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Nuclear Localization Drives α 1-Adrenergic Receptor Oligomerization and Signaling in Cardiac Myocytes

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Abstract

Conventional models of G-protein coupled receptor (GPCR) signaling describe cell surface receptors binding to external ligands, such as hormones or circulating peptides, to induce intracellular signaling and a physiologic response. However, recent studies identify new paradigms indicating that GPCRs localize to and signal at the nucleus and that GPCRs oligomers can influence receptor function. Previously, we reported that endogenous α 1-adrenergic receptors (α 1-ARs) localize to and signal at the nuclei in adult cardiac myocytes. In this study, we examined the mechanisms behind α 1-AR nuclear localization and how nuclear localization impacted receptor function. We verified that endogenous α 1-ARs localized to the nuclear membrane of intact nuclei isolated from wild-type adult cardiac myocytes. Next, we identified and disrupted putative nuclear localization sequences in both the α 1A- and α 1B-adrenergic receptors, which led to mis-localization of α 1-ARs in cultured adult cardiac myocytes. Using these mutants, we demonstrated that nuclear localization was required for α 1-signaling in adult cardiac myocytes. We also found that the nuclear export inhibitor leptomycin B inhibited α 1-AR signaling, indicating α 1-AR signaling must arise in the nucleus in adult cardiac myocytes. Finally, we found that co-localization of the α 1-subtypes at the nuclei in adult cardiac myocytes facilitated the formation of receptor oligomers that could affect receptor signaling. In summary, our data indicate that α 1-AR nuclear localization can drive the formation of receptor oligomers and regulate signaling in adult cardiac myocytes.

Keywords

α 1-adrenergic receptor; G protein-coupled receptor; cardiac myocyte; oligomer; ERK

1. INTRODUCTION

Conventional models of G-protein coupled receptor (GPCR) signaling describe receptor activation at the plasma membrane leading to initiation of downstream signaling within the cell commonly referred to as “outside-in” signaling. However, it has recently become clear that a number of GPCRs localize to and signal at the nuclear membrane in a variety of cell

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types including neurons, hepatocytes, and cardiac myocytes (reviews [1–3]). Endothelin receptors, angiotensin receptors, and β -adrenergic receptors (β -ARs) were all recently reported to localize to the nuclei in adult cardiac myocytes (cardiac myocytes in most species have two nuclei) [4–7]. However, each of these receptors also localizes to the plasma membrane, therefore the functional significance of localization to the nuclei in adult cardiac myocytes remains unclear. Yet, studies using nuclei isolated from adult cardiac myocytes indicated that endothelin receptors induced calcium transients [4], and β -ARs and angiotensin receptors increased gene transcription, suggesting a potential physiologic significance for nuclear GPCR signaling [6, 7].

Targeting of proteins to the nucleus involves nuclear localization sequences embedded in the protein, which consist of mono- or bi-partite basic residues, usually lysines and arginines, or glycine-arginine repeats [8–10]. A family of proteins called importins binds to nuclear localization sequences and facilitates localization to the nucleus. This importin-mediated nuclear localization occurs for proteins that target to the nucleoplasm as well as the inner nuclear membrane [11–13]. This method of nuclear localization was previously described for the type 1 parathyroid hormone receptor [14, 15] and more recently for the gonadotropin-releasing hormone type 1 receptor [16].

Another newly described function of GPCRs is the ability to form oligomers, and receptor hetero-oligomers have been proposed to affect receptor ligand binding, expression, internalization, and signaling. To date, several GPCRs have been shown to form homo- and hetero-oligomers that alter receptor function, including opioid receptors, dopamine receptors, adenosine receptors, angiotensin receptors, and β -ARs (reviews [17–20]). In HEK293 cells, α 1-adrenergic receptors (α 1-ARs) formed homo- and hetero-oligomers, demonstrating the capacity for α 1-ARs to form oligomers [21–24]. Moreover, α 1A- and α 1B-subtype hetero-oligomers altered receptor internalization [23], and α 1B- and α 1D-subtype hetero-oligomers were required for cell surface expression of the α 1D-subtype in HEK293 cells [22]. In cardiac myocytes, β 1- and β 2-ARs formed hetero-oligomers that altered β -AR mediated contractile function, suggesting the possibility that adrenergic receptor oligomers could affect cardiac myocyte function [25].

In general, the lack of validated α 1-AR subtype-specific antibodies [26] has hindered studies attempting to define α 1-AR subcellular localization. However, we overcame this obstacle by using radio-ligand binding assays on fractionated cardiac myocytes and a fluorescent α 1-AR ligand to label receptors in cultured cardiac myocytes, and we localized endogenous α 1-ARs to the nuclei in adult cardiac myocytes [27]. Further, we demonstrated that the downstream α 1-AR signaling partners G α q and phospholipase C β 1 co-localized with α 1-ARs only at the nuclei in adult cardiac myocytes [27]. We also developed a reconstitution system in α 1A- and α 1B-AR double knockout (α 1ABKO) cardiac myocytes, which lack endogenous α 1-ARs, using α 1A- and α 1B-GFP fusion proteins that recapitulated the localization of the two endogenous α 1-subtypes at the nuclei [27]. Finally, we found that organic cation transporter-3 (OCT3) facilitated rapid uptake of catecholamines into adult cardiac myocytes and that this uptake was required for α 1-AR signaling [27]. In short, our data indicated that endogenous α 1-ARs localize to and initiate signaling at the nucleus in adult cardiac myocytes.

In this report, we examined α 1-AR expression in nuclei isolated from adult cardiac myocytes and validated that endogenous α 1-ARs localized to the nuclei in adult cardiac myocytes and that our α 1-GFP constructs reproduced this localization. We identified putative nuclear localization sequences in both the α 1A- and α 1B-subtypes and demonstrated that α 1-AR nuclear localization was required for signaling. Furthermore, we demonstrated that co-localization of the α 1A- and α 1B-subtypes at the nuclei in adult

cardiac myocytes facilitated the formation of receptor oligomers that could regulate receptor signaling in adult cardiac myocytes. Together, our data demonstrate that $\alpha 1$ -AR nuclear localization can drive the formation of receptor oligomers and regulate signaling in adult cardiac myocytes.

2. MATERIAL AND METHODS

2.1 Mice

The generation of $\alpha 1A$ - ($\alpha 1AKO$) and $\alpha 1B$ - ($\alpha 1BKO$) AR single knockout, as well as $\alpha 1A$ -/ $\alpha 1B$ - ($\alpha 1ABKO$) AR double knockout mice, was previously described [28]. 12th-15th generation congenic C57BL/6J mice between 10–15 weeks of age were used in all experiments. The use of all animals in this study conformed to the PHS Guide for Care and Use of Laboratory Animals and was approved by Sanford Research/USD Institutional Animal Care and Use Committee.

2.2 Reagents

All reagents were made using chemicals purchased from Sigma-Aldrich (MO, USA) unless otherwise noted.

2.3 Culture of Adult Mouse Cardiac Myocytes

The procedures for the isolation and culture of adult mouse cardiac myocytes were previously described [29, 30].

2.4 Adenoviral Constructs

The generation of adenoviral constructs of the wild-type $\alpha 1A$ - and $\alpha 1B$ -ARs ($\alpha 1A$ -WT, $\alpha 1B$ -WT) with and without GFP have been previously described [31]. The following additional adenoviral constructs were made using the AdEasy System (Agilent, CA, USA). Adenoviral constructs of $\alpha 1A$ -WT and $\alpha 1B$ -WT tagged with CFP were made similarly to the GFP constructs. To generate nuclear localization sequence mutants in $\alpha 1$ -ARs ($\alpha 1$ -NLSmut), we used the $\alpha 1$ -GFP constructs as templates. DNA mini-genes for mutating the putative nuclear localization sequences (Integrated DNA Technologies, IA, USA), flanked by unique FspAI and AscI restriction sites, were synthesized. For the $\alpha 1A$ -subtype, arginines (R) or lysines (K) were replaced by alanine (A) at positions 334, 335, 342, 348, and 349 corresponding to the human sequence. Similarly, the $\alpha 1B$ -NLSmut contains R/A exchanges at positions 368, 370, and 372 through 380 corresponding to the human sequence. All constructs were sequence verified.

