studies have shown that mutations in the gene *pkd2*, which codes for the protein polycystin 2 (PC2), strongly up-regulate mTORC1 activity promoting autosomal dominant polycystic kidney disease (ADPKD) in humans [6]. Indeed, treatment of patients with rapamycin, which inhibits mTORC1 and promotes autophagosome formation, is an effective therapy for ADPKD, suggesting that dysregulated autophagy may be involved in disease pathogenesis [7,8]. Cardiovascular pathology is often seen in patients with ADPKD, and the role of autophagy in these events is unknown. That said, many studies point to impaired autophagy in a wide range of cardiovascular diseases [2,3,9]. The role of PC2 as a possible regulator of autophagy in cardiac tissue has not been fully elucidated.

PC2 functions as a  $\text{Ca}^{2+}$ -permeable cation channel and participates in maintenance of cytoplasmic  $\text{Ca}^{2+}$  homeostasis [10,11]. Intracellular  $\text{Ca}^{2+}$  handling regulates autophagy, either positively or negatively, depending on the context of time, space, and cell state [12]. Studies have shown that cytosolic  $\text{Ca}^{2+}$  is required to induce autophagy [12,13]. Furthermore, increased cytosolic  $\text{Ca}^{2+}$  levels, induced by agents such as thapsigargin (a chemical inhibitor of ER  $\text{Ca}^{2+}$ -ATPase), ionomycin (a  $\text{Ca}^{2+}$  ionophore) and vitamin  $\text{D}_3$  analogs, trigger autophagic flux in different cell types [14]. Conversely, basal  $\text{Ca}^{2+}$  leak in mitochondria-associated endoplasmic reticulum membrane (MAMs) inhibits autophagy, implicating microdomains of intracellular  $\text{Ca}^{2+}$  in the control of the autophagic cascade [15].

Recently, PC2 has emerged as an important regulator for  $\text{Ca}^{2+}$  homeostasis in adult cardiomyocytes, one which predisposes to dilated cardiomyopathy, as indicated in studies performed in heterozygous “whole body” knockout mice, embryonic knockout cardiomyocytes, and zebrafish models [11,16,17]. Here, we tested the hypothesis that PC2-dependent modulation of intracellular  $\text{Ca}^{2+}$  participates in the control of basal and stress-induced autophagy in cardiomyocytes and other cell types.

## 2. Methods

A detailed Materials and Methods section is available in the *Online Data Appendix A*.

## 3. Results

### 3.1. PC2 is required for stress-induced autophagy in vitro

Cultured cardiomyocytes exposed either to rapamycin (1  $\mu\text{M}$ ), a chemical inhibitor of mTORC1, or to nutrient depletion with Earle's Balanced Salt Solution (EBSS), showed redistribution of a GFP-LC3 fusion protein, transitioning from diffuse cytosolic localization to an autophagic punctate pattern (Fig. 1A), consistent with the expected increase in autophagic activity. To investigate a possible role of PC2, cardiomyocytes were depleted of PC2 by means of siRNA specifically targeting its transcript, and autophagy was evaluated in the setting of

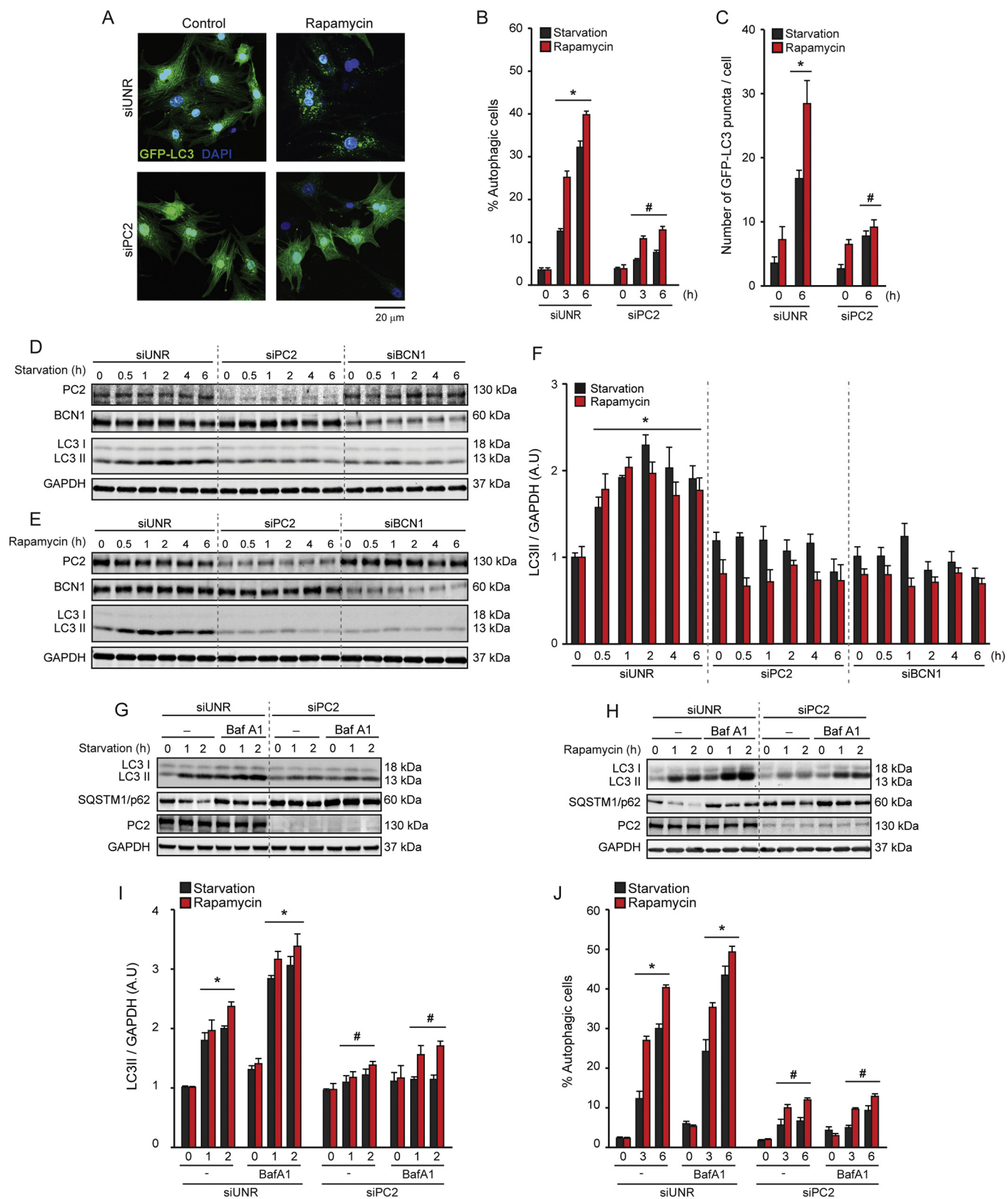
similar autophagy triggers. We observed that PC2 depletion significantly decreased both rapamycin- and starvation-induced autophagic activation as measured by GFP-LC3 puncta formation, percentage of cells manifesting autophagy, and GFP-LC3 puncta/cell (Fig. 1A-C). No changes in total PC2 were observed upon starvation or rapamycin treatment (Fig. 1D-E and Suppl. Fig. 1A). The extent of autophagy inhibition observed following PC2 depletion, as monitored by LC3 I to LC3 II conversion (Fig. 1D-F) and by quantitation of the percentage of autophagic cells, was similar to that seen following depletion of Beclin 1, an essential component of the autophagic machinery (Fig. 1D-F and Suppl. Fig. 1B). Together these results suggest strongly that PC2 is necessary for rapamycin- and starvation-induced autophagy in primary cultured cardiomyocytes.

Increased autophagic flux, defined as the process of autophagosome formation and ultimate fusion with a lysosome, leads to the degradation of both SQSTM1/p62 and LC3 II, while its inhibition promotes the accumulation of both proteins [18]. To determine whether PC2 governs autophagic flux, we exposed primary cultured cardiomyocytes to bafilomycin A1 (BafA1), which suppresses autophagosome-lysosome fusion. Cultured cardiomyocytes were transfected with unrelated (UNR) or PC2 siRNA and then exposed to either rapamycin or nutrient deprivation in the presence or absence of BafA1 (50 nM). As expected, treatment with BafA1 promoted LC3 II and SQSTM1/p62 accumulation following exposure to rapamycin or starvation, whereas LC3 II and SQSTM1/p62 accumulation was significantly reduced in PC2-depleted cardiomyocytes (Fig. 1G–J).

