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The ubiquitin ligase MuRF1 regulates PPAR α activity in the heart by enhancing nuclear export via monoubiquitination

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Abstract

The transcriptional regulation of peroxisome proliferator-activated receptor (PPAR) α by post-translational modification, such as ubiquitin, has not been described. We report here for the first time an ubiquitin ligase (muscle ring finger-1/MuRF1) that inhibits fatty acid oxidation by inhibiting PPAR α , but not PPAR β/δ or PPAR γ in cardiomyocytes in vitro. Similarly, MuRF1 Tg+ hearts showed significant decreases in nuclear PPAR α activity and acyl-carnitine intermediates, while MuRF1 $-/-$ hearts exhibited increased PPAR α activity and acyl-carnitine intermediates. MuRF1 directly interacts with PPAR α , mono-ubiquitinates it, and targets it for nuclear export to inhibit fatty acid oxidation in a proteasome independent manner. We then identified a previously undescribed nuclear export sequence in PPAR α , along with three specific lysines (292, 310, 388) required for MuRF1s targeting of nuclear export. These studies identify the role of ubiquitination in regulating cardiac PPAR α , including the ubiquitin ligase that may be responsible for this critical regulation of cardiac metabolism in heart failure.

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Disclosures

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Keywords

Cardiomyocyte; Peroxisome proliferator-activated receptor α ; Fatty acid metabolism; Cardiac metabolism; Monoubiquitination; Muscle ring finger-1

1. Introduction

Changes in cardiac metabolism occur in response to pathological hypertrophy, characterized by an upregulation of glucose uptake and glycolysis with either no change or a decrease in glucose oxidation (as recently reviewed Kolwicz et al., 2013). In parallel, decreases in fatty acid oxidation are seen with the downregulation of peroxisome proliferator-activated receptor (PPAR) α (Lehman and Kelly, 2002). A shift away from fatty acid oxidation to glucose use is considered beneficial because of the improved oxygen efficiency for ATP synthesis (Korvald et al., 2000; Burkhoff et al., 1991). In chronic ischemic cardiomyopathy, this becomes a critical point since oxygen is limited; however, these changes may also be maladaptive over time to sustain myocardial energetics and function (Kolwicz et al., 2013). In the present study, we describe a novel mechanism by which the ubiquitin ligase muscle ring finger-1 (MuRF1) inhibits myocardial fatty acid oxidation by removing PPAR α from the nucleus in a novel and previously undescribed mechanism.

The PPAR family of transcription factors, including PPAR α , PPAR β/δ , and PPAR γ , regulate fatty acid utilization and oxidation in heart. Cardiomyocyte PPAR isoforms heterodimerize with the retinoid X receptor- α (RXR α), interact with multiple coactivators and repressors, to regulate the gene expression of proteins involved in lipid metabolism and energy regulation (Dowell et al., 1999; Kota et al., 2005; Michalik et al., 2006). Decreased cardiac PPAR activity has been implicated in the regulation of fatty acid utilization in mouse, rat, and dog models of heart failure (Pellieux et al., 2006; Morgan et al., 2006a,b; Barger et al., 2000; Barger and Kelly, 2000). However, the mechanisms by which PPAR is regulated itself in the context of heart failure has not been elucidated. Recent studies have provocatively suggested that post-translational modifications can be found on all three PPAR isoforms. For example, polyubiquitination of PPAR β/δ and PPAR γ 1/2 in cancer and adipocytes cells, respectively, has been identified, but the effects of polyubiquitination on the activity of these PPAR isoforms and whether degradation occurs are not known (Gareau and Lima, 2010; Gopinathan et al., 2009; Wadosky and Willis, 2012). These studies suggest that ubiquitin ligases, proteins that direct specific ubiquitination of substrates (PPARs in this case), may be regulating PPAR activity in some yet to be determined manner.

Muscle-specific ubiquitin ligase MuRF1 expression increases in chronic heart failure (Adams et al., 2007), left ventricular assist device (LVAD) placement in humans (Willis et al., 2009a,b), nutrient deprivation (Baskin and Taegtmeyer, 2011), and ischemic/ dilated human cardiomyopathy (Paul et al., 2004). It was originally identified as a transiently increasing protein in skeletal muscle atrophy (Bodine et al., 2001), mediating cardiac (and skeletal muscle) atrophy in response to glucocorticoids and unloading (Willis et al., 2009; Baehr et al., 2011). MuRF1 regulates cardiomyocyte mass by post-translationally modifying and regulating transcription factors in a disease-specific manner. In pathological cardiac hypertrophy, for example, MuRF1 inhibits cardiomyocyte growth by inhibiting PKC activity

(Arya et al., 2004) and interacting with and inhibiting the transcription factor serum response factor (SRF) activity (Willis et al., 2007). In cardiac ischemia-reperfusion injury, MuRF1 inhibits apoptosis by halting JNK-induced signaling – specifically, MuRF1 polyubiquitinates phosphorylated c-Jun, targeting it for proteasome-dependent degradation, thereby effectively inhibiting downstream AP-1 (c-Jun/Fos) signaling (Li et al., 2011; Wadosky et al., 2014). MuRF1's ability to regulate cell signaling specifically in cardiomyocytes parallels its role in protein quality control and the turnover of key metabolic enzymes, including creatine kinase (Willis et al., 2009a,b). With MuRF1's prominence in interacting with metabolic enzymes regulating energy metabolism (e.g. creatine kinase, pyruvate kinase, 3-hydroxybutyrate dehydrogenase) and our recent identification of PPAR-associated gene expression signatures in microarray analyses (e.g. gene ontology (GO) categories PPAR- γ coactivator (PGC), tricarboxylic acid (TCA) cycle, energy from oxidation) (Willis et al., 2009a,b), we investigated MuRF1 as a potential ubiquitin ligase that regulates cardiomyocyte PPAR activity.

In the present study, we expand on MuRF1's role in cardiac remodeling and cardioprotection by demonstrating that MuRF1 post-translationally regulates PPAR α -mediated fatty acid oxidation. We describe for the first time a novel mechanism whereby MuRF1 specifically inhibits PPAR α , but not PPAR β/δ or PPAR γ activity, by targeting PPAR α for nuclear export through non-canonical ubiquitination (monoubiquitination). Specifically, we demonstrate that MuRF1 interacts with and monoubiquitinates PPAR α and targets nuclear export of PPAR α through its ubiquitin ligase activity. Since ubiquitination occurs on lysines, the specific three lysines involved in ubiquitination were identified (K292, K310, and K358) to surround a nuclear export signal within PPAR α that we identified and validated in the study. Together, these results identify that MuRF1 directs nuclear PPAR α removal without being degraded by a nuclear export-dependent mechanism, driven by monoubiquitination. These studies identify that cardiomyocyte MuRF1 inhibits PPAR α activity by a novel mechanism by which the cardiomyocyte nuclear receptor PPAR α is monoubiquitinated and subsequently removed from the nucleus by the cells nuclear export machinery.