2.5 Biochemical Fractionation of Adult Mouse Cardiac Myocytes

The procedures for biochemical fractionation of freshly isolated or cultured cardiac myocytes were described elsewhere [27]. The purity of each fraction was verified by Western blot using primary antibodies against Na⁺/Ca²⁺ exchanger (plasma membrane; Millipore, MA, USA), GAPDH (cytosol; Fitzgerald Industries International, MA, USA), and LAP2 (nuclear membrane marker; BD Biosciences, CA, USA).

2.6 Localization of $\alpha 1$ -ARs by Confocal Microscopy

In all experiments, cultured cardiac myocytes were infected at a multiplicity of infection (MOI) 1000 for $\alpha 1A$ -constructs or at MOI 3000 for $\alpha 1B$ -constructs [31]. This results in approximately 2.5-fold overexpression of the $\alpha 1A$ -constructs and 4-fold overexpression of the $\alpha 1B$ -constructs relative to endogenous $\alpha 1$ -ARs in WT adult mouse cardiac myocytes [31].

To detect endogenous $\alpha 1$ -ARs in nuclei isolated from WT cardiac myocytes, 50 nM BODIPY-Prazosin (Molecular Probes, OR, USA) was added to the nuclei for 15 minutes, counterstained with the DNA marker Hoechst 33342, and mounted on coverslips. To detect $\alpha 1$ -GFP constructs in nuclei isolated from cultured $\alpha 1$ BKO cardiac myocytes, cardiac myocytes were cultured in 150 mm laminin-coated dishes, infected with $\alpha 1$ -GFP constructs as above, cultured for 40 hrs, fractionated as described above, and mounted on coverslips.

To localize $\alpha 1$ -AR constructs in intact cardiac myocytes, $\alpha 1$ AKO, $\alpha 1$ BKO, or $\alpha 1$ ABKO cardiac myocytes were cultured on coverslips, infected with $\alpha 1$ -GFP constructs as above, cultured for 40 hrs, fixed with 4% paraformaldehyde, and mounted on slides with Fluoromount G (Electron Microscopy Sciences, PA, USA). To stain endoplasmic reticulum (ER), 2 μ M ER Tracker (Molecular Probes, OR, USA) in Tyrode's buffer (140 mM NaCl, 10 mM glucose, 10 mM HEPES, 4 mM KCl, 1 mM MgCl₂, pH 7.45) was added one hour prior to capturing images. Where indicated, cardiac myocytes were counterstained with the DNA marker Hoechst 33342.

All fluorescent images were captured by confocal microscopy using Fluoview software (Olympus BX50 confocal microscope, Olympus, NY, USA). Images were processed for publication using Imaris software (Bitplane Scientific Solutions, MN, USA).

2.7 Sensitized Emission Förster Resonance Energy Transfer (FRET)

$\alpha 1$ ABKO cardiac myocytes were cultured on coverslips as above. At plating, cardiac myocytes were infected with adenovirus expressing wild-type $\alpha 1$ B-CFP. After 16 hours, cardiac myocytes were infected with adenovirus expressing wild-type $\alpha 1$ A-GFP. After 24 hours (40 hours total), cardiac myocytes were fixed and mounted on slides for sensitized emission FRET analysis using an Olympus Fluoview 1000 confocal microscope and Olympus Fluoview 1000 software for FRET analysis. Images were collected for analysis on one scan plane where CFP and GFP were excited with the 405 and 488 lasers respectively. Emission detection for CFP was 470–485 nm and GFP 508–525 nm regardless of excitation source (laser or fluorophore). These narrow bands of detection reduced background auto-fluorescence from cardiac myocytes. Images of cardiac myocytes expressing only the donor (CFP) or acceptor (GFP) fluorophore excited by the donor and acceptor laser were incorporated into the sensitized emission FRET analysis to correct for channel crosstalk. Final image brightness was enhanced post-analysis to aid in visualizing the fluorescence in Figure 6A.

2.8 Western Blot Analysis of Phosphorylated ERK

WT, $\alpha 1$ AKO, $\alpha 1$ BKO, or $\alpha 1$ ABKO cardiac myocytes were cultured as described above. Cardiac myocytes were infected with adenovirus expressing $\alpha 1$ A-WT, $\alpha 1$ A-NLSmut, $\alpha 1$ B-WT, $\alpha 1$ B-NLSmut, or β -galactosidase (β gal, control). After 40 hours, cardiac myocytes were treated with 20 μ M phenylephrine (PE) for 15–20 minutes. In some experiments, cardiac myocytes were pre-treated with 18.5 nM leptomycin B for 20 minutes prior to PE treatment. After treatment, medium was aspirated, and lysates were made using ice-cold 1X RIPA buffer (50 mM Tris pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethanesulfonylfluoride, 1 mM Na₃VO₄, 1 mM NaF, 1% Protease Inhibitor Cocktail). Total protein amounts were quantified by bicinchoninic acid protein assay (Pierce, IL, USA). Samples were prepared and resolved as previously described [27, 31, 32]. Blots were probed with primary antibodies for phosphorylated ERK or total ERK followed by a horseradish peroxidase conjugated rabbit secondary antibody (Cell Signaling Technology, MA, USA). Phosphorylated ERK and total ERK levels were detected by enhanced chemiluminescence (GE Healthcare, NJ, USA), and protein levels were quantified using Quantity One software (Bio-Rad, CA, USA).

2.9 Statistical Methods

Two-way ANOVA with Bonferroni post-test was used to determine differences in fold activation of ERK by densitometry. To test for differences in degree of nuclear localization, a repeated measures ANOVA was used to account for the correlation among technicians due to measuring the same animals. There was no interaction between technician and cell line ($p=0.94$).

3. RESULTS

3.1 Endogenous $\alpha 1$ -ARs Localize to the Nuclei in Adult Cardiac Myocytes

Here, we set out to validate and extend our previous result that endogenous $\alpha 1$ -ARs localize to the nuclei in adult cardiac myocytes [27]. Intact nuclei were fractionated from freshly isolated wild-type adult mouse cardiac myocytes by sucrose density gradient ultracentrifugation. Endogenous $\alpha 1$ -ARs were detected on the nuclear membrane with the fluorescently labeled $\alpha 1$ -antagonist BODIPY-prazosin counterstained with the DNA stain Hoescht 33342 to identify nuclei (Figure 1A). We previously constructed $\alpha 1$ -GFP fluorescent fusion proteins for both the $\alpha 1A$ - and $\alpha 1B$ -subtypes, the two $\alpha 1$ -subtypes expressed in cardiac myocytes, and developed a reconstitution system in cultured $\alpha 1ABKO$ cardiac myocytes [27]. Here, the $\alpha 1B$ -GFP was detected on the nuclear membrane in nuclei isolated from cultured $\alpha 1ABKO$ cardiac myocytes expressing the $\alpha 1B$ -GFP (Figure 1B). The nuclear localization of the $\alpha 1B$ -GFP recapitulated the localization of endogenous $\alpha 1$ -ARs and again validated our reconstitution system [27]. In both experiments, the membrane, cytosolic, and nuclear fractions were validated by Western blots for Na^+/Ca^{2+} exchanger (plasma membrane), GAPDH (cytosol), and LAP2 (nuclear membrane) respectively (Figure 1C).

3.2 The $\alpha 1A$ and $\alpha 1B$ -Subtypes Co-localize to the Nuclei in Adult Cardiac Myocytes

Using our reconstitution system in $\alpha 1ABKO$ cardiac myocytes, we demonstrated that when expressed individually, either the $\alpha 1A$ -GFP or $\alpha 1B$ -GFP localized to the nuclei in adult cardiac myocytes (Figure 1B and 2A and [27, 31]). Here, we found that when we co-expressed the $\alpha 1A$ - and $\alpha 1B$ -subtypes, both receptors co-localized to the nuclei in adult cardiac myocytes (Figure 2A). This suggests the possibility that the receptors might interact at the nuclei to form oligomers that could regulate aspects of $\alpha 1$ -AR biology in adult cardiac myocytes, as previously suggested in HEK293 cells [21–24]. To eliminate the possibility that the nuclear co-localization was due to a defect in synthesis, folding, or targeting causing the receptors to become trapped in the ER, we counterstained of the $\alpha 1ABKO$ cardiac myocytes expressing both $\alpha 1$ -AR subtypes with ER Tracker. Our results suggested that $\alpha 1$ -AR localization to the nuclei was not the result of receptors becoming trapped in the endoplasmic reticulum (Figure 2B).