Similar findings were observed in other cell types, including HeLa cells, human embryonic kidney (HEK) 293 cells, and a human osteosarcoma (U2OS) cell line (Suppl. Fig. 1C-F), suggesting that PC2 is a conserved regulator of autophagic flux. In each cell type, PC2 was required for rapamycin- and starvation-induced autophagy and activation of autophagic flux as evidenced by the accumulation of LC3 II (Suppl. Fig. 1C-D). Further evidence was obtained using an RFP-GFP-LC3 tandem-fluorescence reporter construct, which localizes to autophagosomes (where red plus green fluorescence yields a yellow signal) and to autolysosomes (where GFP fluorescence is quenched by acidic pH, leading to a red signal) (Suppl. Fig. 1E-F). Furthermore, depletion of PC2 exacerbated cell death at both 12 h and 24 h following nutrient withdrawal in cultured cardiomyocytes and HeLa cells, consistent with the prosurvival effects of autophagy in conditions of starvation stress (Suppl. Fig. 1G-H). Together, these results demonstrate that PC2 is required for activation of autophagy triggered by nutrient withdrawal or pharmacological mTOR inhibition with rapamycin.

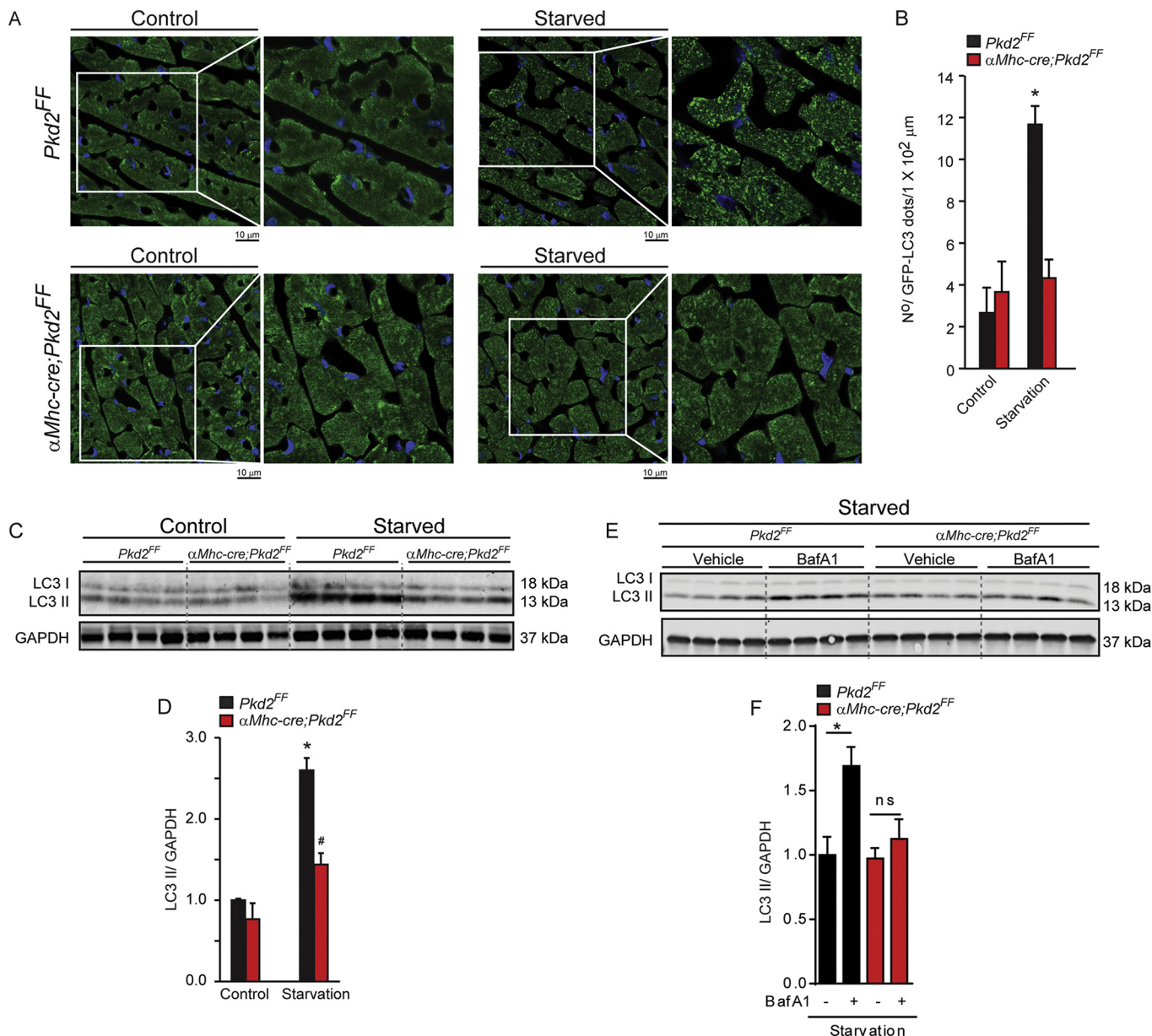
### 3.2. PC2 is required for stress-induced autophagy in vivo

Cardiomyocyte autophagy is required to maintain energy homeostasis in the heart [2,19–21]. Indeed, regulation of autophagic flux participates in the cardiomyocyte metabolic response to changes in demand, including fasting, stress, physical exercise, and disease [20,22]. Given our findings that PC2 regulates nutrient deprivation-induced autophagy in vitro, we evaluated its role in autophagic control in vivo. To this end, the gene coding for PC2 (*Pkd2*) was selectively silenced in cardiomyocytes by crossing mice expressing *Cre* recombinase driven by the  *$\alpha\text{MHC}$*  promoter with mice harboring a “floxed” *Pkd2* allele (*Pkd2<sup>FF</sup>*) [23]. PC2 ablation was confirmed by assessing the recombination between floxed sites and by quantifying PC2 protein levels in isolated adult cardiomyocytes by Western blot (Suppl. Fig. 2A-B). Animals were nutrient deprived for 24 h, which stimulated autophagic vacuole accumulation, as indicated by a punctate pattern of endogenous LC3 [24] (Fig. 2A-B) and by LC3 I to LC3 II conversion in *Pkd2<sup>FF</sup>* mice (Fig. 2C-D). Consistent with our in vitro data, activation of autophagy in cardiomyocytes was significantly reduced in animals



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**Fig. 1.** PC2 is required for starvation- and rapamycin-induced autophagy. Cultured neonatal rat cardiomyocytes were transfected either with an unrelated siRNA (siUNR) or with siRNA specific for PC2 (siPC2) for 48 h, followed by infection with GFP-LC3-expressing adenovirus for 12 h. Subsequently, cultures were treated with rapamycin (1  $\mu$ M) for 3 h. Representative fluorescence microscopy images are shown (A). Nuclei were counterstained with DAPI (10  $\mu$ g/mL). (B, C) Graphics depicting the percentage of cells harboring GFP-LC3 puncta (% of autophagic cells) (B) and the number of GFP-LC3 puncta/cell (C) in cultured cardiomyocytes subjected to starvation or treated with rapamycin (1  $\mu$ M) for 0, 3 and 6 h. (mean  $\pm$  S.E.M., n = 3, \*p < 0.05). (D, E) Cultured cardiomyocytes were transfected with either an unrelated, PC2- or Beclin 1-specific siRNA for 48 h, followed by nutrient deprivation (D) or treatment with rapamycin (1  $\mu$ M) (E). Protein levels of PC2, BCN1 and LC3 were quantified. GAPDH was used as loading control. Images are representative of three independent experiments. (F) illustrates immunoblot quantifications. LC3 II/GAPDH ratios are represented as arbitrary units (A.U.) of cells subjected to starvation or treated with rapamycin (mean  $\pm$  S.E.M., n = 3, \*p < 0.05). (G–J) PC2 regulates autophagic flux. Cultured neonatal rat cardiomyocytes were transfected either with an unrelated siRNA (siUNR) or with siRNA specific for PC2 (siPC2) for 48 h. Alternatively, autophagy was induced either by starvation (G) or rapamycin treatment (1  $\mu$ M) (H) at the indicated times in the presence or absence of bafilomycinA1 (BafA1) (50 nM). Cells were lysed and protein extracts analyzed by immunoblotting for detection of LC3, SQSTM1/p62 and PC2. GAPDH was used as loading control. Images are representative of three independent experiments. Membranes were quantified, and LC3II/GAPDH ratio is depicted in (I) (mean  $\pm$  S.E.M., n = 3, \*p < 0.05). (J) Cultured neonatal rat cardiomyocytes were transfected either with an unrelated siRNA (siUNR) or with siRNA specific for PC2 (siPC2) for 48 h and then infected with GFP-LC3-expressing adenovirus for 12 h. Subsequently, cells were subjected to starvation or treated with rapamycin (1  $\mu$ M) at the indicated times in the presence or absence of bafilomycinA1 (BafA1) (50 nM). Cells were fixed and the percentage of autophagic cells was analyzed by fluorescence microscopy. Percentage of autophagic cells is represented in (J) (mean  $\pm$  S.E.M., n = 3, \*p < 0.05).