2. Materials and methods

Detailed methodology for the following techniques are provided in the Online Supplement: COS7, H9C2, HEK293T, HL-1 cell culturing; FLAG-PPAR α mutant construct generation; qPCR; in vivo and in vitro ubiquitination assays; immunoprecipitation and Western blot analysis; luciferase activity reporter assays; MuRF1 Tg+ and MuRF1 $^{-/-}$ mice generation; glucose and fatty acid oxidation assays; nuclear isolation; PPAR α , PPAR β , and PPAR γ activity assays; acylcarnitine quantification; fatty acid ^{123}I - β -methyl-p-iodophenylpentadecanoic acid (^{123}I -BMIPP) and glucose analogs ^{18}F -2-fluoro-2-deoxy-D-glucose (^{18}F -2-FDG) by single-photon emission computed tomography (SPECT) and computed tomography/positron emission tomography (CT/PET) analysis. Data are expressed as mean $\pm$ SEM. A One-way ANOVA test was used to determine the source of variation. Differences between specific groups were determined using a multiple comparison post-test via the Holm-Sidak method using the all-pairwise procedure. When appropriate, a

Student's t-test was performed to express differences between groups within one variable. Significance was defined as $p < 0.05$.

3. Results

Increasing MuRF1 expression alters cardiomyocyte glucose and fatty acid oxidation. Recent studies have illustrated that car-diomyocyte MuRF1 expression is critical to the cardiac hypertrophic response to pressure overload and the susceptibility to heart failure (Willis et al., 2009a,b, 2007). Like other ubiquitin ligases, MuRF1 interacts with multiple protein substrates, including those directly involved in the cardiac hypertrophic response (e.g. the transcription factor SRF) and in metabolic responses (e.g. creatine kinase) (Willis et al., 2009a,b; Zhao et al., 2008). Since tran-scriptomic analysis has also provided support for MuRF1's role in energy metabolism (Willis et al., 2009a,b), we tested the hypothesis that MuRF1 regulates fatty acid and glucose oxidation at the level of the cardiomyocyte in the heart. To test this hypothesis, we increased MuRF1 expression using adenovirus constructs in neonatal rat cardiomyocytes (NRCMs) and measured the ^{14}C -labeled CO_2 released when cells were allowed to metabolize ^{14}C -labeled glucose or ^{14}C -labeled oleate (Fig. 1). Increasing MuRF1 expression using adenoviral vectors (25 MOI (multiplicity of infection)) resulted in a 62% increase in glucose oxidation and a 58% decrease in fatty acid oxidation in NRCMs (Fig. 1A and B, respectively). While the addition of the adenovirus to the NRCMs did not affect fatty acid oxidation (Fig. 1B, Untreated vs. 5–15 MOI Ad.GFP), it did enhance glucose oxidation (Fig. 1A, untreated vs. all other groups). Similarly, increasing MuRF1 in the HL-1 cardiomyocyte cell line resulted in enhanced glucose oxidation and decreased fatty acid oxidation (Fig. A.1A and B). Conversely, MuRF1 knock-down using siRNA in HL-1 cells resulted in decreased glucose oxidation and enhanced fatty acid (oleate) oxidation (Fig. A.1C and D), suggesting a role for endogenous MuRF1 in regulating fatty acid and glucose oxidation in cardiomyocytes. With increasing MuRF1 expression, fatty acid oxidation is inhibited while parallel increases in glucose oxidation result in an apparent shift of substrate utilization. Based on the amount of fatty acid and glucose oxidation measured, the amount of ATP resulting from the reduced fatty acid oxidation (129/mol) is compensated for by increases in ATP created by the measured enhanced glucose oxidation (38/mol) (Fig. 1C). Since these changes were reminiscent of those seen in heart failure driven by PPAR isoforms (van Bilsen et al., 2004; Madrazo and Kelly, 2008), we investigated the possibility that MuRF1 regulates this substrate shift by regulating PPAR activity.

MuRF1 specifically inhibits PPAR α , but not PPAR β/δ or PPAR γ transcriptional activity. The PPAR family of nuclear transcription factors contains three isoforms expressed in the heart: PPAR α , PPAR β/δ , and PPAR γ (Madrazo and Kelly, 2008). These nuclear receptors dimerize with RXR α , and bind to several co-regulators to interact with the PPAR response-element in the promoters of genes regulated by PPAR family members (Madrazo and Kelly, 2008). These genes encode for proteins that enhance fatty acid oxidation; at the same time, they encode for the glucose inhibitor pyruvate dehydrogenase kinase 4 (PDK4), which enhances its inhibition of glucose oxidation (Kolwicz et al., 2013; Doenst et al., 2013). In this way, inhibiting PPAR results in decreased fatty acid oxidation by transcriptionally down-regulating genes necessary for fatty acid oxidation, while simultaneous removing

PDK4. This would account for PPAR's divergent control of fatty acid and glucose oxidation (Kolwicz et al., 2013; Doenst et al., 2013). To delineate which of the three PPAR nuclear receptors MuRF1 may be inhibiting in cardiomyocytes, we next investigated if expression of MuRF1 affects PPAR transcriptional activity using luciferase reporter gene assays in COS-7 and H9C2 cells (Fig. 2). Since MuRF1 is found in both the nucleus and sarcomere (at the M-line bound to titin), assaying MuRF1's role in regulating PPARs in COS7 cells (without a sarcomere) allowed us first to determine MuRF1's ability to regulate nuclear activity. Assaying MuRF1's regulation of PPARs in HL-1 cells then illustrated the relevance of these findings in a representative cardiomyocyte. In both COS7 and HL-1 cells, co-transfection of a plasmid luciferase reporter driven by a PPAR-response element (PPRE) and a PPAR α expression plasmid resulted in an enhanced PPAR activity; in contrast, co-expression with a MuRF1 expression plasmid resulted in significant inhibition of PPAR α (Fig. 2A and D). To determine the specificity of this response, we next investigated MuRF1's effect on PPAR β/δ and PPAR γ gamma activity. MuRF1 failed to inhibit either PPAR β/δ or PPAR γ induced PPRE-luciferase activity in COS7 or HL-1 cardiomyocyte-derived cells (Fig. 2B,C,E,F).