3.3 Sequences in the C-terminus of the $\alpha 1A$ - and $\alpha 1B$ -Subtypes Drive Nuclear Localization in Adult Cardiac Myocytes

Based on our observation that endogenous $\alpha 1$ -ARs localize to the nuclear membrane in adult cardiac myocytes (Figure 1A and [27]), we hypothesized that both the $\alpha 1A$ - and the $\alpha 1B$ -subtypes contained nuclear localization sequences. As depicted in Figure 3A, we identified a putative bi-partite nuclear localization sequence in the $\alpha 1A$ -subtype and a glycine-arginine (GR) repeat/arginine-rich nuclear localization sequence in the $\alpha 1B$ -AR, with both nuclear localization sequences in the carboxyl tail region of the respective receptors.

To test the functionality of each nuclear localization sequence, we mutated the arginines and lysines in these regions to alanines (Figure 3A). The localization of the $\alpha 1$ -NLSmut

constructs was compared to the localization of the $\alpha 1$ -WT constructs expressed in $\alpha 1$ ABKO cardiac myocytes. Using confocal microscopy, we found that mutation of the putative nuclear localization sequence in either the $\alpha 1$ A-AR (Figure 3B) or the $\alpha 1$ B-AR (Figure 3C) disrupted targeting of $\alpha 1$ -ARs to the nucleus in $\alpha 1$ ABKO cardiac myocytes. Interestingly, neither $\alpha 1$ -subtype appeared to redistribute to the plasma membrane when the putative nuclear localization sequences were mutated. In summary, we identified functional nuclear localization sequences in both $\alpha 1$ A-AR and $\alpha 1$ B-AR, which provided a mechanism for the targeting of $\alpha 1$ -ARs to the nuclei in adult cardiac myocytes.

3.4 Nuclear Localization is Required for $\alpha 1$ -AR Signaling in Adult Cardiac Myocytes

Our previous studies indicated that $\alpha 1$ -ARs co-localize with their downstream signaling partners G α q and PLC β 1 *only* at the nucleus in adult cardiac myocytes, suggesting that $\alpha 1$ -AR signaling must originate at the nucleus [27]. Although our previous results also suggested that $\alpha 1$ -signaling was initiated intracellularly, we were neither able to definitively demonstrate nuclear $\alpha 1$ -AR signaling nor exclude the possibility that a population of $\alpha 1$ -ARs might initiate signaling at the plasma membrane. Therefore, to test the requirement for nuclear localization in $\alpha 1$ -signaling in adult cardiac myocytes, we compared the ability of wild-type $\alpha 1$ -ARs and $\alpha 1$ -NLSmut to induce the phosphorylation of ERK when receptor expression was reconstituted in $\alpha 1$ ABKO cardiac myocytes. In our reconstitution system, both the $\alpha 1$ A-WT and $\alpha 1$ B-WT induced the phosphorylation of ERK upon treatment with the $\alpha 1$ -agonist phenylephrine (PE), whereas neither $\alpha 1$ -NLSmut could (Figure 4A and 4B). It is important to note that the sites mutated in the $\alpha 1$ A- and $\alpha 1$ B-NLSmut do not correspond to previously identified sites required for ligand binding (Figure 3A, residues highlighted in black) or for coupling to G α q (Figure 3A, highlighted in blue) [33–35].

Our previous results also demonstrated that $\alpha 1$ -AR signaling initiated intracellularly, presumably at the nuclei, induced the phosphorylation of ERK at the plasma membrane in adult cardiac myocytes [27]. This suggested an inside-out signaling mechanism, where $\alpha 1$ -AR signaling initiated in the nuclei is transduced to the plasma membrane. Therefore, to confirm the finding that nuclear localization is required for $\alpha 1$ -AR signaling in adult cardiac myocytes, we took the alternative approach of blocking nuclear export with leptomycin B to determine if *endogenous* $\alpha 1$ -AR signaling in WT cardiac myocytes originated in the nucleus. Leptomycin B itself had no effect on the phosphorylation of ERK (Figure 4C). However, leptomycin B completely blocked PE-mediated phosphorylation of ERK in WT adult cardiac myocytes (Figure 4C). In summary, the data in Figure 4 indicated that nuclear localization was required for $\alpha 1$ -AR signaling and suggested that endogenous $\alpha 1$ -ARs activated signaling in the nuclei in adult cardiac myocytes.

3.5 The $\alpha 1$ B-AR Forms Receptor Oligomers in Adult Cardiac Myocytes

Prior reports suggested that $\alpha 1$ -AR receptors form both homo- and hetero-oligomers and that $\alpha 1$ -AR hetero-oligomers can dictate receptor localization in HEK293 cells [21–24]. To determine if $\alpha 1$ -ARs could form receptor oligomers, we assessed the ability of endogenous $\alpha 1$ -ARs to redirect expression of the $\alpha 1$ -NLSmut back to the nuclei in adult cardiac myocytes. In this case, we examined the $\alpha 1$ B-NLSmut because it appeared to display a greater degree of redistribution away from the nuclei than that $\alpha 1$ A-NLSmut. As before (Figure 3C), when expressed in $\alpha 1$ ABKO cardiac myocytes, which lack both receptor subtypes, the $\alpha 1$ B-NLSmut did not localize to the nuclei (Figure 5A). However, expression of the $\alpha 1$ B-NLSmut in $\alpha 1$ AKO cardiac myocytes, which only have the endogenous $\alpha 1$ B-subtype, resulted in a significant degree of nuclear localization ($P=0.006$ vs. $\alpha 1$ ABKO) (Figure 5B). In contrast, expression of the $\alpha 1$ B-NLSmut in $\alpha 1$ BKO cardiac myocytes, which only have the endogenous $\alpha 1$ A-subtype, failed to result in a significant degree of nuclear localization, although a trend was detected ($P=0.141$ vs. $\alpha 1$ ABKO) (Figure 5C).

These data suggest that in $\alpha 1$ AKO cardiac myocytes, the functional nuclear localization sequence in the endogenous $\alpha 1$ B-subtype was sufficient to redirect expression of the $\alpha 1$ B-NLSmut, presumably because of a direct interaction between the receptors. In a similar fashion, the endogenous $\alpha 1$ A-subtype *might* interact with the $\alpha 1$ B-NLSmut, although in this case only a trend was observed. In summary, these data suggest that the $\alpha 1$ B-subtype and possibly the $\alpha 1$ A-subtype can form oligomers in adult cardiac myocytes.

3.6 The $\alpha 1$ A- and $\alpha 1$ B-Subtypes Form Hetero-oligomers that Influence $\alpha 1$ -AR Signaling in Adult Cardiac Myocytes

Our results in Figure 5 suggest the potential for the $\alpha 1$ A- and $\alpha 1$ B-subtypes to form hetero-oligomers, which has been demonstrated in HEK293 cells, but to our knowledge, not in cardiac myocytes. To test this possibility, we created adenoviruses expressing the $\alpha 1$ A-GFP and $\alpha 1$ B-CFP to constitute a Förster Resonance Energy Transfer (FRET) pair and measured receptor interaction using FRET [23]. We reconstituted this FRET pair in cultured $\alpha 1$ ABKO cardiac myocytes, thereby avoiding the potential complications in measuring receptor interaction due to endogenous $\alpha 1$ -ARs. Sensitized emission FRET analysis revealed that the $\alpha 1$ A- and $\alpha 1$ B-subtypes interacted on the nuclear membrane (FRET efficiency ranging from 0.25–0.50 and a FRET distance appropriate for this FRET pair, which is 4.88 nM, Figure 6A). In short, this provides biophysical evidence that $\alpha 1$ -ARs form hetero-oligomers, which to our knowledge is the first such demonstration in adult cardiac myocytes.

