MARCH2 promotes endocytosis and lysosomal sorting of carvedilol-bound β₂-adrenergic receptors


Department of Medicine and Department of Cell Biology, Duke University Medical Center, Durham, NC 27710
Department of Biological Sciences, Tokyo Institute of Technology, Yokohama 226-8501, Japan

Abstract

lyosomal degradation of ubiquitinated β₂-adrenergic receptors (β₂-ARs) serves as a major mechanism of long-term desensitization in response to prolonged agonist stimulation. Surprisingly, the β AR antagonist carvedilol also induced ubiquitination and lysosomal trafficking of both endogenously expressed β₂-ARs in vascular smooth muscle cells (VSMCs) and overexpressed Flag-β₂-ARs in HEK-293 cells. Carvedilol prevented β₂AR recycling, blocked recruitment of Nedd4 E3 ligase, and promoted the dissociation of the deubiquitinases USP20 and USP33. Using proteomics approaches (liquid chromatography–tandem mass spectrometry), we identified that the E3 ligase MARCH2 interacted with carvedilol-bound β₂AR. The association of MARCH2 with internalized β₂-ARs was stabilized by carvedilol and did not involve β-arrestin. Small interfering RNA–mediated down-regulation of MARCH2 ablated carvedilol-induced ubiquitination, endocytosis, and degradation of endogenous β₂-ARs in VSMCs. These findings strongly suggest that specific ligands recruit distinct E3 ligase machineries to activated cell surface receptors and direct their intracellular itinerary. In response to β blocker therapy with carvedilol, MARCH2 E3 ligase activity regulates cell surface β₂AR expression and, consequently, its signaling.

Introduction

Agonist stimulation of cell surface seven-transmembrane G protein–coupled receptors (GPCRs or 7TMRs) leads to heterotrimeric G protein activation and second messenger–mediated cellular responses (Neves et al., 2002; DeWire et al., 2007). Immediately after their activation, 7TMRs are phosphorylated by GPCR kinases (GRKs) leading to the recruitment of cytosolic adaptors called β-arrestins, which terminate G protein signaling and initiate receptor endocytosis (Moore et al., 2007; Shenoy and Lefkowitz, 2011). 7TMR internalization is subsequently coupled to a second wave of signaling via the GRK–β-arrestin signaling and initiate receptor endocytosis (Shenoy et al., 2008). This process is counteracted by β AR deubiquitination, mediated by the deubiquitinases USP20 and USP33; deubiquitination commits the β₂AR to recycle and resensitize at the cell surface (Berthouze et al., 2009). These agonist-dependent processes tightly regulate the magnitude and duration of GPCR signal transduction, thus balancing the downstream cellular responses.

Activation of β₂ARs and α₁ARs in vascular smooth muscle cells (VSMCs) regulates vascular tone and directs blood flow to essential organs. Activation of cardiomyocyte βARs by catecholamines mediates the increase in heart rate and contractility.

7TMR trafficking is substantially influenced by dynamic ubiquitination and deubiquitination of the agonist-activated receptor (Shenoy, 2007; Shenoy and Lefkowitz, 2011). For the β₂-adrenergic receptor (β₂AR), agonist-induced ubiquitination by the HECT domain E3 ligase Nedd4 (neural precursor cell-expressed developmentally down-regulated protein 4) is required for receptor trafficking to the lysosomes and subsequent receptor degradation (Shenoy et al., 2008). This process is counteracted by β₂AR deubiquitination, mediated by the deubiquitinases USP20 and USP33; deubiquitination commits the β₂AR to recycle and resensitize at the cell surface (Berthouze et al., 2009). These agonist-dependent processes tightly regulate the magnitude and duration of GPCR signal transduction, thus balancing the downstream cellular responses.

Correspondence to Sudha K. Shenoy: sudha@receptor-biol.duke.edu

Abbreviations used in this paper: 7TMR, seven-transmembrane G protein–coupled receptor; ANOVA, analysis of variance; β₂AR, β₂-adrenergic receptor; CHF, chronic heart failure; DTME, Dithio-bis-maleimidoethane; GPCR, G protein–coupled receptor; GRK, GPCR kinase; IP, immunoprecipitation; Iso, isoproterenol; KO, knockout; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MDC, monodansylcadaverine; MEF, mouse embryo fibroblast; NS, nonstimulated control; P/S, penicillin/streptomycin; VSMC, vascular smooth muscle cell; WT, wild type.

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associated with stress or exercise. In chronic heart failure (CHF), catecholamine stimulation of βARs leads to pathological responses including myocardial apoptosis and hypertrophy (Xiao et al., 2004). In contrast, βAR antagonists (β blockers) that counteract the binding of catecholamines and block G protein signaling provide survival benefits to patients with CHF (Bristow, 2000). Recent studies have shown that the β blocker carvedilol has unique agonist properties in inducing βAR signaling specifically via β-arrestin while blocking G protein signaling, thus functioning as a β-arrestin–biased agonist (Wisler et al., 2007; Kim et al., 2008a; Shenoy, 2011).

Although carvedilol, metoprolol succinate, and bisoprolol fumarate are used for treating CHF (Hunt et al., 2009; Jabbour et al., 2010), some evidence suggests that the nonselective β blocker carvedilol possesses survival advantages over others (Louis et al., 2001; Domanski et al., 2003). In heart failure, both bisoprolol and metoprolol treatments cause an up-regulation of βAR expression, whereas carvedilol does not, despite being as effective as other β blockers in improving left ventricular function (Heilbrunn et al., 1989; Gilbert et al., 1996; Yamada et al., 1996; Flesch et al., 2001; Kindermann et al., 2004). Therefore, carvedilol could be mechanistically unique in initiating specific itineraries for receptor trafficking and regulating βAR expression as well as signaling. Herein, we report a hitherto unknown molecular mechanism of carvedilol-induced β2AR endocytosis and down-regulation promoted by a novel interaction with an E3 ubiquitin ligase, MARCH2 (membrane-associated RING-CH2).