**Fig. 2.** PC2 is required for starvation-induced autophagy in vivo. *Pkd2<sup>FF</sup>* and *αMhc-cre;Pkd2<sup>FF</sup>* mice were fed a normal chow diet or food-deprived for 24 h prior to sacrifice. Hearts were harvested and cryosectioned. Then, endogenous LC3 levels were quantified in heart tissues by immunofluorescence. Nuclei were stained with DAPI (10  $\mu$ g/mL) (A). GFP-LC3 dots by area were quantified and values represented in (B). *Pkd2<sup>FF</sup>* and *αMhc-cre;Pkd2<sup>FF</sup>* mice were fed a normal chow diet or fasted for 24 h. Subsequently, hearts were harvested and left ventricular heart proteins extracted and resolved by immunoblotting to detect LC3 (C). GAPDH was used as loading control, and images are representative of three independent experiments. Images depicted in (C) were quantified, and LC3 II/GAPDH ratio is depicted in (D) (mean  $\pm$  S.E.M., n = 12/group, \*p < 0.05) (E). Autophagic flux in both *Pkd2<sup>FF</sup>* and *αMhc-cre;Pkd2<sup>FF</sup>* mice after 18 h starvation plus 2 h treatment with vehicle or BafA1 (1.5 mg/Kg i.p. injection). LC3 II levels were measured by Western blot and normalized to GAPDH (F). (mean  $\pm$  S.E.M. n = 4 mice per group, \*p < 0.05).



lacking PC2 ( *$\alpha$ Mhc-cre;Pkd2<sup>FF</sup>* mice) (Fig. 2A–D). Measurements of autophagic flux (BafA1 1.5 mg/kg, i.p. 2 h prior to sacrifice) revealed no increases in autophagic flux triggered by nutrient withdrawal (Fig. 2E–F) upon PC2 ablation. By contrast, robust activation of autophagic flux was observed in wild-type controls (Fig. 2E–F). Together, these results suggest that PC2 is required to induce autophagic flux in the heart in vivo.

### 3.3. Preserved myocardial function in cardiomyocyte-restricted PC2 knockout mice

Of note, we did not observe suppression of basal autophagic activity in cells depleted of PC2 (Fig. 1A–F) but rather robust suppression of stress-induced autophagy. This led us to evaluate ventricular size, structure, and contractile performance. We were further motivated by seemingly conflicting reports in the literature, where heterozygous, global PC2 knockout mice are reported to manifest normal contractile function [16], whereas dilated cardiomyopathy was observed in a zebrafish PC2 knockout model [17].

To evaluate this, we measured ventricular contractile performance in both *Pkd2<sup>FF</sup>* and  *$\alpha$ Mhc-cre;Pkd2<sup>FF</sup>* mice. Echocardiographic measures of left ventricular structure, as well as systolic and diastolic function, were evaluated in both unsedated (systolic parameters) and gently sedated (diastolic parameters) mice. No differences in ventricular chamber size, ejection fraction, stroke volume or cardiac output were observed between *Pkd2<sup>FF</sup>* and  *$\alpha$ Mhc-cre;Pkd2<sup>FF</sup>* mice (Suppl. Tables 1–2). Similarly, analysis of diastolic function revealed no significant differences in left ventricular compliance or relaxation (Suppl. Table 2). Representative echocardiographic recordings from unsedated mice, as well as pulse wave and tissue Doppler recordings from sedated animals are shown (Suppl. Fig. 3A–B). We observed similar findings by

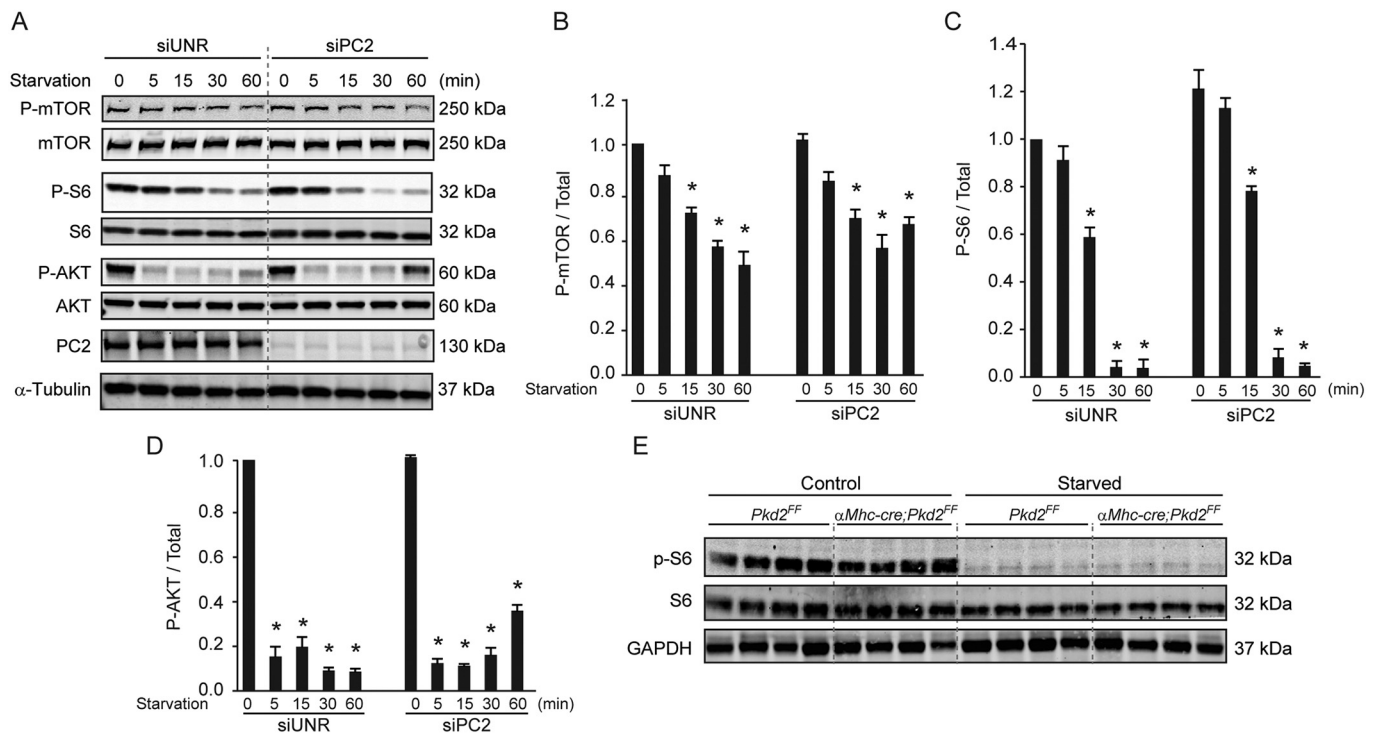
measuring sarcomeric length in isolated adult cardiomyocytes, again detecting no differences between *Pkd2<sup>FF</sup>* and  *$\alpha$ Mhc-cre;Pkd2<sup>FF</sup>* genotypes (Suppl. Fig. 4).