The functional consequence of $\alpha 1$ A- and $\alpha 1$ B-subtype hetero-oligomers is unclear. Previous studies suggested that $\alpha 1$ -AR hetero-oligomers could affect localization and internalization in other cell types [21–23]. However, to our knowledge, an effect of $\alpha 1$ -subtype hetero-oligomers on $\alpha 1$ -AR signaling has not been reported. To test how $\alpha 1$ -AR hetero-oligomers might affect signaling, we measured PE-mediated phosphorylation of ERK, a common $\alpha 1$ -AR signaling pathway [36]. Using our reconstitution system in $\alpha 1$ ABKO cardiac myocytes, we previously reported that the $\alpha 1$ A-subtype facilitated robust PE-mediated ERK phosphorylation, whereas the $\alpha 1$ B-subtype had only a modest effect on PE-mediated ERK phosphorylation [27, 31].

Here, we employed cultured $\alpha 1$ A- or $\alpha 1$ B-single knockout ($\alpha 1$ AKO or $\alpha 1$ BKO) adult cardiac myocytes, reconstituting the missing $\alpha 1$ -subtype to ascertain the potential effect of receptor hetero-oligomers. As a control, we found that PE induced the phosphorylation of ERK in wild-type cardiac myocytes (Figure 6B)

In $\alpha 1$ AKO cardiac myocytes that express only the $\alpha 1$ B-subtype at levels similar to wild type, PE induced modest ERK phosphorylation (Figure 6C). Reconstitution of the $\alpha 1$ A-subtype resulted in robust PE-mediated ERK phosphorylation as expected based on results with the $\alpha 1$ A-subtype in $\alpha 1$ ABKO cardiac myocytes, but increasing $\alpha 1$ B-subtype expression had no significant additive effect (Figure 6C).

In $\alpha 1$ BKO cardiac myocytes that express only the $\alpha 1$ A-subtype at levels similar to wild type, PE had no effect on ERK phosphorylation (Figure 6D). However, increasing $\alpha 1$ A-subtype resulted in robust PE-mediated ERK phosphorylation, as expected, but so did reconstitution of the $\alpha 1$ B-subtype, which was unexpected (Figure 6D). In this case, the ability of the $\alpha 1$ B-subtype to facilitate robust PE-mediated ERK phosphorylation in $\alpha 1$ BKO cardiac myocytes could suggest a functional interaction between the $\alpha 1$ A- and $\alpha 1$ B-subtypes. Combined with our results indicating the formation of $\alpha 1$ A- and $\alpha 1$ B-subtype hetero-oligomers at the nucleus in adult cardiac myocytes, these data suggest that $\alpha 1$ -AR hetero-oligomers might facilitate the phosphorylation of ERK, which to our knowledge is the first demonstration of $\alpha 1$ -AR oligomers regulating signaling in any cell type.

4. DISCUSSION

Here, we examined $\alpha 1$ -AR localization to the nuclei in adult cardiac myocytes, the mechanism behind $\alpha 1$ -AR nuclear localization, and how nuclear localization affected receptor function. In short, we validated our previous findings, clearly demonstrating that endogenous $\alpha 1$ -ARs localized to the nuclear membrane in adult cardiac myocytes. Furthermore, we identified functional nuclear localization sequences in both the $\alpha 1A$ - and $\alpha 1B$ -subtypes that drove nuclear localization and demonstrated that nuclear localization was required for $\alpha 1$ -AR signaling in adult cardiac myocytes. Finally, we found that co-localization of the $\alpha 1$ -subtypes at the nuclei in adult cardiac myocytes facilitated the formation of receptor oligomers that could affect receptor signaling.

These conclusions are supported by the following observations. First, by using nuclei isolated from adult cardiac myocytes and stained with BODIPY-prazosin, we employed a third different technique from our previous work to demonstrate that endogenous $\alpha 1$ -ARs localize to the nuclei in adult cardiac myocytes (Figure 1A). Second, we identified (Figure 3A) and disrupted putative nuclear localization sequences in both the $\alpha 1A$ - and $\alpha 1B$ -subtypes, which led to mis-localization of each subtype when expressed in $\alpha 1$ ABKO adult cardiac myocytes (Figure 3B). Third, we found that disruption of the nuclear localization sequences in either the $\alpha 1A$ - or $\alpha 1B$ -subtypes prevented phosphorylation of ERK upon treatment with the $\alpha 1$ -agonist phenylephrine (Figure 4A-B). Furthermore, based on our finding that $\alpha 1$ -AR signaling activates ERK only at the plasma membrane in adult cardiac myocytes [27], we used leptomycin B to block nuclear export and demonstrate that signaling for endogenous $\alpha 1$ -AR induced phosphorylation of ERK must arise in the nucleus (Figure 4C). Fourth, we showed that endogenous $\alpha 1B$ -ARs can redirect expression of the $\alpha 1B$ -NLSmut back to the nucleus, and observed a similar, although non-significant, trend for the $\alpha 1A$ -AR, suggesting the formation of receptor oligomers. Fifth, we demonstrated direct biophysical interaction between the $\alpha 1A$ - and $\alpha 1B$ -subtypes at the nucleus using FRET (Figure 6A). Finally, we demonstrated that the $\alpha 1B$ -subtype, which alone induces only modest PE-mediated phosphorylation of ERK, could facilitate robust PE-mediated phosphorylation of ERK in $\alpha 1$ BKO cardiac myocytes, which express only the $\alpha 1A$ -subtype that did not respond to PE (Figure 6B-D).

Convention suggests that GPCRs localize to and signal at the plasma membrane. However, several GPCRs have now been localized to the nuclear membrane in a variety of cell types [1–3]. In cardiac myocytes, endothelin receptors, angiotensin receptors, and β -ARs were all detected at the nuclei in adult cardiac myocytes [4–7], and our work indicates that $\alpha 1$ -ARs localized to the nuclei in adult cardiac myocytes as well (Figure 1 and [27, 31]). Yet, the functional relevance of nuclear GPCRs in cardiac myocytes, or any cell, remains unclear. In nuclei isolated from adult cardiac myocytes, endothelin receptors induced calcium transients [4] and angiotensin receptors and β -ARs increased gene transcription [6, 7], but how this might impact overall cardiac myocyte function is uncertain. Here, we found that nuclear localization was required for $\alpha 1$ -AR mediated phosphorylation (activation) of ERK in adult cardiac myocytes (Figure 4). We previously showed that $\alpha 1$ -AR signaling induced phosphorylation of ERK only in caveolae at the plasma membrane in adult cardiac myocytes, describing an inside-out signaling mechanism [27]. Furthermore, we identified an $\alpha 1A$ -subtype-ERK survival-signaling pathway in adult cardiac myocytes, and the absence of this signaling pathway might explain the development of heart failure and increased mortality in $\alpha 1$ ABKO mice subjected to aortic constriction [28, 37]. Therefore, nuclear $\alpha 1$ -AR mediated phosphorylation of ERK at the plasma membrane is an example of nuclear GPCR signaling with significant physiologic consequences.

Although endothelin receptors, angiotensin receptors, and β -ARs all localize to nuclear membrane in adult cardiac myocytes [4–7], our results indicated that unlike these other receptors the majority, if not all, α 1-ARs localized to the nuclei in adult cardiac myocytes [27]. Using saturation radio-ligand binding analysis on nuclei, we demonstrated that at least 80% of α 1-ARs were detected at the nuclei in adult cardiac myocytes, and we failed to detect a functional population of α 1-ARs at the plasma membrane [27]. Subsequently, we performed a similar analysis to measure the distribution of endothelin and angiotensin receptors in adult cardiac myocytes. Our results indicated that 95% of endothelin receptors were detected at the plasma membrane, and only 5% were detected in the nuclei (data not shown), similar to previous studies [4]. However, angiotensin receptor expression levels were below the level of detection in this assay (data not shown). Interestingly, all three receptors signal through G α q, but α 1-AR signaling is cardio-protective (review [36]), whereas chronic endothelin and angiotensin receptor signaling is pathologic (reviews [38, 39]). This might suggest that receptor localization dictates function, or in other words, nuclear Gq-coupled receptor signaling is cardio-protective, whereas plasma membrane Gq-coupled receptor signaling is pathologic. However, this intriguing hypothesis remains to be proven.