We next established a method to quantify PPAR α activity that could be applied to both in vitro and in vivo studies. MuRF1 expression was increased in HL-1 cells using adenovirus (Ad.MuRF1 and control Ad.GFP); parallel assays with MuRF1 knockdown using siRNA MuRF1 cocktail transfection (and a scramble control in parallel) were run. Nuclear protein was isolated and assayed for its ability to bind solid phase PPRE DNA bound, and this was quantified using a colorimetric anti-PPAR α antibody. Increasing MuRF1 expression resulted in a dose-dependent decrease in PPAR α activity (Fig. 2G). Decreasing MuRF1 expression using siRNA enhanced PPAR α expression over 2.5 fold of control levels (Fig. 2H). In parallel studies, increasing, and decreasing MuRF1 altered PPAR α -regulated gene expression (Fig. 2I and J). Taken together, these results suggest that MuRF1 specifically inhibits PPAR α , the most abundant PPAR found in cardiomyocytes (Escher et al., 2001), in both non-cardiomyocyte and at least 2 cardiomyocyte-derived cell lines.

Cardiac MuRF1 expression regulates PPAR α activity and fatty acid oxidation in vivo. With evidence that MuRF1 regulates PPAR α , but not PPAR β/δ or PPAR γ activity in multiple cell types, we next assayed MuRF1's regulation of these factors in vivo. Using previously described MuRF1 cardiac transgenic (Tg+) and MuRF1 $-/-$ mice (Willis et al., 2009a,b, 2007), we assayed isolated cardiac nuclei for PPAR activity (described above in Fig. 2G). Nuclear extracts were placed on plates coated with PPRE DNA, followed by incubation with a capture antibody recognizing PPAR α , PPAR β/δ , or PPAR γ in separate assays (Fig. 3A). Consistent with our in vitro studies, increased α -MHC-driven cardiac MuRF1 expression in the MuRF1 Tg+ hearts resulted in a significant decrease in PPAR α activity, approximately 60% compared with wild-type controls. Conversely, MuRF1 $-/-$ hearts exhibited an approximately 5-fold increase in activity compared with wild-type controls (Fig. 3A). MuRF1 Tg+ and MuRF1 $-/-$ hearts had the same PPAR β/δ and PPAR γ activities as their respective wild-type controls, indicating once again the specificity of MuRF1's effect on PPAR α in vivo.

Previous studies using transgenic mouse models have identified that increasing cardiac PPAR α results in the increase import and oxidation of fatty acids (Madrazo and Kelly, 2008). To determine the amount of imported and oxidized fatty acids, we performed an acylcarnitine profile analysis to quantify fatty acid oxidation intermediates, an assay established to reflect changes in PPAR α activity in vivo (Makowski et al., 2009; Chen et al., 2009; Laghezza et al., 2013; Turer et al., 2009) (Fig. 3C–F). Using this technique, we identified that compared to sibling control mice, MuRF1 Tg+ hearts demonstrated a significant decrease in total acylcarnitine levels (Fig. 3B). Conversely, MuRF1–/– hearts had a significant increase in total acylcarnitine (C2–C18 acyl-CoA species) levels compared with their sibling controls (Fig. 3B). When the total acylcarnitine was divided up into its component long, medium, and short chain acylcarnitines (Fig. 3C–F), similar significant differences were seen in the long and short chain acylcarnitines, reflecting their relative abundance compared with medium chain acylcarnitines. Additional in vivo studies investigating fatty acid and glucose uptake in the heart revealed no differences between MuRF1 Tg+, MuRF1–/–, and their respective wild-type sibling control hearts in studies using the fatty acid analog ^{123}I - β -methyl-p-iodophenylpentadecanoic acid (^{123}I -BMIPP) imaged by single-photon emission computed tomography (SPECT) and the glucose analog ^{18}F -2-fluoro-2-deoxy-D-glucose (^{18}F -2-FDG) imaged by computed tomography/positron emission tomography (CT/PET) (Fig. A.2). These results illustrate that inhibition of fatty acid oxidation in MuRF1 Tg+ mice and enhanced fatty acid oxidation in MuRF1–/– mice, both revealed by significant differences in acyl-carnitine levels, cannot be explained by differences in fatty acid uptake and therefore must be due to regulators of fatty acid oxidation within the cell, such as PPAR α .

We next assayed the expression levels of the PPAR α -regulated genes acetyl-CoA carboxylase 1 (ACC1), aconitase 1 (ACO1), long chain acyl-CoA dehydrogenase (LCAD), and medium chain acyl-coenzyme A dehydrogenase (MCAD) by qPCR. Despite evidence that MuRF1 inhibits PPAR α activity (Fig. 3A and B) and fatty acid oxidation (acyl-carnitine intermediates) (Fig. 3C–F) in vivo, transcription of PPAR α -regulated genes (mRNA) were expressed in the opposite manner from expected. MuRF1 Tg+ hearts demonstrated a transcriptional upregulation of ACC1 and ACO1, whereas MuRF1–/– hearts expressed significantly less ACC1, ACO1, LCAD, and MCAD mRNA (Fig. 3G and H). Given the complexity of the PPAR α complex and its multiple binding partners (RXR and other nuclear receptors), co-repressors, and co-stimulators, it is not surprising to see opposite activities invoked. For example, co-repression has been described when agonist-bound PPARs attract repressors and when PPAR homodimerization occurs (Fan et al., 2011; Flores et al., 2011; Borland et al., 2011). This sort of feedback mechanism common in physiology has only begun to be touched upon in PPAR biology and rarely in vivo.

MuRF1 does not affect the steady state protein levels of PPAR isoforms. Like most ubiquitin ligases reported to date, MuRF1 has largely been reported to polyubiquitinate and degrade its specific substrates, including troponin I, β -myosin heavy chain, cMyBP-c, and c-Jun (Li et al., 2011; Kedar et al., 2004; Fielitz et al., 2007; Mearini et al., 2010). In these previous studies, increasing MuRF1 expression resulted in decreased substrate levels; conversely, reducing MuRF1 resulted in increased substrate protein levels. Since our results demonstrated that MuRF1 inhibits PPAR α activity, we hypothesized that MuRF1 reduces

PPAR α by targeting its degradation. To test this, we performed Western blot analysis on MuRF1 Tg+ and MuRF1 $^{-/-}$ hearts to examine PPAR α , PPAR β/δ , or PPAR γ protein levels (Fig. 3G). Surprisingly, whereas MuRF1 clearly inhibited PPAR α activity, it did not affect its steady state protein level in the heart. These findings led us to hypothesize that MuRF1 inhibits PPAR α activity in a manner independent of proteasome-mediated degradation.