### 3.4. PC2-dependent autophagic control does not involve the mTORC1/AKT pathway or changes in *Atg* transcript abundance

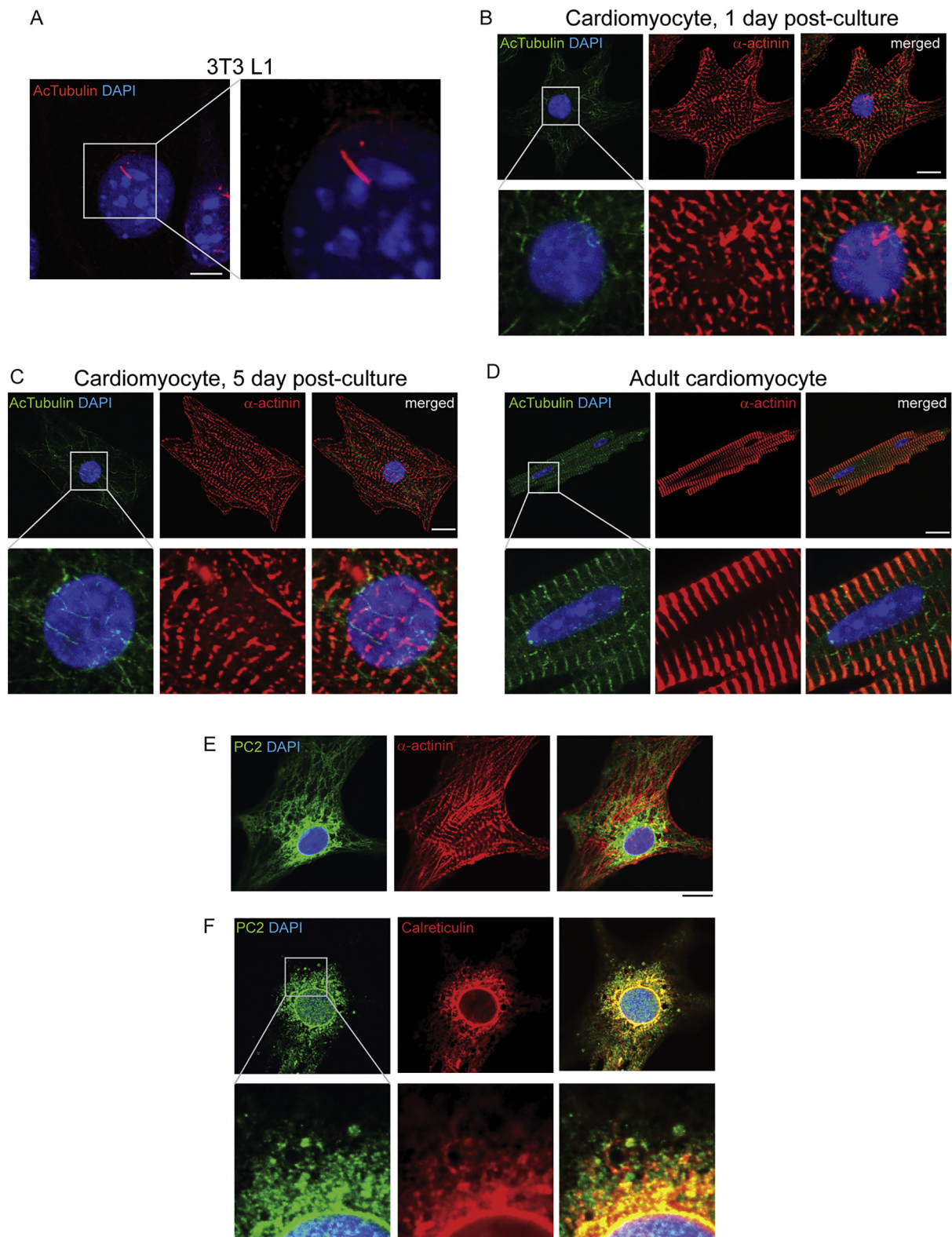
To begin to define mechanisms underlying PC2-dependent control of autophagic activation, we evaluated whether phosphorylation of mTORC1, a master regulator of autophagy, is modulated by PC2. As noted, studies in the kidney have shown that mutations in *Pkd2* are associated with a robust increase in mTORC1 activity which is held to participate in ADPKD disease pathogenesis [6].

As expected, nutrient withdrawal inactivated the mTORC1 pathway, as evaluated by levels of phospho-mTOR and of phosphorylated ribosomal protein S6, both in cultured cardiomyocytes (Fig. 3A–C) and in the heart in vivo (Fig. 3E). As AKT has been implicated in autophagy regulation via signaling pathways that converge on mTORC [25], we also evaluated AKT activity, observing no change as assessed by phospho-AKT (Fig. 3A,D).

Moreover, studies have demonstrated that autophagic activity is governed by the abundance of several essential *Atg* gene products [26,27]. To test this, we evaluated the abundance of several *Atg* transcripts, detecting no significant differences in the levels of *Beclin 1* (*Atg 6*), *Atg3*, *Atg5*, *Atg8*, *Atg12*, *Atg10*, *Ulk1* (*Atg1*) and *Gabarap* (*Atg8*) mRNA, comparing control conditions with PC2-depleted conditions (Suppl. Fig. 5A–I). Collectively, these results suggest that PC2-dependent control of autophagic activity does not involve the canonical mTORC1/AKT signaling pathway or modulation of *Atg* gene expression.



**Fig. 3.** PC2 does not regulate autophagy by modulation of mTORC1, AKT pathways or *atg*-gene expression. Neonatal cardiomyocyte cultures were transfected either with siRNA specific to PC2 (siPC2) or an unrelated siRNA (siUNR) for 48 h. Subsequently, cells were subjected to starvation for 5, 15, 30 and 60 min and whole protein extracts were isolated. mTORC1 and AKT pathways were evaluated by immunoblot of P-mTOR, P-S6 (A–C) and P-AKT (A, D). Total levels of mTOR, AKT and  $\alpha$ -tubulin were used as loading control. PC2 levels (A) were assessed to validate siPC2 efficacy. Images are representative of three independent experiments. Membranes shown in (A) were quantified and depicted in (B–E) (mean  $\pm$  S.E.M.,  $n = 3$ ,  $*p < 0.05$ ). (F) *Pkd2<sup>FF</sup>* and  *$\alpha$ Mhc-cre;Pkd2<sup>FF</sup>* mice were fed a normal chow diet or food-deprived for 24 h. Subsequently, hearts were harvested and left ventricular heart proteins extracted and resolved by immunoblotting to detect S6 phosphorylation. Total S6 and GAPDH were used as loading control. Images are representative of three independent experiments.



**Fig. 4.** Cardiomyocytes are non-ciliated cells, and PC2 localizes to the endoplasmic reticulum in cardiomyocytes. (A) Pre-adipocyte 3T3L1 cultures were fixed and cilia were stained with a specific antibody against acetylated tubulin (red). DAPI (10 µg/mL) was used for nuclei staining. Scale bar: 5 µm. (B, C) Neonatal cardiomyocytes at 1 and 5 days post-culture and adult rat cardiomyocyte cultures (D) were fixed and co-stained with specific antibodies against acetylated tubulin and α-actinin to evaluate the presence of cilia in cardiomyocytes. Nuclei were stained with DAPI (10 µg/mL). Scale bar: 10 µm. Images are representative of three independent experiments. PC2 localizes to the ER (E, F). Neonatal cardiomyocytes were fixed and stained with antibodies specific for PC2 (E, F) or α-actinin (E). ER and nuclei were stained with calreticulin (E) and DAPI (10 µg/mL) respectively, scale bar: 10 µm. Images are representative of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 3.5. PC2 localizes to the endoplasmic reticulum (ER) in cardiomyocytes

PC2 subcellular localization is debated, reported to be localized to the plasma membrane, ER, and primary cilia [28,29]. Also, the subcellular localization of PC2 in cardiomyocytes has never been elucidated. Given that primary cilia have been described as a cellular organelle in which PC2 can be located [30], we tested for the presence of a primary cilium in cultures of neonatal (1 and 5 days post plating) and adult cardiomyocytes. While primary cilia were identified in the 3T3L1 cell line, serving as a positive control (Fig. 4A), they were absent either in neonatal rat or adult mouse cardiomyocytes, as assessed by immunostaining for acetylated tubulin, a specific marker within the ciliary structure [31,32] (Fig. 4B–D). However, despite the absence of cilia, PC2 is unequivocally expressed in neonatal cardiomyocytes in a lattice-work pattern (Fig. 4E). As previous studies have reported that PC2 localizes to the ER [6,33], we evaluated whether this occurs in neonatal cardiomyocytes by co-staining for the ER-specific protein calreticulin. These studies revealed significant overlap and co-localization (Fig. 4F), suggesting PC2 functions within the ER membrane and the perinuclear membrane from which it serves to control autophagy.