Here, we demonstrated the capacity for the α 1B-subtype to form homo-oligomers, and the α 1A- and α 1B-subtypes to form hetero-oligomers in adult cardiac myocytes (Figures 5–6), similar to previous reports in HEK293 cells [21–24]. The ability of the α 1B-subtype to form homo-oligomers was inferred from studies where the α 1B-NLSmut was redirected to the nucleus when expressed in α 1AKO cardiac myocytes that express only endogenous levels of the α 1B-subtype (Figure 5B). Interestingly, the formation of α 1B-homo-oligomers still occurred despite disruption of the nuclear localization sequence (Figure 5B), agreeing in part with previous reports indicating that the carboxyl tail domain of the α 1B-subtype was not required for the formation of receptor oligomers [23]. However, in a similar experiment, endogenous levels of the α 1A-subtype expressed in α 1BKO cardiac myocytes only showed a trend towards redirecting the α 1B-NLSmut to the nucleus (Figure 5C). Whether this is due to differences in expression levels between the receptors (exogenously expressed levels of the α 1B-NLSmut were several fold higher than endogenous α 1A-levels in α 1BKO cardiac myocytes), or that the sequences mutated in the α 1B-NLSmut were required for the formation of hetero-oligomers is unclear. The ability of the α 1A- and α 1B-subtypes to form hetero-oligomers was confirmed using FRET, and interestingly the formation of α 1-AR hetero-oligomers affected α 1-AR mediated phosphorylation of ERK. The ability of hetero-oligomers to affect α 1-AR signaling was inferred from experiments where adding back the α 1B-subtype to α 1BKO cardiac myocytes facilitated robust phosphorylation of ERK. To our knowledge, this is the first demonstration that α 1-AR hetero-oligomers affect signaling, which could have important implications for α 1A-ERK mediated survival-signaling.

In summary, the data presented here demonstrated that α 1-ARs localized to the nucleus, that nuclear localization was driven by nuclear localization sequences identified in both the α 1A- and α 1B-subtypes, that nuclear localization was required for α 1-AR signaling, and that α 1-ARs nuclear localization facilitated that formation of receptor oligomers that impacted receptor signaling. Combined with our previous studies identifying an α 1A-ERK survival signaling pathway that protected the heart from pathologic stress [28, 31, 37], we can for the first time assign a significant physiologic role to nuclear GPCR signaling in cardiac myocytes that might influence the development of novel therapies for heart failure.

HIGHLIGHTS

Endogenous α 1-ARs localize to the nucleus in adult cardiac myocytes

Nuclear localization sequences drive α 1-ARs to the nuclei in adult cardiac myocytes

α 1-AR signaling is initiated in the nucleus in adult cardiac myocytes
 α 1-ARs form oligomers that alter receptor function in adult cardiac myocytes

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ABBREVIATIONS

α1-AR	alpha1-adrenergic receptor
α1A-WT	wild-type alpha1-adrenergic receptor A subtype
α1AKO	alpha1-adrenergic receptor A subtype single knockout
α1B-WT	wild-type alpha1-adrenergic receptor B subtype
α1BKO	alpha1-adrenergic receptor B subtype single knockout
α1ABKO	alpha1-adrenergic receptor A and B subtype double knockout
α1A- or α1B-CFP	GFP, cyan or green fluorescent protein for alpha1-adrenergic receptor subtypes
β-AR	β -adrenergic receptor
βgal	beta-galactosidase
BODIPY	boron-dipyrromethene fluorescent dye
ER	endoplasmic reticulum
ERK	extracellular-regulated kinase
FRET	Förster Resonance Energy Transfer
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GPCR	G-protein coupled receptor
LAP2	lamin-associated protein 2
lepB	leptomycin B
MOI	multiplicity of infection
NCX	Na ⁺ /Ca ²⁺ exchanger
NLSmut	nuclear localization sequence mutant
PE	phenylephrine
P-ERK	phosphorylated extracellular-regulated kinase
T-ERK	total extracellular-regulated kinase
WT	wild-type

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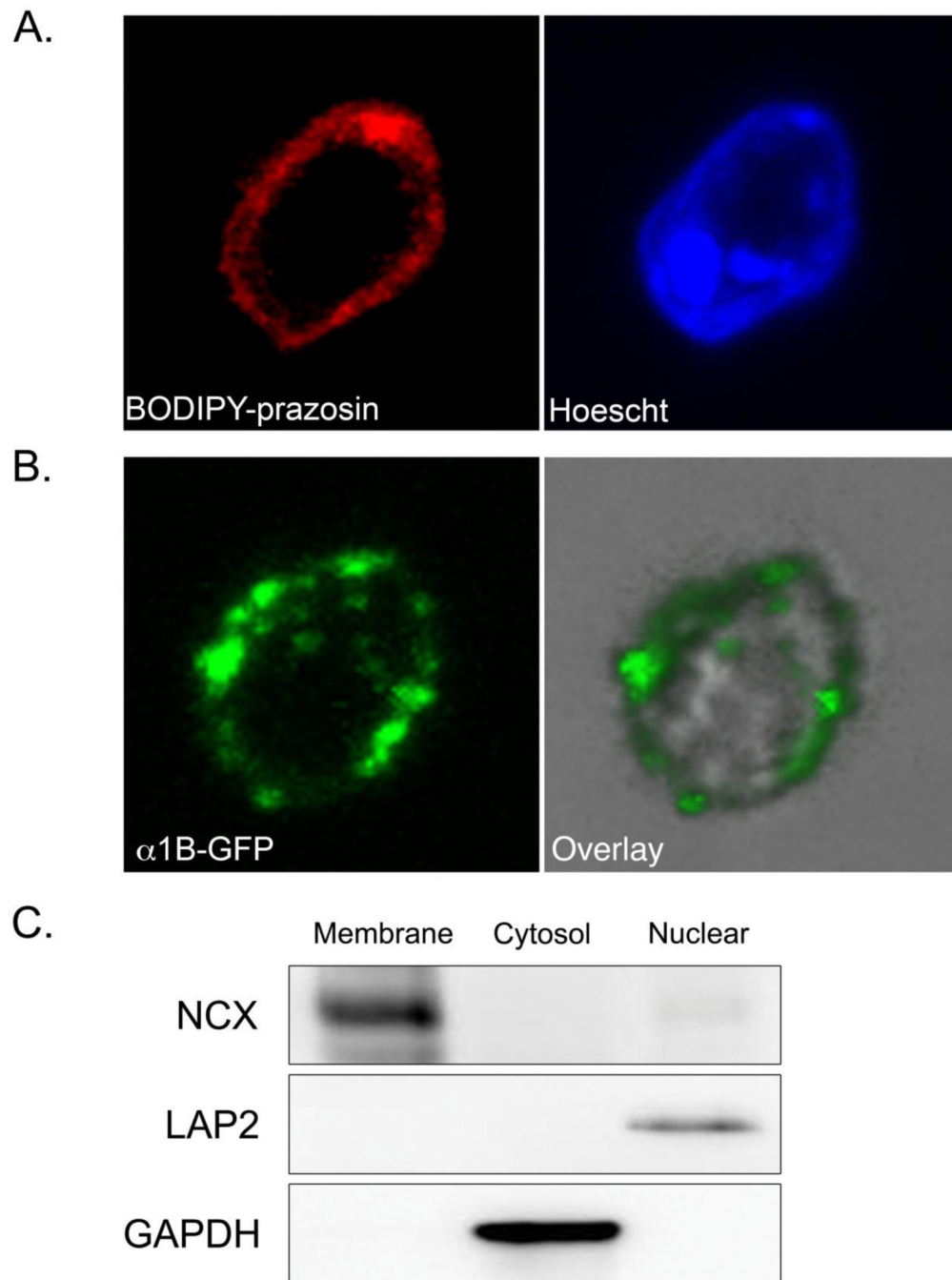


Figure 1. Endogenous α 1-ARs Localize to the Nuclei in Adult Cardiac Myocytes

(A) Freshly isolated wild-type adult cardiac myocytes were fractionated by homogenization and sucrose density gradient ultracentrifugation. Isolated nuclei were stained with BODIPY-prazosin, counter-stained with Hoechst 33342, mounted on coverslips, and visualized by confocal microscopy. Original magnification = 1200x. (n=3 separate isolations) (B) Cultured α 1ABKO adult cardiac myocytes were infected with adenovirus expressing α 1B-GFP and, after 40 hrs, cardiac myocytes were fractionated as in (A). Isolated nuclei were mounted on coverslips and visualized by confocal microscopy. Original magnification = 1200x. (C) Membrane, cytosol, and nuclear fractions (30 μ g) from freshly isolated adult

cardiac myocytes were validated by Western blots for Na⁺/Ca²⁺ exchanger, GAPDH, or LAP2 respectively. Representative Western blots are shown. Similar results were obtained from cultured cardiac myocytes as we previously described [27].