MuRF1 interacts with PPAR α to regulate its nuclear localization. We next determined if MuRF1 interacted with PPAR α , given the possibility that MuRF1's regulation of PPAR α could occur by indirect means since PPAR α is only one component of a larger complex, including RXR α and PGC-1. Using HL-1 cells, we immunoprecipitated MuRF1 and were able to pull down and immunoblot endogenous PPAR α (Fig. 4A). Similarly, when we immunoprecipitated endogenous PPAR α we were able to pull down MuRF1 in HL-1 cells (Fig. 4B). To determine how this might occur, we next performed confocal microscopy analysis in HL-1 cells to examine the location of endogenous PPAR α in the presence and absence of increased MuRF1 expression (Fig. 4C). As expected, endogenous PPAR α expression was found throughout the cell in control cells, both in the cytosol and nucleus (Fig. 4C). Surprisingly, in cells with increased MuRF1 expression, endogenous PPAR α was cleared from the nucleus (Fig. 4C). When larger a larger cell samplings were made, endogenous nuclear PPAR α was present in 99.2% of control HL-1 cells (Fig. 4D). In contrast, increasing MuRF1 expression resulted in only 29.2% of the cells retaining endogenous nuclear PPAR α (Fig. 4D). We hypothesized that MuRF1 may post-translationally modify PPAR α in such a way that it becomes excluded from the nucleus. This hypothesis is consistent with the fact that MuRF1 inhibits PPAR α 's (nuclear) activity without degrading protein substrate itself.

MuRF1 mediates PPAR α nuclear export to inhibit its activity. Recent studies have identified that ubiquitination of nuclear proteins by ubiquitin ligases can target nuclear export in the context of cancer. For example, the ubiquitin ligase MDM2 mono-ubiquitinates p53, which results in nuclear export of p53 in cancer cells (Li et al., 2003; Carter et al., 2007). While this mechanism occurs in the context of cancer (e.g. in the H1299 human non-small cell lung carcinoma cell line and the U2OS osteosarcoma cell line), similar mechanisms have not been described in muscle cells. Therefore, we investigated if MuRF1's ability to remove nuclear PPAR α in cardiomyocytes was related to nuclear export. We used the potent and specific nuclear export inhibitor leptomycin B (LMB) (Kudo et al., 1998). Leptomycin B alkylates and inhibits the CRM1/ exportin 1 (XPO1) protein required for the nuclear export of proteins containing a nuclear export signal (NES), by glycosylating a cysteine residue (Kudo et al., 1999). MuRF1's ability to remove endogenous nuclear PPAR α was inhibited in the presence of LMB treatment, indicating a role for the NES machinery in this process. In the absence of LMB, increasing MuRF1 expression resulted in endogenous nuclear PPAR α localization in 11.3% cells, compared with 82.3% in control cells, respectively (Fig. 5A, upper vs. lower rows). In parallel experiments using LMB, MuRF1's ability to alter the nuclear localization of PPAR α was inhibited, with 95.7% of the nuclei having endogenous PPAR α , compared with 96.4% in control cells (Fig. 5B, upper vs. lower rows). We next quantified how the nuclear transport effect of MuRF1 affected endogenous PPAR α activity by assaying the amount of nuclear PPAR α that could bind to a solid phase DNA with a PPRE. Compared to control cells, an 80% decrease in binding activity was found when

MuRF1 expression was increased (Fig. 5C). Consistent with our confocal analysis, we identified that LMB inhibited MuRF1's ability to decrease endogenous PPAR α activity in cardiac-derived cells (Fig. 5D). Whereas MuRF1 is rarely found in the nucleus, LMB treatment "captured" MuRF1 in the nucleus of approximately 25% of the cells (Fig. 5D). It is possible that MuRF1's nuclear import and export is so swift under normal conditions that noticeable accumulation of MuRF1 in the nucleus is not seen, but can be uncovered in the presence of LMB. MuRF1's ability to enter and exit the nucleus (albeit briefly) would also explain MuRF1's inhibition of other nuclear substrate activity, including SRF shown previously (Willis et al., 2007), and PPAR α in the current study. Taken together, these findings indicate MuRF1 enters and then rapidly moves out of the nucleus to remove endogenous PPAR α in a manner dependent upon the cell's nuclear export machinery.

MuRF1 monoubiquitinates PPAR α in vitro and in vivo. As a muscle-specific ubiquitin ligase, MuRF1's ability to regulate interacting substrates has been linked to its ability to ubiquitinate the protein at specific lysines. MuRF1 polyubiquitinates and degrades cTnI, c-Jun, and β -MHC in a proteasome-dependent manner (Li et al., 2011; Kedar et al., 2004; Fielitz et al., 2007). In contrast, other substrates, such as PKC ϵ and SRF, localization and activity are regulated without protein degradation (Arya et al., 2004; Willis et al., 2007). Since MuRF1 interacts with PPAR α , inhibits its activity, but does not change the steady state protein levels when it is increased, we next detailed MuRF1's interaction and ubiquitination of PPAR α . Using MuRF1 mapping constructs, we identified that MuRF1 binds PPAR α through its B-box and MuRF-family conserved (MFC) regions (Fig. 6A). We next performed an in vitro ubiquitination assay where we added recombinant E1, E2 (UbcH5c), MuRF1, PPAR α , and ubiquitin. Western blot analysis of this reaction yielded both a predominant unmodified form halfway between the 52 and 76 kDa molecular weight markers (Fig. 6B). A single additional band was seen in the full reaction (far right lane), but not in the control reactions missing just 1 component (E1, E2, MuRF1, PPAR α , OR ubiquitin, respectively). This specific band (i.e. not formed without all the reaction components present) was found between the PPAR α (~54 kDa) and below the 76 kDa marker. Since a single ubiquitin has a molecular mass of 8.5 kDa, it represents a monoubiquitination (estimated to be 62.5 kDa = 54 kDa + 8.5 kDa). This is not the canonical polyubiquitination of PPAR α , since these post-translationally modified chains appear as a smear. For example, MuRF1 simultaneously autoubiquitinated itself by this type of poly-ubiquitin chain (Fig. 6B, lower anti-GST blot), revealing that the reaction stoichiometry drove MuRF1 to monoubiquitinated PPAR α in vitro. Prior studies have revealed MuRF1's ability to autopolyubiquitinate through all of the possible ubiquitin lysine chains (K6, K11, K27, K29, K33, K48, K63) (Kim et al., 2007), whereas mono- and multi-monoubiquitination patterns are not associated with ubiquitin chain formation (Sadowski et al., 2012).