**Results**

**The β blocker carvedilol induces β2AR ubiquitination and promotes lysosomal trafficking**

Because the β2AR agonist isoproterenol (Iso) induces ubiquitination of the receptor (Shenoy et al., 2001, 2008; Liang and Fishman, 2004; Berthouze et al., 2009; Xiao and Shenoy, 2011), one would expect β blockers to function as antagonists and block this effect. Contrary to this premise, the β blocker carvedilol induced dose-dependent ubiquitination of the β2AR in VSMCs, as can be seen with β2AR immunoprecipitation (IP; Fig. 1, A and B). To corroborate the identity of the β2AR as the immunoprecipitated, carvedilol-responsive protein species from VSMCs, we took several approaches. First, the reactivity of the anti-β2AR IgG toward either purified recombinant β2AR or to endogenously expressed receptor protein was eliminated by preblocking the antibody with purified β2AR protein (Fig. S1 A). Second, transfection of β2AR-specific siRNA decreased the immunoblotted signals for endogenous β2AR by 55% in rat VSMCs (Fig. S1 B). Third, we obtained equivalent results with epitope-tagged β2ARs overexpressed in HEK-293 cells, challenged with either the antagonist carvedilol or the agonist Iso, but not the antagonist propranolol (Fig. 1, C and D). Thus, although their SDS-PAGE migration differs from the polydisperse, hyperglycosylated β2AR bands detected in overexpression systems (Fig. 1, A and C, compare β2AR blots), the sharp bands detected in the immunoprecipitated samples from VSMCs are authentic β2AR bands. Detection of similarly “sharp” bands for endogenously expressed β2ARs has been previously shown in cardiac extracts isolated from wild-type (WT) but not β2AR knockout (KO) mice (Rohrer et al., 1996). Thus, the β blocker carvedilol, used to treat CHF, acts at the β2AR to induce ubiquitination in a manner similar to the agonist Iso but completely distinct from the antagonist propranolol.

To determine the fate of β2ARs ubiquitinated in a carvedilol-responsive manner, we labeled cell surface endogenous β2ARs in quiescent cells using an IgG (I3D6) that recognizes β2AR extracellular domains. We then traced the intracellular destination of the I3D6-bound β2ARs after treatment with different ligands. This approach proved practicable with endogenous β2ARs in VSMCs (Fig. 2, A and B; and Fig. S1, C and D), as well as with overexpressed Flag-β2ARs in HEK-293 cells (Fig. 2 C). Upon stimulation with either carvedilol or Iso, β2ARs internalize and colocalize with the Lysotracker dye that labels late endosomes and lysosomes (Fig. 2, A–C; and Fig. S1 D). These colocalization patterns were confirmed by determining Pearson’s correlation...
blocker induces β2AR degradation • Han et al.

(3D6-β2AR) along with LysoTracker red. After 1 h uptake, the antibody was washed out and cells were stimulated at 37°C for 1 h with 1 µM carvedilol, Iso, or propranolol (Prop) diluted in serum-free media. At the end of stimulation, cells were fixed, permeabilized, and labeled with a secondary antibody conjugated to Alexa Fluor 488. Images were obtained with a confocal microscope and a 100× oil objective. (C) Lysosomal trafficking of overexpressed Flag-β2ARs in HEK-293 cells assessed by the same procedure used in A and B. Bars, 10 µm. Arrows indicate colocalization of receptors and LysoTracker dye. (D–F) The bar graphs [mean ± SEM] represent Pearson’s correlation coefficients that were calculated for β2AR and LysoTracker Red colocalization in the respective cells for NS, carvedilol and Iso-stimulated conditions and were significant, as determined by one-way ANOVA [***, P < 0.001; ** P < 0.01; *, P < 0.05 versus NS; n > 20 cells for all conditions].

Effect of endocytosis inhibitors and lysine mutations on carvedilol-induced internalization and ubiquitination

Generally, agonist-induced internalization of the β2AR involves clathrin- and dynamin-dependent mechanisms (Zhang et al., 1996; Gagnon et al., 1998; Ahn et al., 1999; Pierce et al., 2000; Shenoy and Lefkowitz, 2003). To examine if carvedilol-induced internalization proceeds via these trafficking mechanisms, we treated VSMCs and HEK-293 cells with monodansylcadaverine (MDC), which inhibits receptor clustering into clathrin-coated pits (Haigler et al., 1980; Nandi et al., 1981; Phonphok and Rosenthal, 1991), and dynasore, which inhibits dynamin GTPase activity and dynamin-dependent endocytosis (Macia et al., 2006). We then determined the amount of cell surface receptor by surface anti-body labeling followed by ELISA (see Materials and methods). Both MDC and dynasore inhibited β2AR endocytosis induced by Iso (Fig. 3, A–C). In contrast, β2AR endocytosis induced by carvedilol was inhibited by dynasore, but not MDC (Fig. 3, A–C). Thus, carvedilol-induced β2AR endocytosis appears to involve clathrin-independent yet dynamin-dependent internalization mechanisms (Fig. 3, A–C).

Both MDC and dynasore augmented the levels of ubiquitinated β2ARs seen after 1 h of Iso stimulation; however, only dynasore exerted this effect on β2ARs in carvedilol-challenged cells (Fig. 3 D). These data suggest that both Iso and carvedilol promote ubiquitination of the β2AR at the plasma membrane: if ubiquitination occurred after internalization, endocytosis inhibitors would engender a decline in ubiquitinated species, rather than the increase we observed.

Previous studies indicate that Iso-stimulated ubiquitination and lysosomal degradation are ablated in a β2AR mutant in which all Lys residues are mutated to Arg (0K-β2AR; Shenoy et al., 2001; Liang et al., 2008; Xiao and Shenoy, 2011), even though
Carvedilol stimulation blocks recruitment of Nedd4

Agonist-induced lysosomal degradation of the β2AR follows its ubiquitination by the E3 ubiquitin ligase Nedd4 (Shenoy et al., 2008). Consistent with this earlier finding, Iso stimulation increased the association of endogenous Nedd4 with the β2AR (Fig. 4, A and B). In contrast, carvedilol treatment dramatically diminished the association of Nedd4 with the β2AR (Fig. 4, A and B). To test whether Nedd4 effected carvedilol-induced β2AR degradation, we used Nedd4 RNAi in VSMCs. In the presence of cycloheximide that inhibits protein synthesis, carvedilol promoted significant degradation of the β2AR within 6–24 h, and this degradation was not affected by Nedd4 knockdown (Fig. 4, F–E). In contrast, Iso-stimulated degradation was completely blocked upon Nedd4 knockdown in VSMCs (Fig. 4, F–H). These data strongly suggest that carvedilol can induce degradation of β2AR in VSMCs through mechanisms independent of Nedd4.