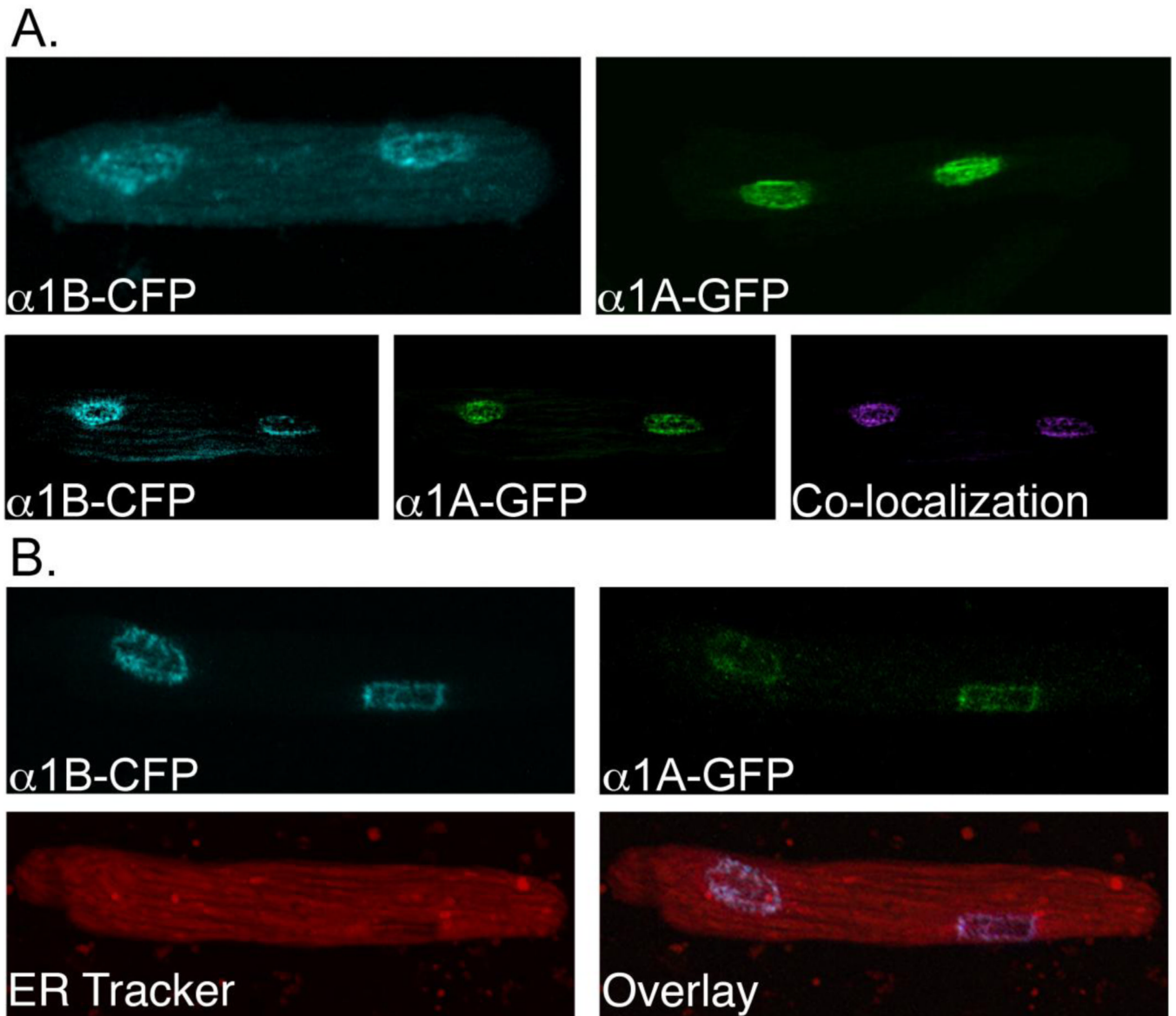


Figure 2. The $\alpha 1A$ and $\alpha 1B$ -Subtypes Co-localize to the Nuclei in Adult Cardiac Myocytes

(A) Upper panel: representative confocal images of a $\alpha 1B$ KO cardiac myocytes expressing only the $\alpha 1B$ -CFP (upper left) or only $\alpha 1A$ -GFP (upper right). Lower panel: representative confocal images of an $\alpha 1B$ KO cardiac myocyte expressing both the $\alpha 1B$ -CFP (left) and the $\alpha 1A$ -GFP (middle). Co-localization is pseudo-colored magenta (right). (B) Representative confocal images of an $\alpha 1B$ KO cardiac myocyte expressing both the $\alpha 1B$ -CFP (upper left) and the $\alpha 1A$ -GFP (upper right) and counterstained with 2 μ M ER Tracker (lower left) and the overlay image (lower right). Final magnification = 600x (n=6 separate cultures).