Like many cell free phenomena, these in vitro ubiquitination results needed confirmation in the more complex milieu of the cell. Therefore, we next sought to confirm the relevance of MuRF1's apparent monoubiquitination of PPAR α in the more complex milieu of a cell, with multiple other proteins present, including multiple E2s known to drive the complexity and type of MuRF1 ubiquitin chains (Kim et al., 2007). Interestingly, MuRF1 similarly monoubiquitinated PPAR α in cells demonstrating that increasing MuRF1 expression results in increased post-translational modification of PPAR α in both HL-1 cells and HEK293T

cells, which results in the addition of one or two ubiquitin modifications (di-mono-ubiquitination) being added (Fig. 6C and D, respectively). Both mono- and di-monoubiquitin modifications have been reported to mediate changes in cellular localization in various cancer cell lines (Haglund et al., 2003; Lee et al., 2008; Liu and Subramani, 2013). These studies illustrate that increasing MuRF1 expression induces a multi-monoubiquitination of PPAR α , which may be responsible for the resulting nuclear export observed.

An interesting finding related to nuclear export machinery inhibited by LMB is that it is dependent upon recognition of nuclear export signals (NES). However, the sequences that make up the NES(s) for PPAR α have not been described to date. Nuclear export signals are made up of hydrophobic residues, often leucines (L), arranged in a LXXXLXXLXL motif, where X is any other amino acid. Given the critical nature of the leucines in NESs, we first scanned the PPAR α amino acid sequence for leucine clusters and found three near each other at amino acid residues 300–308 (Fig. A.3B and C). In addition to this leucine rich region (NES1), we next used an algorithm to identify any additional potential NES sequences in PPAR α . Based on published analysis of a wide range of nuclear export signals, we used a bioinformatics approach to predict other potential leucine-rich nuclear export signals in PPAR α (<http://www.cbs.dtu.dk/services/NetNES/>) (la Cour et al., 2004). This analysis identified a cluster of 3 predicted NES amino residues in an 8 amino acid cluster at residues 368–377 (lelddsdisl), suggesting a putative nuclear export sequence, which we named NES2 (Fig. A.3B and C). These sequences, identified as NES1 (ldlndqvltl) and NES2 (leldds-disl), are located in the ligand-binding domain (domain E/F) in PPAR α , detailed in Fig. A.3A. Using standard published procedures (Simon-Areces et al., 2013; Lin et al., 2005; Durchschlag et al., 2004; Brown et al., 2004; Hake et al., 2000), we next examined whether these putative NES1 and NES2 regions were functional, by mutating the Leucine to Alanine in the NES1 and NES2 regions. If NES1 and/or NES2 were actual nuclear export sequences, these mutations would prevent their nuclear export and confirm their role in targeting PPAR α for nuclear export in cardiomyocytes. HL-1 cells were transfected with 1) wild-type PPAR α ; 2) NES1 mutant PPAR α ; or 3) NES2 mutant PPAR α in parallel (Fig. 6A). Compared to wild-type PPAR α (Fig. 7A, top panels), NES1 mutant PPAR α demonstrated an increased nuclear localization in experiments run in parallel (Fig. 7A, middle panels). In contrast, the NES2 mutant PPAR α did not have an altered nuclear localization compared with wild-type PPAR α (Fig. 7A, bottom panels). These studies identify for the first time that the novel NES1 is a bone fide NES sequence in PPAR α . Despite NES1's ability to maintain its nuclear localization, increased MuRF1 expression was able to specifically export both the NES1 and NES2 mutant PPAR α (Fig. 7B and Fig. A.3D).

MuRF1's PPAR α monoubiquitination requires 3 lysine groups flanking the newly identified NES1. To determine which lysines in PPAR α are monoubiquitinated, and to illustrate MuRF1's dependence upon this ubiquitination for nuclear export of PPAR α , we created PPAR α mutant proteins that lacked specific lysine groups (by mutations changing lysine to arginine) surrounding the 2 putative NES sites that we identified in the experiment described above (Fig. A.3A). Six PPAR α mutants, named A1, A2, A3, A4, A5, and A6 were created, each with only 1 lysine altered. When cells were transfected with A1–A6 and MuRF1

expression was increased, we found that PPAR α A1, PPAR α A2, and PPAR α A3 were not removed from the nucleus as was wild-type PPAR α when MuRF1 expression was increased (Fig. 8). PPAR α A4–A6 mutants were removed from the nucleus to the same extent as wild-type PPAR α (Fig. A.4) and parallel control cells (Fig. A.5). These studies illustrate the necessity of the three individual lysines surrounding the novel NES1 PPAR α , which result in the mono- and/or di-mono-ubiquitination that we identified in cells and in vitro ubiquitination assays.

4. Discussion

In the present study, we identified the striated muscle-specific MuRF1 ubiquitin ligase as a direct regulator of PPAR α activity in the heart in vivo. Increasing MuRF1 expression decreased PPAR α activity while decreasing MuRF1 expression using shRNA or mouse models lacking cardiac MuRF1 resulted in significant increases in PPAR α activity. MuRF1's inhibition of PPAR α occurred using a mechanism requiring nuclear export, evidenced by its inhibition by leptomycin B. After identifying the first NES in PPAR α using mutational analysis, we next demonstrated that MuRF1 is mono-ubiquitinated using in vitro ubiquitination assays. In cell models in vivo, we found that increasing MuRF1 monoubiquitinated or di-monoubiquitinated PPAR α . When lysines A1, A2, and A3 surrounding the novel NES1 were mutated to arginine, MuRF1's ability to induce nuclear export was prevented. We proposed that increasing MuRF1 results in MuRF1's translocation to the nucleus where PPAR α is monoubiquitinated and preferentially recognized by CRM1 and the nuclear export complex, mediating its subsequent export and inhibition of activity (transcription of PPAR α -regulated genes). These studies parallel recent findings in cancer, whereby the ubiquitin ligase MDM2 was found to monoubiquitinate p53 to target its nuclear export (Li et al., 2003). In contrast to MDM2 and p53, we have not found that increased MuRF1 levels poly-ubiquitinate and degrades PPAR α like increased MDM2 levels have been reported. This novel pathway possibly regulates the newly identified NES1 sequence-targeted nuclear export. These findings are also the first description of a pathway by which cardiac PPAR isoform levels are regulated and may be critical in the substrate switching the heart utilizes to adapt in cardiac diseases, such as cardiac hypertrophy and heart failure.