Carvedilol stimulation promotes dissociation of deubiquitinases and blocks receptor recycling

Ubiquitination of the β2AR is reversed by the deubiquitinases USP20 and USP33, which tonically associate with the β2AR (Berthouze et al., 2009). Because carvedilol enhanced β2AR ubiquitination and degradation independently of the known β2AR E3 ligase Nedd4, we reasoned that carvedilol might diminish β2AR deubiquitination by reducing the association of the β2AR with USP20 and/or USP33. To test this hypothesis, we assayed β2AR/USP association by coIP. Whereas USP20 and USP33 showed the expected stable association with the Iso-bound β2AR, they both dissociated from the carvedilol-bound β2AR (Fig. 5, A–D). Because USP20/33-mediated β2AR deubiquitination regulates recycling of internalized β2ARs (Berthouze et al., 2009), we asked whether this process was also affected by carvedilol. Cells were labeled with anti-β2AR (I3D6) IgG, challenged with either Iso or carvedilol for 1 h, washed, and then allowed to recover for 1 h at 37°C. Subsequent confocal microscopy revealed that both Iso and carvedilol challenged engendered β2AR internalization. However, whereas Iso-treated cells showed complete recovery of cell surface β2ARs upon inducing recycling, carvedilol-treated cells showed persistent internalization of the majority of β2ARs (Fig. 5 E). To complement these confocal microscopy studies, we also assayed cell surface β2ARs by ELISA for the extracellular domain anti-β2AR IgG. As shown in Fig. 5 F, both carvedilol and Iso induced β2AR internalization, but only Iso stimulation caused recycling of the internalized receptors. Together, these data indicate major differences between Iso- and carvedilol-triggered β2AR trafficking and protein–partner interaction, even though both ligands induce β2AR ubiquitination and endocytosis.

Agonist- and β blocker-induced trafficking involve different E3 ubiquitin ligases

Because Nedd4 is not involved in β2AR trafficking (Fig. 4), we hypothesized that the carvedilol-bound conformation of the β2AR might engage novel protein partners to mediate ubiquitination.
In contrast, Iso did not promote colocalization of MARCH2 and subsequent lysosomal trafficking. To identify \( \beta_2 \)AR colocalization as both markedly increased MARCH2/\( \beta_2 \)AR plasma membrane in unstimulated cells. Carvedilol challenge ment (Fig. S3 A).

E3 ubiquitin ligase, MARCH2, as well as several other protein partners that interact with the cytoplasmic compartment and some expression is detected at the plasma membrane (Fig. 6 A). However, we detected a small amount of colocalization of \( \beta_2 \)AR and MARCH2 at the plasma membrane in unstimulated cells. Carvedilol challenge markedly increased MARCH2/\( \beta_2 \)AR colocalization as both proteins translocated to endosomes (Fig. 6 A and Fig. S3 B). In IP assays, MARCH2 and the \( \beta_2 \)AR showed constitutive association that was augmented by carvedilol treatment (Fig. 6 B). A similar carvedilol-induced increase in association was also detected between HA-MARCH2 and \( \beta_2 \)AR (Fig. S3, B and C). In contrast, Iso did not promote colocalization of MARCH2 and \( \beta_2 \)AR (Fig. 6 A and Fig. S3 B). Unfortunately, we could not detect MARCH2 protein at endogenous levels of expression despite using four different commercially available MARCH2 antibodies (see Materials and methods) and three custom-generated ones (Nakamura et al., 2005)—all of which detected overexpressed MARCH2.

To evaluate whether MARCH2 E3 ligase activity affects carvedilol-induced binding with the \( \beta_2 \)AR and to determine if MARCH2 catalytic activity is required for its cotrafficking with the receptor, we generated and tested a MARCH2 mutant (MARCH2\(^{CCH}\)). This construct carries mutations within the \( \text{RING} \) domain (at cysteines 64 and 67 as well as histidine 90) that are critical for coordinating zinc ions (Fig. 7 A). Alteration of the zinc coordinating residues or deletion of the \( \text{RING} \) domain generally ablates enzymatic activity of \( \text{RING} \) domain E3 ligases (Joazeiro and Weissman, 2000). To assess if WT and the putative catalytically inactive MARCH2 would have differences in their subcellular distributions, we examined WT MARCH2 and MARCH2\(^{CCH}\) for their colocalization with the early endosomal marker (early endosomal antigen 1) and LysoTracker. As shown in Fig. S4, both WT and mutant constructs colocalized similarly with these vesicular markers and this did not change despite using four different commercially available MARCH2 antibodies. The bottom panel shows equivalent amounts of whole cell extracts were immunoblotted for endogenous \( \beta_2 \)AR and subsequently the blots were stripped and reprobed for \( \beta_2 \)AR (bottom). The extent of Ned4 knockdown is shown in D and G. (E and H) \( \beta_2 \)AR protein bands were quantified and normalized to respective actin levels and plotted as bar graphs. The amount of \( \beta_2 \)AR in NS cells represents 100% in each set of samples. Data shown are mean ± SEM from five independent experiments. Indicated samples were significantly decreased as compared with respective NS samples; one-way ANOVA, \( **, P < 0.001; **, P < 0.01 \). Additionally, the control and Ned4 siRNA groups were significantly different only for Iso-stimulated samples as analyzed by two-way ANOVA.

Carvedilol recruits MARCH2 to promote ubiquitination and internalization of cell surface \( \beta_2 \)ARs

In unstimulated cells, MARCH2-GFP is mostly distributed in the cytoplasmic compartment and some expression is detected at the plasma membrane (Fig. 6 A). However, we detected a small amount of colocalization of \( \beta_2 \)AR and MARCH2 at the plasma membrane in unstimulated cells. Carvedilol challenge markedly increased MARCH2/\( \beta_2 \)AR colocalization as both proteins translocated to endosomes (Fig. 6 A and Fig. S3 B). In IP assays, MARCH2 and the \( \beta_2 \)AR showed constitutive association that was augmented by carvedilol treatment (Fig. 6 B). A similar carvedilol-induced increase in association was also detected between HA-MARCH2 and \( \beta_2 \)AR (Fig. S3, B and C). In contrast, Iso did not promote colocalization of MARCH2 and \( \beta_2 \)AR (Fig. 6 A and Fig. S3 B). Unfortunately, we could not.
MARCH2CCH inhibited both ubiquitination and internalization 60 min after carvedilol treatment (Fig. 7 F). Thus, coexpression of membrane, and the protein complexes remained there even at Flag-

methods) was used to quantitate cell surface I3D6 IgG (labeling at 4°C; see Materials and Iso. Bars, 20 µm. (F) Cell surface ELISA for the only in cells that were previously treated with incubation (1 h, 37°C) to promote recycling (right, top and bottom); and after removal of I3D6 IgG, washing with culture medium, and further (middle bottom); and after removal of I3D6 at 37°C with either Iso (middle top) or Carv USP33 instead of USP20. (E) Confocal images of cells stained with 13D6 IgG depict the Flag-

USP33 instead of USP20. (E) Confocal images on NS cells. Plotted are the means ± SEM from three independent experiments: **, P < 0.01; *, P < 0.05 versus NS.