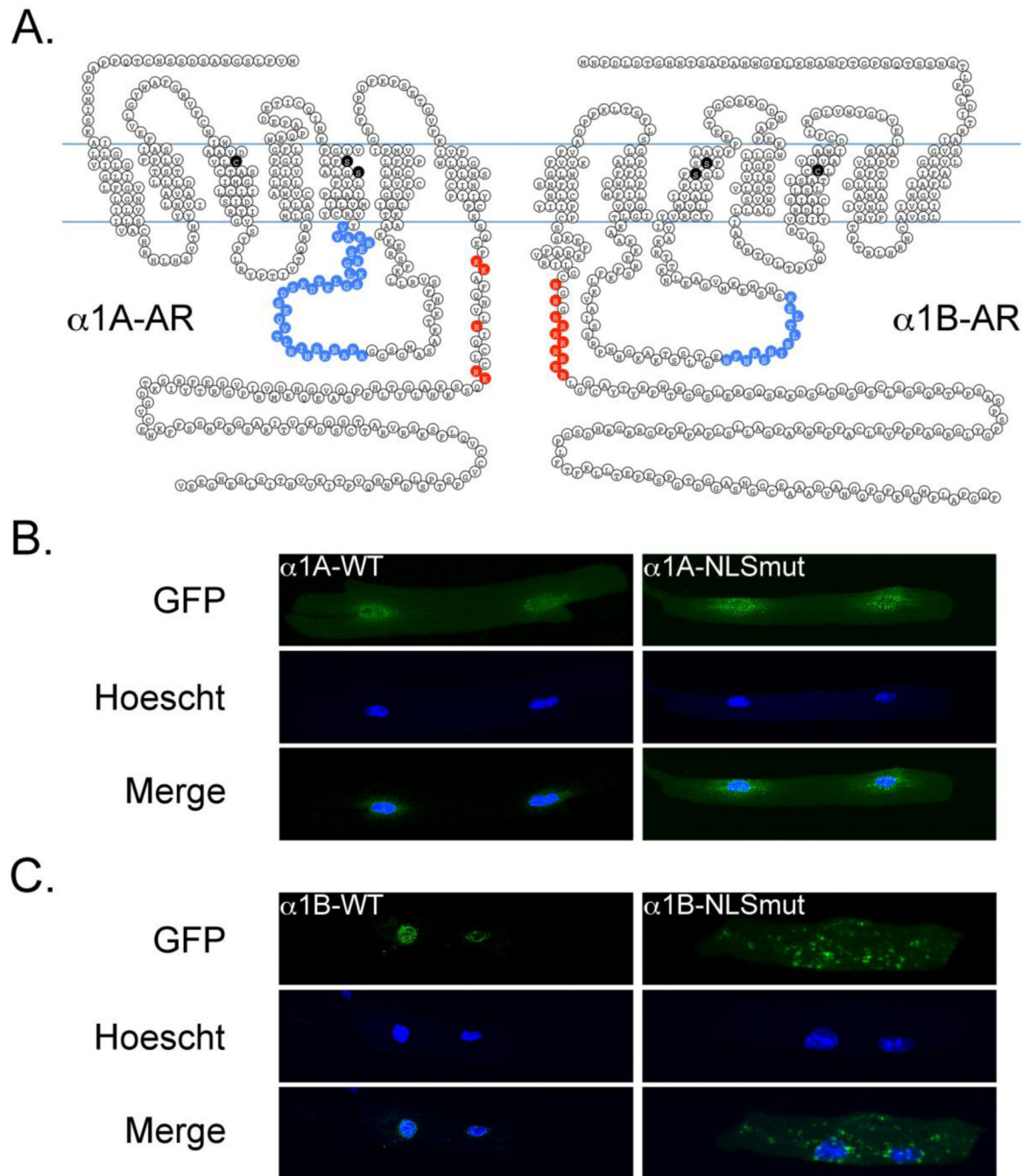


Figure 3. Sequences in the C-terminus of the $\alpha 1A$ - and $\alpha 1B$ -Subtypes Drive Nuclear Localization in Adult Cardiac Myocytes

(A) Amino acid schematic diagram of the $\alpha 1A$ - and $\alpha 1B$ -ARs. Lysine (K) and arginine (R) residues mutated to alanine within the nuclear localization sequence for each receptor are indicated in red. Amino acid residues critical for ligand binding are indicated in black and regions for $G\alpha q$ binding are indicated in blue [33–35]. (B) Representative confocal images of the $\alpha 1A$ -WT (left) and the $\alpha 1A$ -NLSmut (right) adrenergic receptors expressed in $\alpha 1ABKO$ cardiac myocytes (n=6 separate cultures). (C) Representative confocal images of the $\alpha 1B$ -WT (left) and $\alpha 1B$ -NLSmut (right) adrenergic receptors express in $\alpha 1ABKO$ cardiac myocytes (n=6 separate cultures).

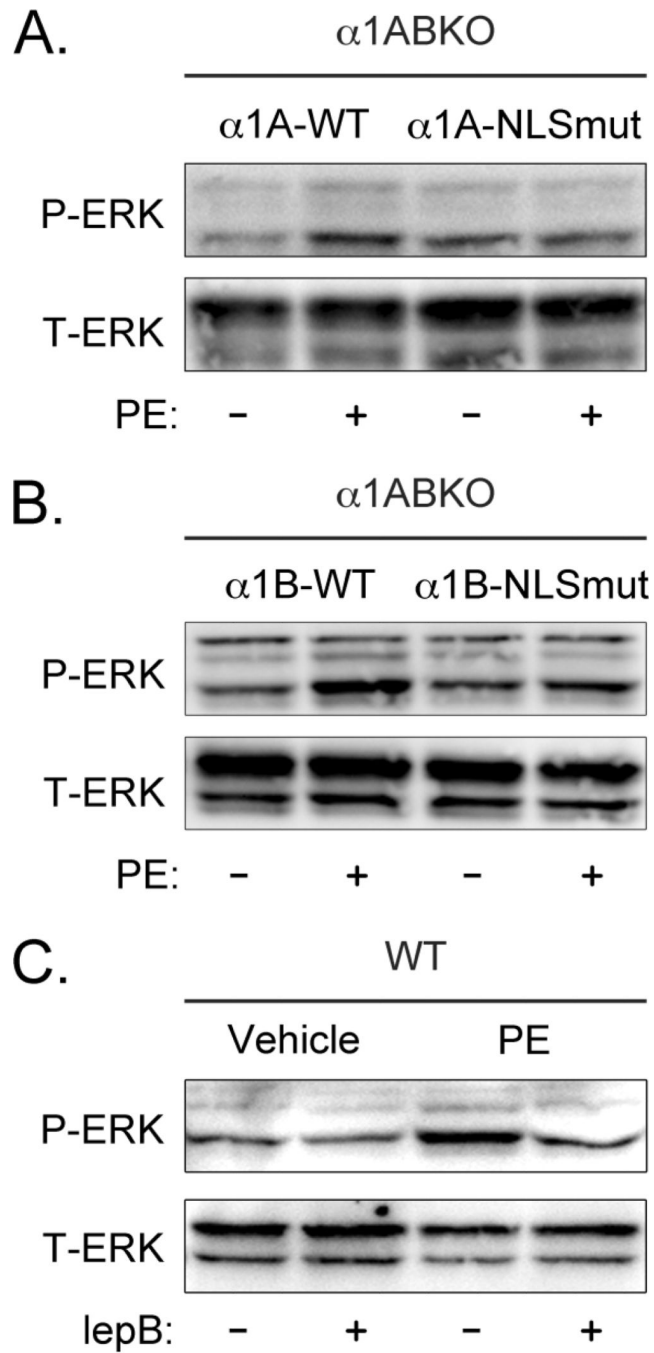


Figure 4. Nuclear Localization is Required for $\alpha 1$ -AR Signaling in Adult Cardiac Myocytes
 Representative Western blot for phosphorylated ERK (P-ERK) and total ERK (T-ERK) from $\alpha 1$ ABKO cardiac myocytes expressing (A) $\alpha 1$ A-wild-type or $\alpha 1$ A-NLSmut or (B) $\alpha 1$ B-wild-type or $\alpha 1$ B-NLSmut treated with 20 μ M phenylephrine (PE) for 20 minutes (n=3). (C) Representative Western blot for P-ERK and T-ERK in wild-type cardiac myocytes treated with 20 μ M PE for 20 minutes in the absence or presence of the nuclear export inhibitor leptomycin B (lepB) (n=6).