Furthermore, we determined the localization of PC2 in adult cardiomyocytes. PC2 immunostaining manifested a reticular pattern, similar to the established distribution of SR membranes, with only modest colocalization with a RyR2 marker (Suppl. Fig. 6A–B). Moreover, the distribution of PC2 emerged as parallel line doublets around the z-line which were co-labeled with  $\alpha$ -actinin (Suppl. Fig. 6C). To corroborate our immunostaining specificity, we repeated this staining in the presence of PC2 control antigen, which completely neutralized the PC2 staining (Suppl. Fig. 6D).

### 3.6. Intracellular $Ca^{2+}$ is required to trigger autophagy

PC2 is an ER  $Ca^{2+}$  channel that modulates intracellular  $Ca^{2+}$  in cardiomyocytes and other cell types [2,11,29]. In light of this, we hypothesized that PC2-dependent control of autophagic activity involves changes in  $Ca^{2+}$  homeostasis. Specifically, we tested whether alterations in intracellular  $Ca^{2+}$  are required to induce autophagy following mTOR suppression by rapamycin or starvation. We observed that chelation of intracellular  $Ca^{2+}$  (BAPTA-AM) significantly reduced stress-induced autophagy, as assessed by quantification of autophagic cells (Fig. 5A–D) and LC3 I to LC3 II conversion in the presence/absence of BafA1 (Fig. 5E–G). Conversely, depletion of extracellular  $Ca^{2+}$  using EGTA, a non-cell-permeable chelating agent, did not affect rapamycin- or starvation-induced autophagy, suggesting that intracellular but not extracellular  $Ca^{2+}$  is required for stress-induced autophagy in cultured cardiomyocytes and HeLa cells (Fig. 5A–H).

Next, we evaluated whether a massive  $Ca^{2+}$  release elicited by caffeine (RyR agonist) has an impact on autophagic flux. NRVM were stimulated with caffeine (20 mM) for 2 h in the presence or absence of 50 nM BafA1 to measure autophagic flux. Interestingly, caffeine treatment completely blocked autophagic flux in NRVM (Suppl. Fig. 7). These results suggest wholesale increases in cytosolic  $Ca^{2+}$  induced by caffeine are not sufficient to activate autophagy.

### 3.7. PC2 $Ca^{2+}$ channel function is required to trigger autophagy

To test for a link between PC2-modulated intracellular  $Ca^{2+}$  handling and autophagy, we assessed the effect of PC2 over-expression on autophagic control. Adenoviral-mediated PC2 over-expression stimulated autophagy in a dose-dependent manner in both cultured cardiomyocytes and in HeLa cells, as assessed by increased numbers of autophagic cells and by the number of autophagic vacuoles per cell (Fig. 6A–F). PC2 over-expression also promoted LC3 I to LC3 II conversion in cardiomyocytes (Fig. 6G). Importantly, these effects were abolished in cells expressing a  $Ca^{2+}$  channel activity-deficient PC2 mutant harboring a single amino acid substitution (D509V) in the third

membrane-spanning domain [34]. Adenoviral over-expression of the D509V PC2 mutant did not stimulate autophagy in cultured cardiomyocytes or in HeLa cells (Fig. 6A–F,H), indicating that PC2 requires a functional  $Ca^{2+}$  channel to govern autophagy. Consistent with this, PC2 over-expression-mediated autophagy was inhibited significantly in cardiomyocytes pretreated with BAPTA-AM, indicating that intracellular  $Ca^{2+}$  is required in PC2-dependent autophagic control (Fig. 6A–F). In aggregate, these data suggest strongly that alterations in intracellular  $Ca^{2+}$ , occurring by means of PC2 ion channel function, participate in PC2-dependent governance of autophagic activity.

### 3.8. PC2 modulates $Ca^{2+}$ cycling in cardiomyocytes

Organism-wide silencing of PC2 leads to alterations in  $Ca^{2+}$ -handling in both embryonic and adult cardiomyocytes [11,16]. In light of this, we aimed to characterize  $Ca^{2+}$ -handling in adult cardiomyocytes isolated from our cardiomyocyte-restricted PC2 knockout mice.  $Ca^{2+}$  transients measured with Fluo-8 ( $K_d$  389 nM) and elicited by field electrical stimulation at 0.5 Hz were significantly reduced in adult cardiomyocytes isolated from  $\alpha Mhc$ -cre;*Pkd2*<sup>FF</sup> mice compared to *Pkd2*<sup>FF</sup> (Fig. 7A–D). Sarcoplasmic reticulum (SR)  $Ca^{2+}$  stores were evaluated by caffeine challenge (10 mM). We observed that the caffeine-induced cytosolic  $Ca^{2+}$  transient measured with Fluo-8 was significantly reduced in PC2 KO cardiomyocytes, indicative of partial SR  $Ca^{2+}$  store depletion (Fig. 7E–F). Similar results were observed using an SR  $Ca^{2+}$  loading indicator (Fluo-5N) which revealed significant signal reduction following caffeine challenge in mutant cardiomyocytes (Fig. 7G–H).

Moreover, we studied the levels of calsequestrin-2 (CASQ2), a major  $Ca^{2+}$  buffering protein, in isolated adult cardiomyocytes, detecting no difference between *Pkd2*<sup>FF</sup> and  $\alpha Mhc$ -cre;*Pkd2*<sup>FF</sup> mice (Suppl. Fig. 8A–B). In addition, levels of the ER proteins BiP/GRP78 and calreticulin were similar in cardiomyocytes depleted of PC2 as compared with control cells (Suppl. Fig. 8C–E). Collectively, these data suggest that PC2 silencing induces SR  $Ca^{2+}$  store depletion and that ER-localized PC2 modulates  $Ca^{2+}$  handling in cardiomyocytes with consequent autophagic control.