To assess the physiological relevance of MARCH2-mediated effects on the β2AR, we used siRNA-mediated knockdown of endogenously expressed MARCH2. Three different siRNA oligonucleotides targeted to mRNA sequences conserved in both mouse and rat were tested in VSMCs isolated from both species and each led to a significant decrease in mRNA levels (MARCH2-1: >80%; MARCH2-2 and MARCH2-3: 50–70% decrease; Fig. 8 A and Fig. S5 A). Upon depletion of MARCH2, carvedilol-induced β2AR ubiquitination (Fig. 8, B and C; and Fig. S5 B) was abolished in both rat and mouse VSMCs. In addition, carvedilol-induced internalization of the β2AR was also completely eliminated (Fig. 8, D and E; and Fig. S5, C and D), reiterating the link between MARCH2-induced ubiquitination and β2AR endocytosis. To determine if MARCH2 knockdown affects β2AR degradation, we performed degradation assays in the presence of cycloheximide and assessed protein levels after 6 and 24 h of carvedilol stimulation as shown in Fig. 4 C. Additionally, to confirm that these effects are specific for MARCH2 activity, we performed siRNA rescue by transfecting a plasmid encoding human MARCH2 cDNA into rat or mouse VSMCs along with the siRNA. Carvedilol-induced β2AR degradation was blocked upon down-regulating MARCH2 (Fig. 8, F and G; and Fig. S5, E and F). Furthermore, rescue of MARCH2 expression by cDNA transfection reversed this effect of MARCH2 knockdown and significant β2AR degradation was detected (Fig. 8, F and G; and Fig. S5, E and F). These data provide convincing evidence that MARCH2 mediates carvedilol-induced ubiquitination of endogenous β2ARs in VSMCs and that this ubiquitination is required for β2AR endocytosis and trafficking to the lysosomes to promote degradation of the β2AR protein.

stimulation, whereas WT MARCH2 overexpression promoted β2AR ubiquitination to the same extent as endogenous MARCH2 (Fig. 7, D and E). Contrastingly, Iso-stimulated ubiquitination was unaffected by WT MARCH2, but augmented by MARCH2CCH (Fig. 7, D and E). These data suggest that a low level of association of MARCH2 and agonist-activated β2ARs occurs, and removal of this “inhibitory” MARCH2 activity (by overexpression of MARCH2CCH) likely potentiates Nedd4-mediated ubiquitination of agonist-bound receptors.

Consistent with the binding and ubiquitination studies shown in Fig. 7 (B–E), stimulation with carvedilol, but not Iso, engendered MARCH2CCH/β2AR colocalization at the plasma membrane, and the protein complexes remained there even at 60 min after carvedilol treatment (Fig. 7 F). Thus, coexpression of MARCH2CCH inhibited both ubiquitination and internalization of carvedilol-bound β2ARs. In marked contrast to the effect of MARCH2CCH on β2AR endocytosis by carvedilol (Fig. 7 F), WT MARCH2 coexpression promoted robust internalization of the β2AR at 60 min of carvedilol stimulation (Fig. 7 G). This suggests that carvedilol-induced ubiquitination mediated by MARCH2 is critical for promoting β2AR endocytosis. These data also suggest that MARCH2 catalytic activity is not required for its interaction with the β2AR and loss of activity creates a “substrate trap” leading to a stable complex formation between the two molecules.

MARCH2 functions as a critical regulator of carvedilol-induced ubiquitination and lysosomal degradation

To assess the physiological relevance of MARCH2-mediated effects on the β2AR, we used siRNA-mediated knockdown of
Carvedilol but not propranolol promoted β2AR internalization upon carvedilol stimulation (Fig. 9 E). The internalized β2AR was colocalized with the LysoTracker dye, indicating that the internalized carvedilol-bound β2AR is sorted to lysosomes (Fig. 9, E and F). In these β-арrestin1/2 double KO MEFs, both WT MARCH2 and MARCH2(C173S) were recruited to the β2AR upon carvedilol stimulation with kinetics similar to those observed in HEK-293 cells, which express normal amounts of endogenous β-арrestins (Figs. 6 B, 7 B, and 9 G). Together, these results suggest that unlike the agonist-induced effects on the β2AR, which are dependent on β-арrestin expression, carvedilol-induced MARCH2/β2AR association, β2AR ubiquitination, internalization, and lysosomal trafficking occur in the absence of β-арrestins.

Together with previous studies, this work supports a paradigm in which carvedilol can evoke parallel and independent molecular effects by binding to the β2AR (Fig. 10). These effects include blockade of G protein coupling (Ruffolo et al., 1990), stimulation of β-арrestin–dependent signaling (Wisler et al., 2007), and induction of MARCH2-mediated β2AR ubiquitination and subsequent lysosomal degradation. Moreover, carvedilol-induced β2AR internalization requires MARCH2-mediated ubiquitination of the β2AR, suggesting that β blockers engage ubiquitin as a signal for β2AR endocytosis.

**Discussion**

We report the molecular and cellular effects of a clinically relevant β blocker, carvedilol, on its pharmacologic target β2AR in a physiologically relevant cell system. Our data reveal a novel molecular mechanism by which carvedilol (a βAR antagonist that reduces mortality in CHF) induces persistent βAR downregulation. Previous findings have shown that acute treatments with carvedilol stimulate GRK6-mediated receptor phosphorylation of the β2AR- and β-арrestin2-dependent MAPK signaling in HEK-293 cells (Wisler et al., 2007; Kim et al., 2008a; Nobles et al., 2011). In the current work, we demonstrate that prolonged treatment with carvedilol causes lysosomal trafficking and degradation of endogenous β2AR in primary VSMCs. Further, carvedilol-induced internalization occurs independent of β-арrestin binding and proceeds via clathrin-independent, yet dynamin-dependent, mechanisms. We have also discovered that carvedilol-bound β2AR is a physiological substrate for the E3 ubiquitin ligase MARCH2, and the resulting ubiquitin modification of the receptor is required for both internalization and lysosomal sorting of the β2AR.