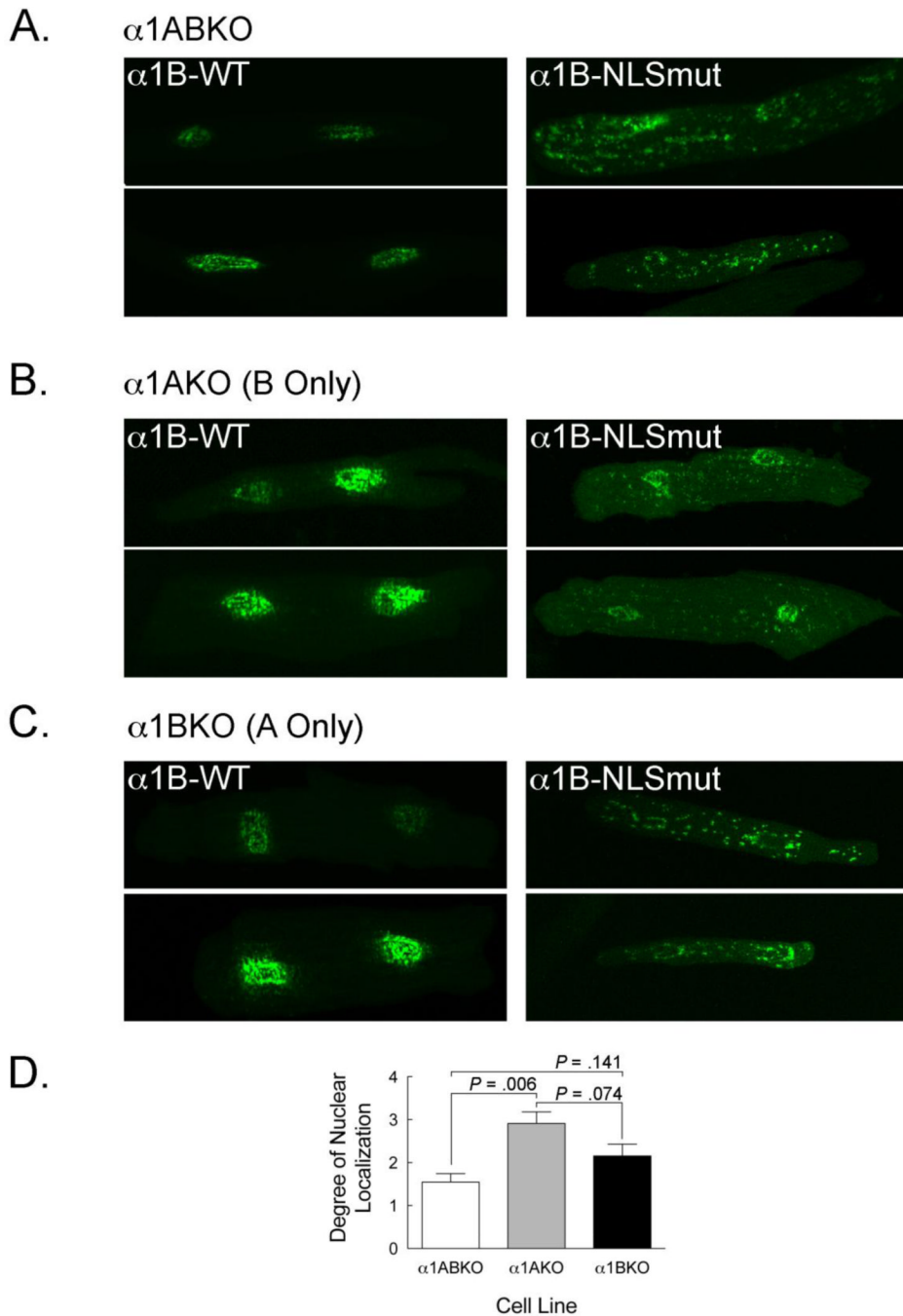


Figure 5. The α 1B-AR Forms Receptor Oligomers in Adult Cardiac Myocytes

(A) Representative confocal images of α 1B-WT (left) and α 1B-NLSmut (right) in α 1ABKO cardiac myocytes. (B) Representative confocal images of α 1B-WT (left) and α 1B-NLSmut (right) in α 1AKO cardiac myocytes which express only endogenous α 1B-ARs. (C) Representative confocal images of α 1B-WT (left) and α 1B-NLSmut (right) in α 1BKO cardiac myocytes which express only endogenous α 1A-ARs. Final magnification = 600x. (D) Comparison of the degree of nuclear localization of the α 1B-NLSmut in α 1ABKO, α 1AKO, and α 1BKO myocytes. The degree of nuclear localization was determined by scoring each image on a scale from 0 to 5 with higher values representing

increased nuclear fluorescence. Cells with nuclear and non-nuclear were scored 0 = no nuclear fluorescence, 1 = up to 25% of the nuclear membrane was identifiable, 2 = 26–50% of the nuclear membrane was identifiable, 3 = 51–75% of the nuclear membrane was identifiable, 4 = 76–100% of the nuclear membrane was identifiable. A score of 5 was given to cardiac myocytes with nuclear fluorescence and no non-nuclear fluorescence. Scores from three blinded technicians were included in the statistical analysis (n=6 for each genotype).

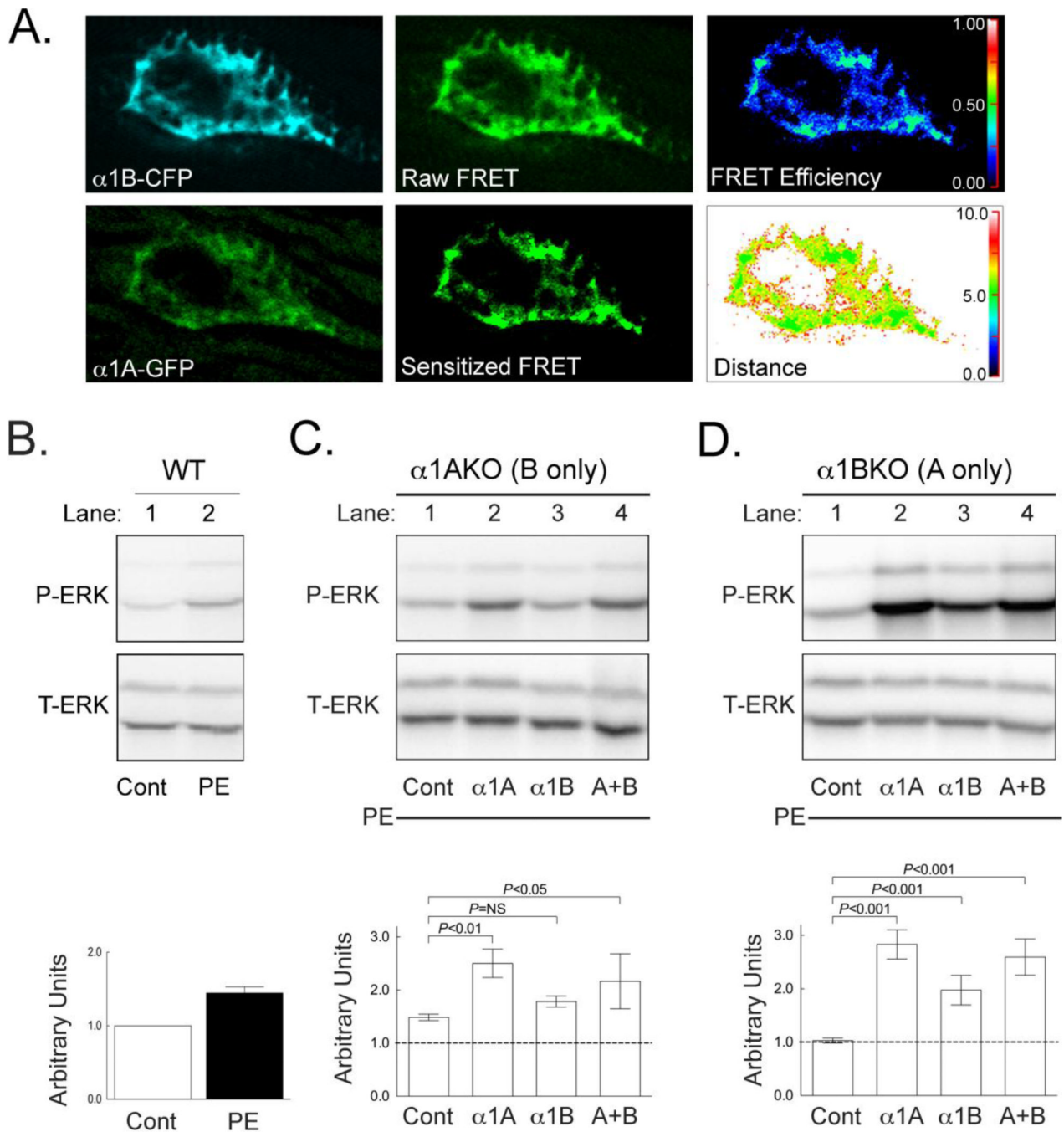


Figure 6. The α 1A- and α 1B-Subtypes Form Hetero-oligomers that Influence α 1-AR Signaling in Adult Cardiac Myocytes

(A) Representative confocal images of a nucleus from an α 1BKO cardiac myocyte expressing both the α 1B-CFP (upper left) and α 1A-GFP (lower left). The resulting raw FRET image (top center) and sensitized FRET image corrected for crosstalk between CFP and GFP emission (bottom center). FRET efficiency between α 1B-CFP and α 1A-GFP in the nucleus (upper right) and calculated FRET distances between α 1B-CFP and α 1A-GFP (bottom right) indicating hetero-oligomerization (n=3 separate cultures). The brightness of the fluorescent images was enhanced to aid visualization of color against the black background. (B) Representative Western blot for P-ERK and T-ERK in wild-type cardiac

myocytes treated with 20 μ M PE. **(C)** Representative Western blots for P-ERK and T-ERK in α 1AKO cardiac myocytes infected with adenovirus expressing the α 1A- alone (lane 2), the α 1B- alone (lane 3) or both (lane 4) and treated with 20 μ M PE. **(D)** Representative Western blots for P-ERK and T-ERK in α 1BKO cardiac myocytes infected with adenovirus expressing the α 1A- alone (lane 2), the α 1B- alone (lane 3) or both (lane 4) and treated with 20 μ M PE. Semi-quantitative analysis of Western Blots shown are shown in B, C and D. Replicate experiments (n=3 separate cultures) were quantified by densitometry and treatments were compared by ANOVA with a Bonferonni post-test. NS = not significant.