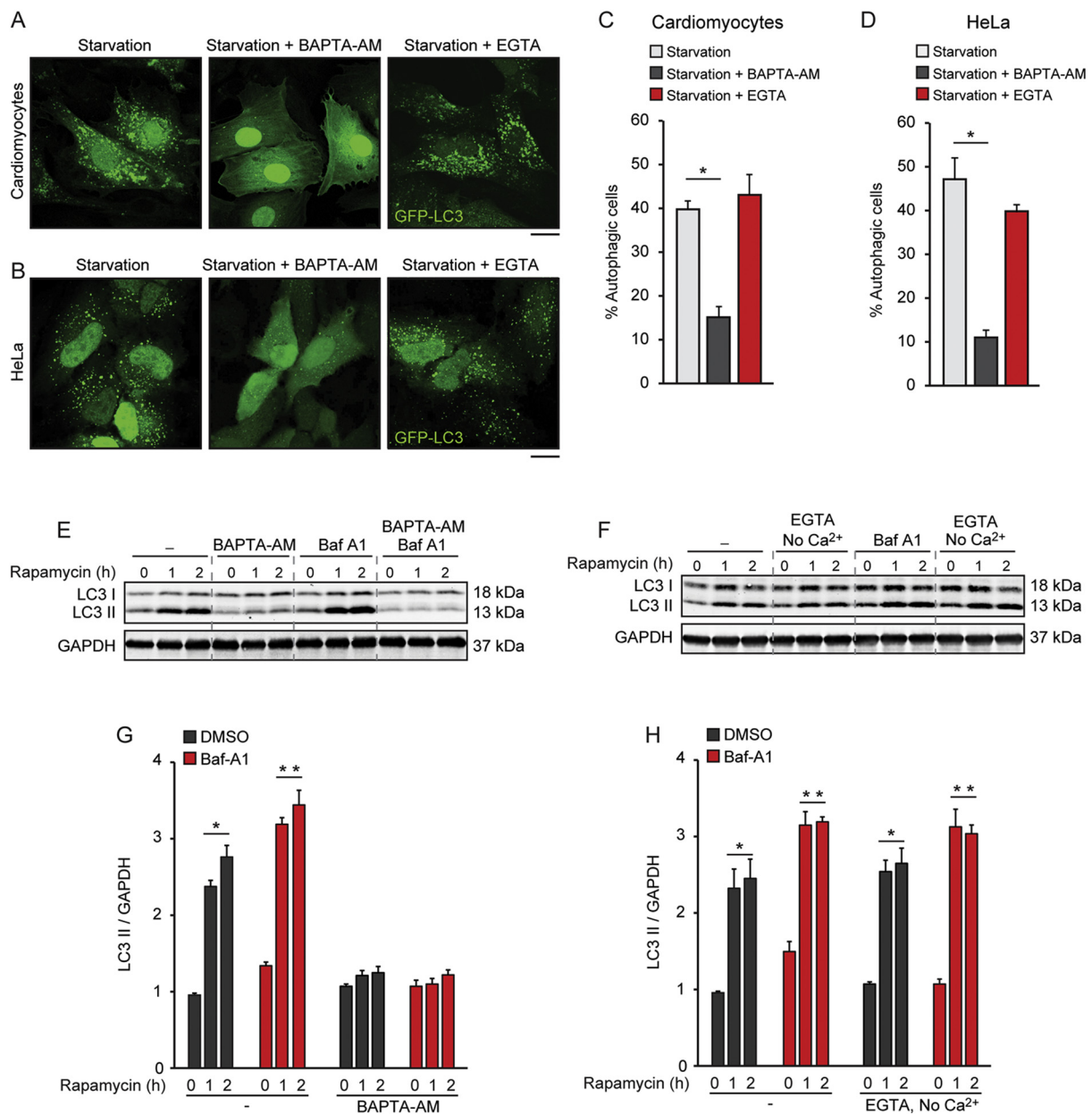
## 4. Discussion

Autophagy is a highly conserved process of cellular catabolism, recycling, and organelle turnover. Its maintenance at basal levels of activation is required for cell survival. Further, levels of autophagic flux vary tremendously in the setting of cellular stress, participating in many instances in disease pathogenesis. As a result, there is great interest in unveiling mechanisms that govern autophagic activation in hopes of titrating it for therapeutic benefit.

Here, we set out to test for a role of PC2 in the governance of cardiomyocyte autophagy. We report that PC2 is required for activation of autophagic flux in a wide range of cell types. In contrast, PC2 does not appear to play a major role in regulating basal levels of autophagy. Consistent with that observation, mice that harbor cardiomyocyte-restricted silencing of PC2 manifest normal cardiac structure and contractile performance. We report that PC2-dependent control of autophagy does not involve changes in the activation of mTOR or AKT or alterations in the abundance of *atg* transcripts. As PC2 functions as a  $Ca^{2+}$ -permeable ion channel, we tested for a role of PC2-dependent alterations in intracellular  $Ca^{2+}$  handling. In aggregate, our findings point to PC2 as a novel regulator of autophagy acting through ion channel-dependent control of intracellular  $Ca^{2+}$  homeostasis.

Recently, Lu et al. reported that PC2 is required for glucose starvation-induced autophagy in human embryonic stem cell-derived cardiomyocytes (hESC-CM) [35]. Depletion of PC2 blunted glucose starvation-induced autophagy, whereas PC2 overexpression had the opposite effect. This report also showed that RyR2 interacts with PC2 in hESC-CM and that PC2 knockdown reduced caffeine-sensitive SR stores,





**Fig. 5.** Intracellular Ca<sup>2+</sup> is required to trigger autophagy. Representative images of neonatal cardiomyocytes (A) and HeLa cells (B) transfected with GFP-LC3 and serum starved for 6 h in the presence or absence of BAPTA-AM (20 μM) or EGTA (100 μM). Nuclei were stained with DAPI (10 μg/mL). Scale bar: 10 μm. GFP-LC3 dots were analyzed by fluorescence microscopy and the % of autophagic cells quantified in (C-D) (mean ± S.E.M., n = 3, \*p < 0.05). Neonatal cardiomyocytes (E, G) were treated with rapamycin (1 μM) for 0, 1 and 2 h with or without BAPTA-AM (20 μM) in the presence or absence of bafilomycin A1 (BafA1) (50 nM). In (F), cells were treated with rapamycin as in (G), but maintained in standard medium (with CaCl<sub>2</sub>) or Ca<sup>2+</sup> free medium (without CaCl<sub>2</sub>) supplemented with EGTA (100 μM) either with or without Baf A1 (50 nM). Cells were lysed, and proteins analyzed by immunoblot to evaluate LC3 I to LC3 II conversion. GAPDH was used as loading control (E, F). Images shown in (E) and (F) are representative of three independent experiments. LC3 II levels were quantified using GAPDH as loading control. Quantifications are depicted in (G) and (H) (mean ± S.E.M., n = 3, \*p < 0.05).

findings similar to previously reported evidence in embryonic mouse cardiomyocytes derived from whole-body PC2 KO mice [11]. Our data demonstrate that PC2 ablation disrupts autophagic flux induced by either starvation or rapamycin. These observations were confirmed in vivo by measuring autophagic flux in PC2 cKO mice. Thus, our findings suggest that PC2 is an essential component in the control of cardiomyocyte autophagy, thereby confirming and extending findings reported by Lu et al. [35].

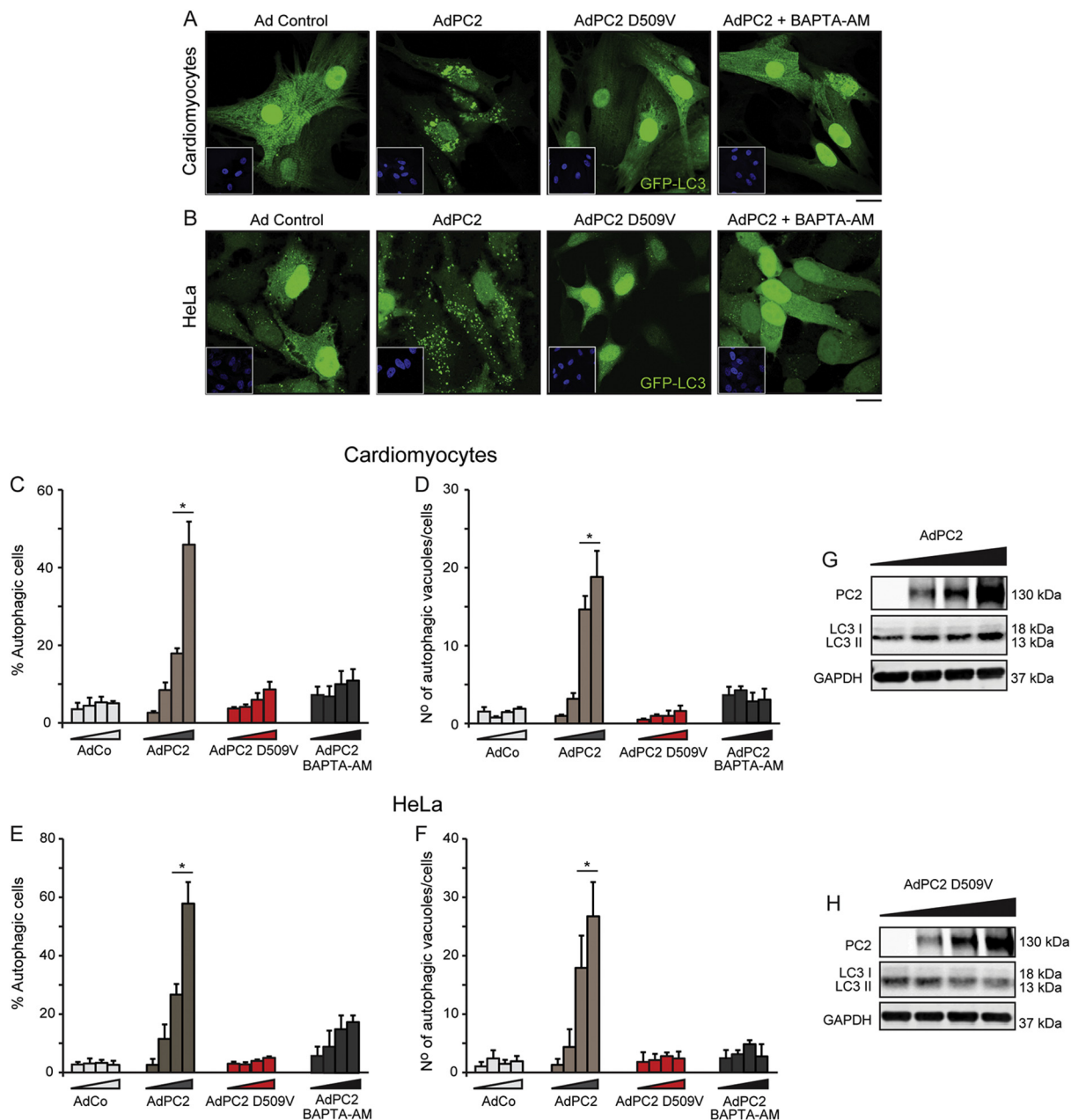
In the present study, we provide new insight into the control of autophagic flux in cardiomyocytes via PC2. Previous evidence has shown that Ca<sup>2+</sup> chelation impairs autophagic flux [36], and we have shown that intracellular Ca<sup>2+</sup> is a key regulator in both starvation- and rapamycin-induced autophagy. Additionally, we demonstrate that PC2 overexpression is sufficient to induce autophagy in a manner dependent