Carvedilol-induced ubiquitination pattern of both endogenous and Flag-β2ARs is similar and detected as high molecular mass bands as reported before for agonist-stimulated ubiquitination (Shenoy et al., 2001). Because both Iso- and carvedilol-induced ubiquitination are detectable with the anti-ubiquitin antibody clone FK1 that preferentially detects polyubiquitin chains (Fujimuro and Yokosawa, 2005), both Iso and carvedilol lead to the accumulation of high molecular mass bands on Western blotting. This suggests a novel role for β-арестin1 and/or β2AR association.
Figure 7. A catalytically inactive MARCH2 mutant binds β2AR and blocks carvedilol-induced ubiquitination and internalization. (A) Schematic of WT and mutant MARCH2 constructs. RING-CH, transmembrane domains (TMD), dileucine (LL) motifs, and PDZ-interacting domains are indicated. In the MARCH2<sup>CH</sup> mutant, three residues within the catalytic domain are mutated: Cys64 and Cys67 to Ser and His90 to Gln. (B) HEK-293 cells with stable expression of Flag-β2AR were transfected with either pCDNA3 vector or HA-MARCH2<sup>CH</sup>, and Flag immunoprecipitates were analyzed for bound MARCH2 after carvedilol or Iso stimulation for different times. (C) The signals for MARCH2 in β2AR immunoprecipitates were normalized to the respective β2AR amounts in the IP and plotted as mean ± SEM quantified from three independent experiments.**, P < 0.01; *, P < 0.05 versus NS sample. (D) HEK-293 cells with stable expression of Flag-β2AR were transfected with either WT MARCH2 or MARCH2<sup>CH</sup> and challenged ±1 µM carvedilol or Iso stimulation for 1 h, and then solubilized and analyzed for ubiquitination. Flag-β2AR immunoprecipitates were immunoblotted serially for ubiquitin (top) and the β2AR (middle). Cell lysates were immunoblotted for MARCH2 (bottom). (E) The bar graph presents β2AR ubiquitination normalized to cognate β2AR band density as mean ± SEM quantified from three independent experiments.**, P < 0.01; *, P < 0.05 versus NS sample from vector transfection. [F] The confocal panels show the subcellular distribution of Flag-β2AR (red) and MARCH2<sup>CH</sup>-GFP (green) alone or together (Merge) in quiescent cells and after stimulation with either 1 µM carvedilol or Iso for 2 or 60 min. (G) Confocal images show robust internalization of Flag-β2AR and colocalization (arrows) with WT MARCH2-GFP after 60-min carvedilol treatment. Bars, 10 µm.

To polyubiquitination of the β2AR. We recently reported that Iso-induced β2AR ubiquitination involves 5 of the 14 intracellular lysines located in the third intracellular loop and carboxyl tail regions of the human β2AR (Xiao and Shenoy, 2011). However, whether Iso and carvedilol target the same intracellular lysines for ubiquitination remains to be determined. Intriguingly, because carvedilol induces ubiquitination of the lysineless β2AR mutant (OK-β2AR) and because the viral counterparts of MARCH2 RING domain ligases have been shown to target noncanonical sites for ubiquitination (Cadwell and Coscoy, 2005), carvedilol-induced ubiquitination might involve cysteines and/or lysines or possibly other noncanonical sites (serines or threonines) within the β2AR.

Although both Iso and carvedilol stimulate comparable β2AR ubiquitination, the regulatory components and cellular consequences are distinct. Agonist-dependent ubiquitination requires β-arrestin2 (Shenoy et al., 2001, 2008), whereas carvedilol-induced ubiquitination does not; agonist-dependent ubiquitination is mediated by the HECT domain E3 ligase Nedd4 (Shenoy et al., 2008; Nabhan et al., 2010), whereas carvedilol-induced ubiquitination involves MARCH2 and not Nedd4; agonist-dependent internalization occurs in the absence of ubiquitination (Shenoy et al., 2001, 2008; Liang et al., 2008; Xiao and Shenoy, 2011), whereas carvedilol-induced internalization occurs only upon receptor ubiquitination by MARCH2; agonist-occupied β2ARs can recycle and engage USP20 and USP33 activities, whereas carvedilol binding blocks recycling and binding of these USPs. These differences are likely because of the distinct receptor conformations induced by each ligand (Galandrin et al., 2007; Rosenbaum et al., 2009; DeWire and Violin, 2011), which leads to binding of specific protein partners to mediate distinct cellular consequences. Recently arrestin-like proteins have been shown to be important for mediating the trafficking of plasma membrane receptors and channels in yeast and mammalian systems (Lin et al., 2008; Nikko et al., 2008; Polo and Di Fiore, 2008; Nabhan et al., 2010; Becuwe et al., 2012). However, these proteins may not play an important role in carvedilol-induced β2AR trafficking because they act mainly as protein partners for HECT domain E3 ligases and may not critically regulate the RING domain–containing MARCH family ligases.