A recent study has identified that PPAR γ is exported rapidly from the nucleus after being phosphorylated in cancer cell lines, including MCF-7 breast adenocarcinoma and HEK293T embryonic kidney cell lines (Burgermeister and Seger, 2007). This mitogen-induced nuclear export is through its direct interaction with the MAPK/ERK-kinases 1/2 (MEKs) (Burgermeister and Seger, 2007). Of particular interest in this study was that the NES that interacts with CRM1/exportin-1 was found in MEK1, not PPAR γ . This suggests that MEK1's interaction with PPAR γ was critical in its nuclear export and possibly otherwise independent of any phosphorylation of PPAR γ itself. Recent studies have found that MEK1 similarly directs PPAR α , although with PPAR α MEK1 interacts with the C-terminal end, where a proposed, but not confirmed LXXLL NES motif was hypothesized (el Azzouzi et al., 2012). These studies were particularly interesting as they were performed in the NKL-Tag ventricular muscle cell line and MEK1 inhibitors in wild-type mice appeared to parallel these in vitro studies (el Azzouzi et al., 2012).

The regulation of PPAR α by post-translational modification with ubiquitin has been demonstrated in rat hepatoma and the HepG2 cell lines (Gopinathan et al., 2009). The E3 MDM2 has been associated with increased poly-ubiquitination of PPAR α ; interestingly, the MDM2 to PPAR α ratio determined if MDM2 activated (MDM2: PPAR α <1) or inhibited (MDM2: PPAR α >1) (Gopinathan et al., 2009). Parallel studies in another hepatoma cell line (HepG2) illustrated that MDM2's inhibition of PPAR α activity was due to its degradation (Blanquart et al., 2002; Blanquart et al., 2004; Hirotani et al., 2001). Both sets of studies identified that MDM2-dependent modulation of PPAR α activity was dependent upon the presence of the PPAR α ligand Wy14643 (Gopinathan et al., 2009; Blanquart et al., 2002, 2004; Hirotani et al., 2001). Interestingly, in the present study, we did not see MuRF1 poly-ubiquitinate or degrade PPAR α no matter how low or high the ratio of MuRF1:PPAR α was (data not shown). Similarly, all experiments were performed in the presence of serum, which included endogenous PPAR α ligands. We did not find the addition of Wy14643 altered the results of our studies, so we did not include them after our initial evaluation.

Monoubiquitination, like that found on PPAR α resulting from MuRF1 ubiquitination, has been associated with directing changes in sub-cellular location. Classically, this has been described at the plasma membrane and associated with endosomal targeting of receptors after ligand engagement. One example involves receptor tyrosine kinases (RTKs), where by interaction with their cognate ligand results in monoubiquitination at the plasma membrane (Haglund et al., 2003). Upon monoubiquitination, these RTKs move to the endosomal compartment to be shuttled to the lysosome, not recycled to the plasma membrane, in multiple cancer cells, including NIH 3T3 cells, B82L, and HeLa (Haglund et al., 2003). The ubiquitin ligase MDM2, with ubiquitin ligase, has been reported to ubiquitinate p53, with monoubiquitination of p53 being sufficient for nuclear export (Carter et al., 2007). Interestingly, MDM2 ubiquitination of p53 contributes to two different steps of p53's nuclear export: 1) ubiquitination exposes a carboxyl-terminal NES, and 2) promotes the disassociation of MDM2 (Carter et al., 2007). Further studies indicated that MDM2's monoubiquitination of p53 promotes the interaction of the SUMO E3 ligase PIAS γ with p53, enhancing SUMOylation and nuclear export in U2OS osteosarcoma cells and MEF cells (Carter et al., 2007). Monoubiquitination of nuclear PTEN in cancer cells has demonstrated that the mono-ubiquitination of PTEN is essential for nuclear import (Trotman et al., 2007). In contrast to p53 and the present study, the post-translational modification of a nuclear protein targets the substrate to move into the nucleus, illustrating how nuclear localization signals (NLS) can be regulated by monoubiquitination in the same manner as NES's can.

The mismatch between MuRF1's effects on PPAR α activity/ localization and on PPAR α -regulated gene expression was unexpected. But this may be the result of the multiple roles MuRF1 has in cardiomyocyte metabolism and the multiple levels that PPAR α may be regulated. MuRF1 may be directly (or indirectly) affecting compensatory gene regulation at numerous levels, resulting in complex and possibly contradictory relationships. For example, MuRF1 regulates metabolism by regulating the turnover of creatine kinase (critical for ATP shuttling to the sarcomere) (Willis et al., 2009a,b), localizes in the mitochondria and reduces ROS production in vivo (Mattox et al., 2014). MuRF1 itself interacts (in Y2H systems) with numerous metabolic proteins, such as enoyl coenzyme A hydratase, malonyl-

CoA decarboxylase, and multiple other proteins involved in glycolysis and the Krebs cycle (e.g. Aldolase A, Pyruvate kinase, Pyruvate dehydrogenase) (Willis et al., 2009a,b; Witt et al., 2005). Taken together, MuRF1's combined effects on metabolism, in addition to the regulation of PPAR α described here, is complex. There, the unique findings on the overall metabolic responses here are not completely surprising. The transcriptional complex made up of PPAR α and RXR α includes a myriad of other proteins, including co-repressors, some of which have been described to bind agonist-bound PPARs, some just recently described (Borland et al., 2011). Having these multiple players has made predicting the effects of reducing PPAR protein on PPAR transcription. The ratio of PPAR α :RXR α in cardiomyocytes, for example, may be more important in determining PPAR activity than the levels of PPAR α alone. Recent studies have shown that enhanced PPAR expression can change the ratio of PPAR:RXR α , favoring PPAR homodimers that can act as repressors (opposite of what would be predicted), despite more PPAR being present and located on PPAR response elements in the promoter of PPAR-transcribed genes (Fan et al., 2011; Flores et al., 2011). Overall, our understanding of post-translational regulation of the PPAR complex itself remains limited and should be expected to be complex and unpredictable, but nonetheless critical to our understanding of PPARs in metabolism and the pathophysiology of disease.