on both its Ca<sup>2+</sup> channel activity and the presence of intracellular Ca<sup>2+</sup>. Together, these results suggest a specific role for PC2 in regulating autophagy in cardiomyocytes via its Ca<sup>2+</sup>-conducting properties.

#### 4.1. Modulation of autophagic activity

Autophagy is characterized by the formation of double membrane structures called autophagosomes that engulf cytosolic cargo, such as proteins, organelles, and lipid droplets. Autophagosomes fuse with lysosomes forming autophagolysosomes where the luminal content is degraded by lysosomal enzymes. Cargo degradation generates new essential metabolites that are distributed to the cytosol for reuse. Several essential autophagy-related proteins (ATG) have been identified and





**Fig. 6.** PC2  $Ca^{2+}$  channel function is required to trigger autophagy. Cardiomyocytes (A) and HeLa (B) cells were co-transfected with GFP-LC3 plus PC2-expressing or PC2 D509V-expressing adenoviruses for 24 h. Cells co-transfected with PC2 were also exposed to BAPTA-AM (20  $\mu$ M) for 6 h. Empty adenovirus (AdCMV) was used as control. Cells were fixed, and autophagy evaluated by fluorescence microscopy. Nuclei were stained with DAPI (10  $\mu$ g/mL) and representative images are shown. Scale bar: 10  $\mu$ m. The percentage of autophagic cells and the number of autophagic vacuoles per cell observed in (A) and (B) are depicted in the graphics in (C–D, E–F), respectively (mean  $\pm$  S.E.M., n = 3, \*p < 0.05). Cardiomyocyte (G) and HeLa cell (H) cultures were transfected with adenoviruses expressing PC2 WT or PC2 D509V in the presence and absence of BafA1 (50 nM) for 24 h. Cells were lysed, and protein analyzed by immunoblot to detect PC2 and LC3 I to II conversion. GAPDH was used as loading control. Results are representative of three independent experiments.

organized into functional complexes that participate in the formation of autophagosomes [37].

Autophagy is a vital mechanism of degradation of proteins and organelles in eukaryotic cells [1]. Autophagy is not only central to the control of proteostasis, serving to maintain cellular and tissue health, but it has also been widely implicated in the development of several pathological conditions, including cardiovascular diseases [2,3,38]. Although mechanisms regulating autophagy have been partially elucidated, important steps toward detailed understanding of this process are needed.

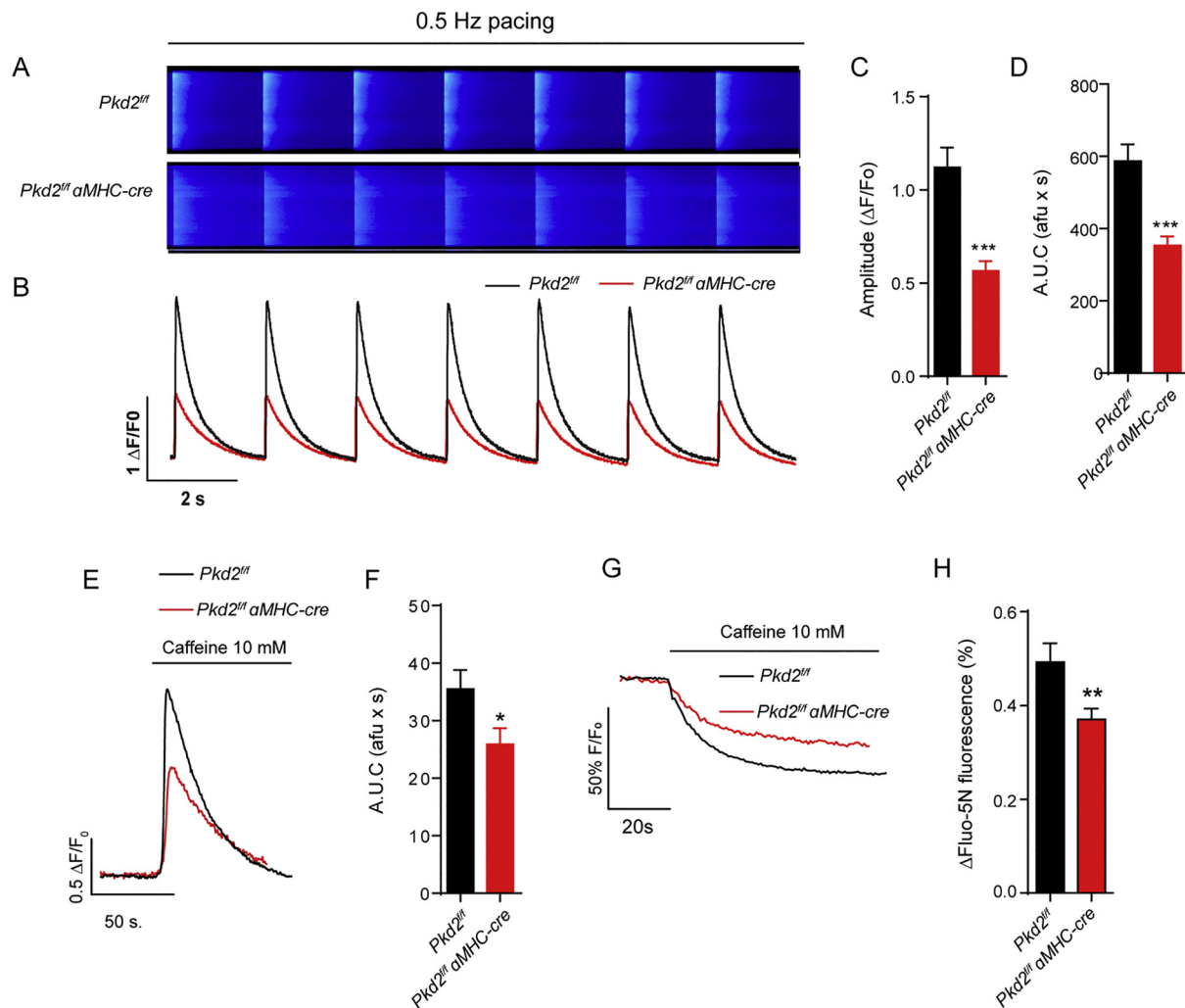
It is of particular interest to identify means of modulating stress-induced changes in autophagic activity without squelching basal levels, as the latter is cytotoxic [38]. Findings reported here pointing to a role

of PC2 in governing stress-induced autophagy without significant change in basal autophagic flux are a step toward this goal.