Carvedilol, is currently defined as a β-arrestin–biased agonist because it can stimulate β-arrestin–dependent signaling while blocking G protein coupling (Wisler et al., 2007). Our findings...
show that this β-arrestin–biased agonist can lead to β-arrestin–
independent pathways: recruitment of MARCH2 and β-arrestin–
independent endocytosis of the β2AR. Notably, both early endocytosis and postendocytic sorting effects induced by this 
bias agonist are dependent on MARCH2. This reveals yet
another tier of complexity of receptor–ligand interaction, where 
a ligand can be biased toward a particular protein recruitment
and is often associated with endosomal membranes. MARCH2
is a recently characterized E3 ubiquitin ligase that is ubiquitously expressed
in human heart and vasculature. At therapeutic doses, the peak
immediate activity and is also a blocker of
nonselective β-blocker induces β2AR degradation
and may even increase the incidence of heart failure in hy-
pertensive patients (Lasagna, 2000). Carvedilol has also been
shown to reduce myocardial infarction and morbidity in ani-
mals models of coronary artery occlusion, where propranolol
alone does not confer an advantage in CHF (Cohn et al., 1986)
and other
β-blockers had no significant effect (Hamburger et al.,
1992). Whether MARCH2 is recruited by other PDZ domain
proteins such as NHERF1 and 2 that regulate
2AR trafficking, and degradation of endogenous
β2ARs. (A) Rat VSMCs were transfected with either control (CTL) or MARCH2 siRNA; total RNA was extracted 48 h later and subjected to
RT-PCR for MARCH2 or GAPDH, as indi-
cated. There was an 80 ± 6% knockdown of MARCH2 mRNA (n = 6). (B) Rat VSMCs
transfected with CTL or MARCH2 siRNA
were stimulated with carvedilol (1 h, 37°C),
and βAR immunoprecipitates were probed
with anti-Ub (top) or anti-β2AR (bottom) IgG.
(C) The ubiquitin signals were quantified, nor-
malized to the β2ARs in each IP sample, and
plotted as bar graphs (mean ± SEM). Compared
with NS: *, P < 0.05; **, P < 0.01; ***, P < 0.001;
n > 20 VSMCs for all conditions. (F) Rat
VSMCs were transiently transfected with con-
trol or MARCH2 siRNA along with either vec-
tor (HA-pCDNA3) or HA-MARCH2. Cells were then
stimulated with 1 μM carvedilol for indi-
tanced times in the presence of 20 μM cyclo-
heximide, and then solubilized. VSMC lysates
were immunoblotted serially with IgG specific
for the β2AR, HA, and β-actin. (G) The signals
for β2AR were quantified from eight indepen-
dent experiments, normalized to β-actin levels,
and plotted as mean ± SEM. As analyzed
by two-way ANOVA, only carvedilol-treated
MARCH2 siRNA + pCDNA3 samples were
significantly different from the counterparts in
all other groups. Statistical analysis per one-
way ANOVA within each group is displayed.
Compared with cognate NS VSMCs: *, P < 
0.05; **, P < 0.01; ***, P < 0.001.
plasma level of carvedilol is 100 ng/ml and the drug is mostly bound to plasma proteins. However, it accumulates in extra-vascular tissues, which could affect βAR cell surface expression and lead to prolonged blockade of βAR signaling. Other recent studies also suggest that in heart failure models, where β1AR is down-regulated, β2ARs are not and become redistributed and reprogrammed to carry out β1-AR-like functions, thus contributing to progression of heart failure (Nikolaev et al., 2010). This suggests that down-regulating the redistributed β2ARs with carvedilol may be beneficial in heart failure patients. We believe that in this scenario MARCH2 activity might be critical in mediating the beneficial effects exerted by carvedilol in orchestrating cell surface levels of β2AR and consequently its signaling.

Materials and methods

Cell lines
Human embryonic kidney cells (HEK-293) were purchased from American Type Culture Collections and maintained in minimal essential medium containing 10% FBS and 1% penicillin/streptomycin (P/S). HEK-293 cells stably transfected with Flag-β2AR or Flag-β1AR-YFP have been described previously (Shenoy et al., 2008; Berthouze et al., 2009). These cells were generated by transfecting early passage HEK-293 cells with 1 µg of plasmid DNA: Flag-β2AR/pcDNA3 or Flag-β1AR-mYFP/pcDNA3 and positive clones were selected against 1 mg/ml G418. Stable cells were further maintained by the addition of 400 µg/ml of G418 to the culture media. Aortic VSMCs were isolated from adult rats or mice using protocols reported previously and were maintained in DMEM supplemented with 10% FBS and 1% P/S (Kim et al., 2008b). To isolate these primary cells, aortas were dissected, washed with saline buffer, separated from the adventitia and endothelial cells, and then digested at 37°C for 1.5 h with 0.1% collagenase II, 15 U/ml elastase, and 0.1% soybean trypsin inhibitor mixed in PBS. Released smooth muscle cells were cultured in DMEM with 20% FBS and 1% P/S (Life Technologies) immediately after isolation and subsequent subcultures were maintained in DMEM supplemented with 10% FBS and 1% P/S. All experiments were repeated in cells isolated at least three independent times. Animals (C57/Bl6 mice or Sprague-Dawley rats) were purchased from vendors and housed in Duke University animal facilities. All animal procedures were performed according to protocols approved by Duke University Institutional Animal Care and Use Committee.

Plasmids and transfections
Human MARCH2/pCMV6-XL5 plasmid was purchased from OriGene. HA-MARCH2/pcDNA3 and MARCH2-pEGFPN1 were generated using standard cloning methods. To generate MARCH2CH, three amino acid mutations (cysteines 64 and 67 to serines and histidine 90 to glutamine) were introduced by QuikChange site-directed mutagenesis protocol. HEK-293 cells transfected with Flag-β2AR along with vector, β-arrestin1+HA or β-arrestin2+HA plasmids. Flag immunoprecipitates were immunoblotted with anti-Flag and anti-β2AR antibodies. Bars, 10 µm. (F) The bar graph (mean ± SEM) represents Pearson’s correlation coefficients that were calculated for β2AR and LysoTracker red colocalization for NS and carvedilol-stimulated conditions. ***, P < 0.0001; n > 20 cells for each condition. (G) β-Arrestin1/2 KO MEFs with stable expression of the Flag-β2AR (600 fmol/mg of cellular protein) were transfected with either MARCH2CH-GFP or MARCH2CH-GFP stimulated with carvedilol for the indicated times. Flag immunoprecipitates and MEF lysate blots were immunoblotted serially for ubiquitinated receptors. Bars, 10 µm. (A) β-Arrestin1/2 double KO MEFs transfected with the Flag-β2AR were pretreated with ±1 µM ICI 118,551 and then stimulated with ±10 µM carvedilol (1 h, 37°C). Flag immunoprecipitates were isolated and probed for ubiquitinated β2ARs (top) and total β2ARs (second panel). The bottom two panels show expression levels of β2AR and β-actin as detected in solubilized extracts. The ubiquitin signal in each IP was quantified and normalized to the corresponding Flag-immunoprecipitates and MEF lysate blots were immunoblotted serially for ubiquitinated receptors. Bars, 10 µm. (B) The ubiquitin signal in each IP was quantified and normalized to the corresponding Flag-immunoprecipitates and MEF lysate blots were immunoblotted serially with anti-Ub and anti-β2AR antibodies. **, P < 0.01; *, P < 0.5 versus pCDNA3-NS. (C) β-Arrestin ubiquitination was quantitated as in Fig. 1 D and plotted as the means ± SEM from four independent experiments. ***, P < 0.001; *, P < 0.5 versus pCDNA3-NS. (D) Subcellular distribution of Flag-β2AR as detected by surface labeling (as in Fig. 2) is shown in green and LysoTracker in red. After 1 h of carvedilol stimulation, receptors are seen in endocytic vesicles and partially colocalized with LysoTracker [arrows]. Bars, 10 µm. (F) The bar graph (mean ± SEM) represents Pearson’s correlation coefficients that were calculated for β2AR and LysoTracker red colocalization for NS and carvedilol-stimulated conditions. ***, P < 0.0001; n > 20 cells for each condition. (G) β-Arrestin1/2 KO MEFs with stable expression of the Flag-β2AR (600 fmol/mg of cellular protein) were transfected with either MARCH2CH-GFP or MARCH2CH-GFP stimulated with carvedilol for the indicated times. Flag immunoprecipitates and MEF lysate blots were immunoblotted serially for MARCH2 and the β2AR, as indicated. Shown are results from a single experiment, representative of three independent experiments.