5. Conclusions

Muscle ring finger-1 (MuRF1) is the first muscle-specific ubiquitin ligase reported that directs nuclear PPAR α inhibition in a proteasome-independent manner. These studies demonstrated that increasing MuRF1 inhibits fatty acid oxidation, while enhancing glucose oxidation and inhibiting PPAR α . Mechanistically, these studies report that cardiomyocyte MuRF1 inhibits PPAR α activity by a novel mechanism by which the cardiomyocyte nuclear receptor PPAR α is mono-ubiquitinated (adjacent to a novel NES also identified in these studies) and subsequently removes it from the nucleus by the cells nuclear export machinery. Furthermore, the present study identified the first confirmed NES in PPAR α , along with three specific lysines (292, 310, 388) required for MuRF1s targeting of nuclear export. These studies identify the role of ubiquitination in regulating cardiac PPAR α in vivo, including the cardiac-specific ubiquitin ligase that may be responsible for this critical regulation of cardiac metabolism in heart failure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard abbreviations

MuRF1	Muscle ring finger-1
PPAR	Peroxisome proliferator-activated receptor
RXRα	RXR retinoid X receptor- α
LVAD	Left ventricular assist device
SRF	Serum response factor
GO	Gene ontology
PGC	PPAR-gamma coactivator
TCA	Tricarboxylic acid
^{123}I-BMIPP	^{123}I - β -methyl-p-iodophe-nylpentadecanoic acid
^{18}F-2-FDG	^{18}F -2-fluoro-2-deoxy-D-glucose
NRCMs	Neonatal rat cardiomyocytes
MOI	Multiplicity of Infection
SPECT	Single-photon emission computed tomography
PET	Positron emission tomography

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.mce.2015.06.008>.

Fig. 1.

Increasing MuRF1 expression enhances primary cardiomyocyte glucose oxidation and inhibits fatty acid (oleate) oxidation. A. ^{14}C -Glucose and B. ^{14}C -Oleate oxidation in neonatal rat cardiomyocytes (NRCMs) with increasing MOI of Ad.GFP or Ad.Myc-MuRF1. Etomoxir, a commercially available fatty acid oxidation inhibitor was used as a control to demonstrate that fatty acid oxidation inhibition could be detected. C. Using the glucose and fatty acid (oleate) oxidation determination, shifts in calculated ATP resulted in no net change of ATP (based on $^{14}\text{CO}_2$ released from the cells, assuming 1 mol glucose = 38 ATP; 1 mol oleate = 129 ATP). Data represent 3 independent experiments run in triplicate. D. Immunofluorescence microscopy detection of GFP (from Ad.GFP or Ad.MuRF1) vs. non-infected cells run in parallel with ^{14}C -Glucose/ ^{14}C -Oleate replicates. A One-Way ANOVA was used to determine significance. # $p < 0.05$ vs. media alone. * $p < 0.05$ vs. Ad.GFP; ** $p < 0.05$ vs. untreated control cells.

Fig. 2.

MuRF1 regulation of PPAR α , PPAR β/δ , and PPAR γ activity in vitro. PPAR α , PPAR β/δ , and PPAR γ activity was determined using PPRE-driven luciferase plasmids, co-transfected with a PPAR α , PPAR β/δ , and PPAR γ expression plasmid, in the presence or absence of MuRF1. A. In Cos7 cells, MuRF1 significantly inhibited PPAR α , but not B. PPAR β/δ , or C. PPAR γ activity. Similarly, MuRF1 significantly inhibited D. PPAR α , but not E. PPAR β/δ , or F. PPAR γ activity in cardiac-derived HL-1 cardiomyocyte cell line. G. Determination of nuclear PPAR α binding to PPRE DNA using a capture ELISA platform demonstrates the increasing MuRF1 expression results in decreased nuclear PPAR α activity; conversely H. decreasing MuRF1 expression with siRNA results in increased nuclear PPAR α activity in HL-1 cardiomyocytes. I. Quantitative RT-PCR analysis reported cardiac PPAR α -activated mRNA expression in HL-1 cardiomyocytes with increased MuRF1 (transduced with Ad.GFP-Myc-MuRF1) or J. decreased MuRF1 expression (Ad.GFP-shMuRF1). Expression of cells without treatment was set at 1. Data are mean $\pm$ SEM from at least 3 independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. Ad.GFP or Ad.scMuRF1 infected control cells.

Fig. 3.

Cardiomyocyte MuRF1 expression regulates PPAR α , PPAR β/δ , and PPAR γ activity in vivo without altering protein levels of PPAR α . A. The increased cardiomyocyte MuRF1 in MuRF1 Tg+ hearts results in decreased nuclear PPAR α binding activity, but not PPAR β/δ , or PPAR γ activity for binding PPRE DNA using a capture ELISA platform. B. Conversely, MuRF1-/- hearts lacking cardiomyocyte MuRF1 have a >5 fold increase in nuclear PPAR α binding activity, but no effect on PPAR β/δ , or PPAR γ activity using a capture ELISA assay. C-F. Quantitative metabolomics analysis of acyl-carnitine (AC) in MuRF1 Tg+ and MuRF1-/- hearts. Normalized concentrations of C. total acylcarnitines (Total AC). Further breakdown to these total ACs by D. long chain (C14-C22), E. medium chain (C6-C12), and F. short chain (C2-C5), illustrates MuRF1's regulation of fatty acid oxidation significantly affects long and short chain ACs, while medium ACs are not affected. Subcategories of acylcarnitines classified by chain length measured in mouse heart homogenates from the indicated strain and represented by box and whiskers plotting the median and min to max, respectively (n = 3-4 biological replicates per strain). G. Western immunoblot analysis MuRF1 Tg+ (N = 4/group) and MuRF1-/- (N = 3/group) hearts have PPAR α , PPAR β/δ , and PPAR γ expression that do not differ from strain-matched control hearts (N = 4 and N = 3, respectively), with the exception of PPAR β which was decreased in MuRF1-/- hearts. H. qPCR analysis of reported cardiac PPAR α -regulated mRNA illustrates that the increased MuRF1 in MuRF1 Tg+ hearts increases the expression of ACC1 and ACO1 while I. MuRF1-/- hearts have increased levels of all ACC1, ACO1, LCAD, and MCAD. Data are mean $\pm$ SEM. A Student's t-test was performed to determine significance between groups (Panels A, B, G, H, *p < 0.05, **p < 0.01, ***p < 0.001). Changes in total, long chain, and

short chain were significant at an FDR <5% (*) (Panels C–F, post-tests: * $p < 0.001$ comparing MuRF1+/+ versus MuRF1–/–, and Wild-type^{MuRF1Tg} vs. MuRF1 Tg+, respectively. ** $p < 0.01$ and *** $p < 0.001$ MuRF1–/– vs. MuRF1 Tg+). n.s. =not significant.

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Fig. 4.