#### 4.2. $Ca^{2+}$ -dependent control of autophagy

The mTORC1 and AKT signaling axes are widely recognized regulators of the autophagic pathway, and their activation is generally associated with autophagy inhibition [5,39]. Findings reported here demonstrate that PC2-dependent control of autophagy does not involve either pathway, nor does it involve changes in Atg levels [40]. Rather, PC2-dependent control of autophagy occurs downstream of the mTOR pathway.

As noted, our findings point to a novel role of intracellular  $Ca^{2+}$



**Fig. 7.** PC2 modulates  $\text{Ca}^{2+}$  cycling and promotes partial SR  $\text{Ca}^{2+}$  store depletion. Adult cardiomyocytes were paced at 0.5 Hz, and Fluo-8 fluorescence was measured by line scan and confocal microscopy (A). Representative  $\text{Ca}^{2+}$  transients elicited by electrical stimulation in  $\text{Pkd2}^{\text{fl/fl}}$  and  $\text{aMhc-cre};\text{Pkd2}^{\text{fl/fl}}$  cardiomyocytes (B). Quantification of amplitude and area under the curve of fluorescence transients (C–D). Cytosolic  $\text{Ca}^{2+}$  transients elicited by 10 mM caffeine in adult cardiomyocytes (E–F). SR  $\text{Ca}^{2+}$  levels were measured using Fluo5N and caffeine challenge (G–H). Data are expressed as mean  $\pm$  SEM from 3 to 4 different cultures.

handling in the regulation of autophagy. Previous studies in a variety of cell types have shown that cytosolic  $\text{Ca}^{2+}$  is required to activate gene transcription to enhance autophagy [41]. For example,  $\text{Ca}^{2+}$  influx increases autophagy through Beclin 1 and Atg5 in HEK-293 T cells exposed to  $\text{Ca}^{2+}$  phosphate transfection [42]. Recently, it has been proposed that TRPM7 (transient receptor potential melastatin 7) governs basal autophagy, suggesting that  $\text{Ca}^{2+}$  entry regulates this process [43]. On the other hand, evidence suggests that modulation of intracellular  $\text{Ca}^{2+}$  is necessary for autophagy to occur [44].

Consistent with our data, prior studies have shown that agents that increase cytosolic  $\text{Ca}^{2+}$  by ER-mediated  $\text{Ca}^{2+}$  release, such as ionomycin, ATP, and vitamin  $\text{D}_3$  analogs are potent inducers of autophagy, and chelation of intracellular  $\text{Ca}^{2+}$  suppresses autophagic activation [13,14]. Our findings, in both excitable and nonexcitable cells, are consistent with this and extend these data by demonstrating that PC2  $\text{Ca}^{2+}$  channel activity is required in this novel pathway of autophagic control.

#### 4.3. PC2 localization and function

PC2, also known as TRPP2 (transient receptor potential polycystin 2), belongs to the transient receptor potential (TRP) family of ion channels [45]. PC2 has six transmembrane domains, a pore-forming region, a ZF domain for  $\text{Ca}^{2+}$  binding, and cytoplasmic N- and C-

terminal tails [45–47]. Its subcellular localization remains controversial [28]. Whereas some studies point to localization of PC2 in the membrane of the ER, others provide evidence for its trafficking to the plasma membrane and to primary cilia [29]. However, despite these differing reports, there is widespread agreement that PC2 functions as a  $\text{Ca}^{2+}$ -permeable ion channel, the activity of which can be modulated by interaction with other proteins and which can generate  $\text{Ca}^{2+}$  microdomains within the cell [11,48]. Indeed, fluid flow-mediated mechanical stimulation of primary cilia in kidney cells is held to elicit PC2  $\text{Ca}^{2+}$  channel activation, allowing  $\text{Ca}^{2+}$  entry from the extracellular space into the cytoplasm, a response which is necessary to maintain cellular homeostasis in the kidney [45]. Of note, this observation was called into question by a recent report which provided strong evidence that primary cilia are not mechanosensors [49]. We report here that neither neonatal nor adult cardiomyocytes harbor primary cilia and that in both cell types, PC2 is localized to the ER, where it regulates  $\text{Ca}^{2+}$  flux from the ER to the cytoplasm. Thus, PC2-dependent governance of cardiomyocyte autophagy is cilia-independent.

#### 4.4. Cardiomyocyte-specific PC2 knockout

This is the first report documenting PC2-dependent control of cardiomyocyte  $\text{Ca}^{2+}$  handling and contractility, and ventricular contractile performance. Our data are consistent with previous studies

obtained in embryonic cardiomyocytes from homozygous “full body” PC2 knockout mice and in zebrafish models showing smaller  $\text{Ca}^{2+}$  transients in cardiomyocytes lacking PC2 [11,17]. We report that adult cardiomyocytes isolated from cardiomyocyte-restricted PC2 KO mice elicit reduced  $\text{Ca}^{2+}$  transients triggered by membrane depolarization due to partially depleted SR  $\text{Ca}^{2+}$  load, similar to previous reports [11].

PC2 and RyR2 can physically interact, and PC2 can negatively regulate the RyR2 open state [11]. We have observed increased spark frequencies (spontaneous  $\text{Ca}^{2+}$  release events mediated by RyR2 opening) in PC2-deficient cardiomyocytes, which may underlie, at least in part, depletion of SR  $\text{Ca}^{2+}$  stores (data not shown). At the present time, the relative contribution of RyR2-mediated  $\text{Ca}^{2+}$  leak versus PC2 function as a  $\text{Ca}^{2+}$  channel in these events is unknown, but caffeine experiments (RyR agonist) suggest that  $\text{Ca}^{2+}$  release via RyR2 itself is insufficient to increase flux.

A link between  $\text{Ca}^{2+}$  and autophagy was discovered using the  $\text{Ca}^{2+}$  phosphate method for transfection in HEK293-T.  $\text{Ca}^{2+}$  influx increases autophagy through Beclin 1 and Atg5 [42]. In addition, increased  $\text{Ca}^{2+}$  elicited by several stimuli (ionomycin, ATP, thapsigargin) is associated with increased  $\text{Ca}^{2+}$ /calmodulin kinase-beta and AMP-activated protein kinase and mTOR inhibition in MCF-7 cells [14]. Thus, it seems that  $\text{Ca}^{2+}$  can have a dual effect on autophagy [50]. For example, Decuyper et al. showed that rapamycin increases ER  $\text{Ca}^{2+}$  content, decreases ER  $\text{Ca}^{2+}$  leak, and increases  $\text{Ca}^{2+}$  release through  $\text{IP}_3\text{R}$  receptors [36]. Moreover, these investigators demonstrated that BAPTA blunted rapamycin-induced autophagy. Altogether, these data suggest that intracellular  $\text{Ca}^{2+}$  is required for mTOR-dependent autophagic flux.

Although  $\text{Ca}^{2+}$  cycling is impaired in PC2-deficient cardiomyocytes, no alterations in contractility or heart function were observed in adult mice. This is consistent with our observations of no change in basal levels of autophagic flux. These data, collected in mice of 2–4 months of age, agree with a recently published study showing that “whole body” PC2<sup>+/-</sup> mice manifest defects in heart function only after 9 months of age [51]. Moreover, this study reported alterations in  $\text{Ca}^{2+}$ -contractile proteins, with increased RyR2 levels and decreased SERCA2a and phospho-PLB levels [51].

#### 4.5. Summary and perspective

In summary, the present report uncovers a novel mechanism of cellular control of autophagy, one which is driven by PC2 and its governance of intracellular  $\text{Ca}^{2+}$  homeostasis. This novel cascade functions independently of mTORC1, AKT or Atg transcript levels. Rather, our findings point to a novel cascade of intracellular ion channel-dependent control of  $\text{Ca}^{2+}$  homeostasis in the regulation of stress-induced, but not resting, autophagic flux.

#### Disclosures

None.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yjmcc.2018.03.002>.

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