Antibodies
Anti-β2AR (M20, H20, and I3D6) antibodies were obtained from Santa Cruz Biotechnology, Inc. Anti-Nedd4 WW domain (EMD Millipore) was used to detect endogenous Nedd4 in HEK-293 cells. Anti-Nedd4 antibody (Cell Signaling Technology) was used for Western blots of rat VSMCs. Custom-generated anti-MARCH2 antibodies, anti-MAR2-Δ41, anti-MAR2-Δ51 (immunoblotting shown in Western blot panels), and anti-MAR2-Δ384, have been reported previously (Nakamura et al., 2005). Anti-peptide antisera (anti-MAR2-Δ41 and anti-MAR2-Δ51) were generated in rabbits against synthetic peptides corresponding to residues 212–230 and to 42–61
Published November 19, 2012

- Carvedilol induces distinct molecular effects. Upon binding to the β2AR, carvedilol prevents G protein coupling, recruits β-arrestin transiently to induce pERK signaling, and forms a stable complex with MARCH2 to promote ubiquitin-dependent endocytosis and lysosomal degradation. Carvedilol also blocks the association of the β2AR with Nedd4, and USP20 and USP33, all of which are recruited to the β2AR when it is activated by iso.

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**Figure 10.** Carvedilol induces distinct molecular effects. Upon binding to the β2AR, carvedilol prevents G protein coupling, recruits β-arrestin transiently to induce pERK signaling, and forms a stable complex with MARCH2 to promote ubiquitin-dependent endocytosis and lysosomal degradation. Carvedilol also blocks the association of the β2AR with Nedd4, and USP20 and USP33, all of which are recruited to the β2AR when it is activated by iso.

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**Carvedilol induces distinct molecular effects.** Upon binding to the β2AR, carvedilol prevents G protein coupling, recruits β-arrestin transiently to induce pERK signaling, and forms a stable complex with MARCH2 to promote ubiquitin-dependent endocytosis and lysosomal degradation. Carvedilol also blocks the association of the β2AR with Nedd4, and USP20 and USP33, all of which are recruited to the β2AR when it is activated by iso.

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**IP and immunoblotting**

Rat or mouse VSMCs were serum deprived for 1 h and then stimulated with vehicle or carvedilol for 1 h. At the end of incubation, cells were harvested in a lysis buffer containing 50 mM Hepes, pH 7.5, 0.5% NP-40, 250 mM NaCl, 2 mM EDTA, and 10% (vol/vol) glycerol. All buffers were supplemented with protease inhibitors. Harvested cells were further solubilized by adding n-Dodecyl β-maltoside (1% final) and incubating on a rotator at 4°C for 1–2 h. After this, samples were centrifuged, soluble extracts were prepared, and protein concentrations were determined by Bradford analysis. Equal amounts of lysates were mixed with 2 μg anti-β2AR M20 or normal rabbit IgG along with protein A/G agarose beads and rotated overnight at 4°C. Nonspecific binding in the immunoprecipitate was eliminated by repeated washes with lysis buffer, and bound protein was eluted with sample buffer containing SDS. Samples were incubated at 37°C for 30 min before SDS-PAGE. The eluted proteins were separated on a gradient gel (4–20%; Invitrogen) and transferred to nitrocellulose membrane (0.2 μm; Bio-Rad Laboratories) for Western blotting. Anti-ubiquitin antibody was first used to probe the blots for ubiquitinated receptor bands, after which membranes were stripped (stripping solution; Thermo Fisher Scientific) and reacted with an anti-β2AR antibody (M-20; Santa Cruz Biotechnology, Inc.) to detect receptors in the immunoprecipitates. Protein A-HRP was used instead of an anti–rabbit secondary antibody to minimize signals from IgG bands (Lal et al., 2005). Chemiluminescence detection was performed using SuperSignal West Pico or Femto reagent (Thermo Fisher Scientific). Signals were detected using a charge-coupled device camera (Chemidoc XR5: Bio-Rad laboratories) and quantified using ImageLab 3.0 software (Bio-Rad Laboratories). Analysis of Nedd4/β2AR and MARCH2/β2AR interactions were performed using Dithio-bis-maleimidoethane (DTME; Thermo Fisher Scientific) as described previously (Shenoy et al., 2007). Cells plated on poly-l-lysine-coated 100-mm dishes were stimulated at 37°C in PBS containing 10 mM Hepes, pH 7.5, with vehicle or agonist. Stimulations were terminated by the addition of DTME to a final concentration of 2 mM, and plates were rocked for 40 min at room temperature. Cells were washed three times with PBS/ Hepes to remove unreacted DTME and lysed in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, 1% NP-40, and 0.5% deoxycholate) and receptors were immunoprecipitated.