MuRF1 interacts with PPAR α by co-immunoprecipitation and increasing MuRF1 expression removes nuclear localization. **A.** Immunoprecipitation of MuRF1 in HL-1 cells (forward reaction), followed by immunoblot analysis of endogenous PPAR α detected a specific interaction between MuRF1 and PPAR α . **B.** Immunoprecipitation of endogenous PPAR α in HL-1 cells (reverse reaction) also detected a specific interaction between PPAR α and MuRF1. **C.** Confocal microscopy analysis of HL-1 cells revealed that at the single cell level, increasing MuRF1 expression (Ad.MuRF1 25 MOI) caused endogenous PPAR α to move out of the nucleus (middle panel) compared with controls (top). **D.** The removal of endogenous PPAR α by MuRF1 (25 MOI) was found in most of the cells compared with controls, which rarely lacked nuclear endogenous PPAR α . **E.** Analysis of at least 3 independent experiments from >5 fields of view demonstrated that increasing MuRF1 (AdMuRF1) decreased the nuclear endogenous PPAR α 70% (99.2%–29.2%), while increasing the number of cells with cytoplasmic endogenous PPAR α 70% (70.8%–0.8%). N =244 (Ad.GFP), N =228 (Ad.myc-MuRF1). A Student's t-test was performed to determine significance between groups. *p < 0.01.

Fig. 5.

MuRF1's nuclear removal and inhibition of PPARα activity is dependent on the nuclear export machinery. A. The MuRF1-mediated removal of endogenous PPARα, found in cells with increased MuRF1 (Ad.myc-MuRF1 vs. Ad.GFP, top two rows) B. is inhibited when cells are treated with Leptomycin B (LMB), an inhibitor of exportin (CRM1)-mediated nuclear export. By recognizing leucine-rich nuclear export signals (NESs) in target proteins like PPARα, CRM1 mediates the nuclear export and inhibition. C. In parallel experiments measuring nuclear PPARα activity (ability of isolated nuclear PPARα to bind PPRE-coated ELISA plates), increasing MuRF1 inhibited PPARα activity ~60% compared with controls. D. Paralleling MuRF1's removal of nuclear PPARα in confocal microscopy experiments, LBM inhibition of nuclear export in HL-1 cells prevented MuRF1's inhibition of PPARα activity. E. The presence of MuRF1 is rarely found in the nucleus in normal conditions (e.g. Fig. 4). However, in the presence of LMB, MuRF1 was found in greater than 25% of HL-1 cells 4 h after treatment indicating that MuRF1 movement in and out of the nucleus is dependent upon nuclear export of it or other interacting proteins necessary for MuRF1 to interact with nuclear receptors. A Student's t-test was performed to determine significance between Ad.GFP and Ad.MuRF1. *p < 0.05. n.s. =not significant.

Fig. 6.

MuRF1 binds PPAR α through its MFC domain and promotes PPAR α monoubiquitination through its ubiquitin ligase activity. A. Immunoprecipitation of PPAR α (anti-FLAG) in cells co-transfected FLAG-PPAR α and myc-MuRF1 deletion mutants identified that MuRF1's MFC (MuRF Family Conserved) region is required for its interaction with PPAR α . B. Western immunoblot analysis of in vitro ubiquitination reactions containing recombinant E1, E2, MuRF1, PPAR α , and ubiquitin (= Full reaction) demonstrates that MuRF1 monoubiquitinates PPAR α in a specific manner (compare to No E1, No E2, No MuRF1, No PPAR α , No ubiquitin lanes). C. Determination of MuRF1's ubiquitination pattern in vivo similarly reveals the presence of monoubiquitination. Specifically, HL-1 cells transfected with FLAG-PPAR α and HA-Ubiquitin (HA-Ub) with increased MuRF1 (Ad.Myc-MuRF1 vs. Ad.GFP, 4 h) demonstrates that PPAR α is multi-monoubiquitinated. D. Similarly, PPAR α is multi-monoubiquitinated in HEK293 cells in a RING finger-dependent manner. Compared to MuRF1's monoubiquitination of PPAR α , MuRF1 RING (MuRF1 lacking the N-terminal RING finger domain with ubiquitin ligase activity) is unable to similarly monoubiquitinate PPAR α despite equal expression levels of MuRF1 (IB: MuRF1 [cMyc]).

Fig. 7.

Identification and confirmation of PPAR α 's first nuclear export sequence targeting nuclear export. Three PPAR α constructs were made to confirm if putative NESs identified here (strategy outlined in Fig. A.3A, in blue): 1) a FLAG-tagged wild-type PPAR α ; 2) a full length FLAG-tagged NES1 mutant PPAR α , whereby all 4 leucines in the NES1 region were replaced with alanine; and 3) a full length FLAG-tagged NES2 mutant PPAR α , with all 3 leucines in the NES2 region were replaced with alanine. A. Compared to wild-type PPAR α (top row), NES1, but not NES2 transfected cardiomyocytes, have increased nuclear staining consistent with an impaired nuclear export. B. To determine if the enhanced nuclear concentration of the NES1 mutant PPAR α impaired MuRF1's ability to remove it, parallel studies with increased MuRF1 were performed to test MuRF1's ability to remove PPAR α from the nucleus. Increasing MuRF1 (Ad.myc-MuRF1 25 MOI) reduced nuclear NES1 mutant PPAR α compared with control (Ad.GFP 25 MOI) by 63.2% (83.9%–20.7%), consistent with the 70% reduction seen in endogenous PPAR α in previous experiments (Fig. 5). Despite the importance of the NES1 for nuclear export, modification of MuRF1's modification and nuclear export of this mutant PPAR α is unaffected.

Fig. 8.

Three specific lysines surround NES1 are required for MuRF1's nuclear export of PPARα. Since ubiquitin ligases, including MuRF1, place ubiquitin on protein lysines, we next determined the lysines required for MuRF1's nuclear export of PPARα. Six additional FLAG-tagged PPARα constructs were created with the six most proximal lysines to the NES1 and NES2 mutated to arginine, preventing ubiquitination from occurring (see Fig. A.3 for details). Compared to controls (row 1), MuRF1 clears wild-type PPARα in HL-1 cardiomyocytes (row 2). However, full length PPARα with a single lysine to arginine mutation at surrounding NES1 (A1, A2, and A3) is not cleared from the nucleus. With increased MuRF1 (Ad.MuRF1), PPARα mutants A1, A2, and A3 localize in the nucleus (rows 3–5, right table – 89.4%, 77.4%, and 78.3%, respectively) to the same extent as Ad.GFP controls (row 1, 81.1%). In contrast, increased MuRF1 (Ad.MuRF1) results in nuclear removal of wild-type PPARα, remaining in only 26.4% of the cells (row). PPARα mutants A4, A5 and A6 were localized only in cytoplasm as PPARα control (Fig. A.4), indicating that the lysines A4, A5, A6 are not potential ubiquitination sites needed for MuRF1's nuclear export of PPARα.