**Immunostaining of endogenous β2AR in VSMCs**

Early passage (<6) VSMCs were plated on collagen-coated glass-bottomed dishes (MatTek Corporation). When needed, the cells were transfected with siRNA using Lipofectamine 2000 as described in the previous section. Cells were washed once with DMEM containing 0.1% BSA and 10 mM Hepes (DMEM-BSA), pH 7.5, and covered with the mouse monoclonal β2AR antibody I3D6 in DMEM-BSA (dilution 1:100). LysoTracker red was added to the antibody solution (1:1,000 dilution) for simultaneous uptake of the dye. The antibody feeding was performed at room temperature for 1 h. After, the cells were stimulated with vehicle or carvedilol and returned to a 37°C incubator for desired times, and then, at the end of stimulation, the cells were washed with PBS and fixed with 5% formaldehyde for 30 min. Secondary antibody (anti–mouse IgG) conjugated to Alexa Fluor 488 was added and the cells were labeled at 4°C overnight. Immunostained cells (on the glass-bottom dish) covered with PBS buffer were imaged at room temperature with a confocal microscope (LSM 510 META; Carl Zeiss) using a Plan-Apochromat 100× NA 1.4 objective lens (Carl Zeiss). All confocal analyses were performed on samples from three to five independent experiments. In each experiment, several cells or groups of cells were analyzed. Image acquisition used the LSM 510 operating software and images were later exported as TIFF files. Further processing (resizing, addition of text, etc.) was performed using Adobe Photoshop software (CS2) and any change in brightness/contrast was applied to the images and images were later exported as TIFF files. Further processing (resizing, addition of text, etc.) was performed using Adobe Photoshop software (CS2) and any change in brightness/contrast was applied to the entire image. Pearson’s correlation coefficients for quantification of β2AR-LysoTracker colocalization was performed in ≥20 cells from multiple independent experiments using ImageJ software (National Institutes of Health).

**Internalization by ELISA**

To quantify cell surface β2ARs, cells were plated in either 24- or 96-well dishes. Cell surface receptors were prelabeled with the mouse monoclonal 1D6-β2AR antibody (Santa Cruz Biotechnology, Inc.) diluted at 1:300 in serum-free media containing 10 mM Hepes, pH 7.4, and 0.01% BSA at 4°C for 1 h. Cells were then washed and exposed to either...
carvedilol or Iso (both at 1 μM) for 1 h at 37°C. Subsequently, cells were washed with PBS and labeled with alkaline phosphatase–conjugated goat anti–mouse antibody for 1 h at 4°C. After this, unbound antibodies were removed by repeated washing with PBS and color development was induced by adding one-step nitrophenyl phosphate disodium salt (Thermo Fisher Scientific). After 10 min of development, the reaction was stopped by adding 2N NaOH. Absorbance was measured at 405 nm on a plate reader (Bio-Rad laboratories). Samples labeled with secondary antibody alone were used as background controls. The amount of cell surface receptors is presented as a percentage of cell surface receptors in unstimulated cells. For the experiments involving endocytosis inhibitors, either vehicle (DMSO), 100 μM MDC, or 80 μM dynasore were added 5 min before stimulation of the cells. For experiments involving recycling, ligands were removed by washing and adding fresh media (warmed at 37°C) containing 10 mM Hepes, pH 7.4, and 0.01% BSA and incubation at 37°C for 1 h.

Proteomics analysis HEK-293 cells stably transfected with Flag-β2AR were used for preparation of the β2AR complexes. Cells were grown to ~75% confluency and treated with buffer, 10 μM Iso or 10 μM carvedilol for 6 h before harvesting. The harvested cells were solubilized with Lysis buffer (50 mM Hepes, 0.5% NP-40, 250 mM NaCl, 10% Glycerol, and 2 mM EDTA). Next, n-Dodecyl β-c-maltoside (1% final) was added and samples were rotated at 4°C for 1–2 h. The solubilized β2AR complexes were isolated with anti-Flag [M2] affinity agarose beads, eluted with sample buffer, and separated by SDS-PAGE [4–20%, gradient gel; Invitrogen]. Each sample lane on the SDS-PAGE gel was demarcated in to four to five sections, excised, chopped into small pieces, and subjected to in-gel trypsin digestion. In brief, the gel pieces were destained by 25 mM of ammonium bicarbonate in 50% acetonitrile. The samples were reduced by 2 mM dithiothreitol, alkylated by 10 mM iodoacetamide, and then subjected to trypsin [final concentration of 5 ng/μL] digestion at 37°C overnight. Tryptic peptides were subjected to LC/MS/MS analyses on an LTQ Orbitrap XL (Thermo Scientific) with a Finnigan Nanospray II electrospray ionization source. Tryptic peptides were injected onto a 75 μm × 150 mm BEH C18 column (particle size 1.7 μm; Waters) and separated using a nanoACQUITY Ultra Performance LC system (Waters; Xia and Shenoy, 2011). The LTQ Orbitrap XL was operated in the data-dependent mode using the TOP10 strategy (Hoa et al., 2006). Each scan cycle was initiated with a full MS scan of high mass accuracy (400–2,000 m/z; acquired in the Orbitrap XL at 6 × 104 resolution setting and automatic gain control target of 106). This was followed by MS/MS scans (automatic gain control target of 5,000; threshold 3,000) in the linear ion trap on the 10 most abundant precursor ions. Selected ions were dynamically excluded for 30 s. Singly charged ions were excluded from MS/MS analysis. MS/MS spectra were searched by using the Mascot (Matrix Sciences, Inc.) algorithm against a composite database containing the SwissProt Homo sapiens [human] protein sequences and their reverse sequences. Search parameters allowed two missed tryptic cleavages, a mass tolerance of ±10 ppm for precursor ion, a mass tolerance of ±0.02 Da for product ion, a dynamic modification of 57.02146 D containing a chloroform extraction and isopropanol precipitation. To confirm the reduction of MARCH2 RNA levels by treatment of MARCH2-specific siRNA, RT-PCR analyses were performed using Prism software (version 5; GraphPad Software). P < 0.05 at the 95% confidence level was considered significant.

Online supplemental material Fig. S1 shows immunoblotting and immunostaining specificity and negative controls for the detection of endogenously expressed β2ARs in VSMCs. Fig. S2 shows carvedilol-stimulated trafficking of YFP-tagged β2ARs. Fig. S3 includes identification of novel regulators of β2AR trafficking using proteomics. Fig. S4 shows subcellular distribution of MARCH2 and MARCH2ΔCterminus a carvedilol. Fig. S5 demonstrates that carvedilol-induced ubiquitination, internalization, and degradation of endogenous β2ARs in mouse VSMCs are mediated by MARCH2. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201208192/DC1.

We thank Dr. R.J. Leffkowitz for insightful comments and for providing β-adrenergic receptor reagents. We also thank Drs. Arthur Moseley, Will Thompson, and Erik Sodeblom for their input in the proteomics experiments; and Ms. Vidya Venkat for technical help.

We acknowledge support from the National Institutes of Health (HL 080525-15 S.K. Shenoy, HL 077185 to N.J. Freedman, and HL 075443; Proteomics Core support to K.H. Xiao). S. Han was supported by the American Recovery and Reinvestment Act stimulus award (HL 080525-04S1).

Submitted: 31 August 2012
Accepted: 18 October 2012
