Han et al., http://www.jcb.org/cgi/content/full/jcb.201208192/DC1

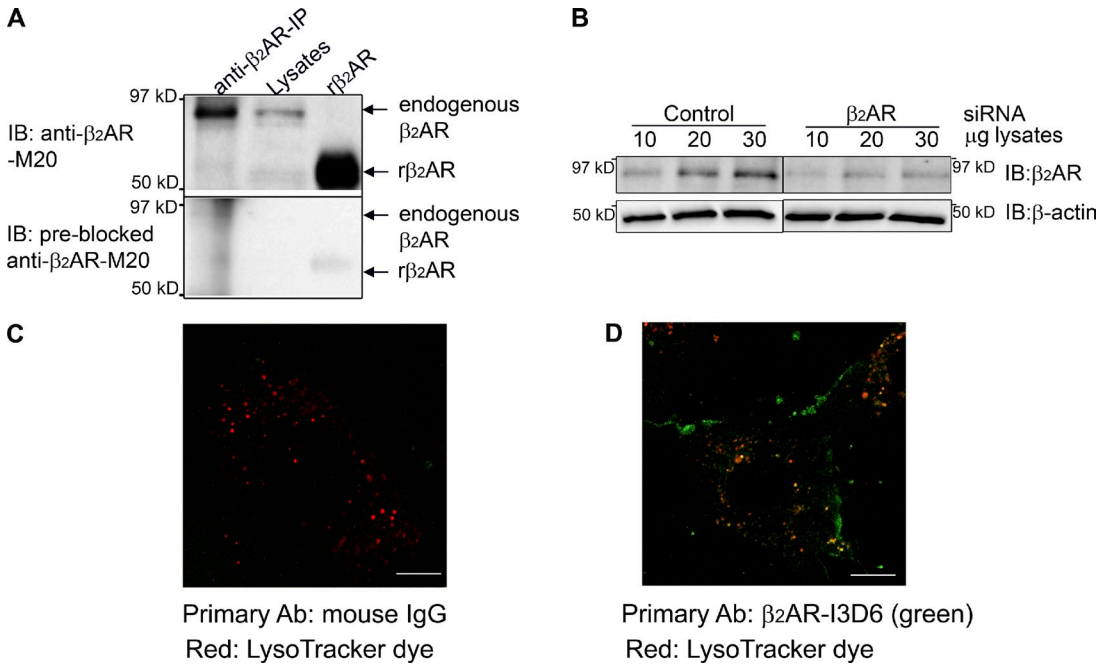


Figure S1. **Immunoblotting and immunostaining specificity of endogenous β_2ARs in VSMCs.** (A) Endogenous β_2ARs (rat VSMCs) immunoprecipitated with anti-β_2AR IgG, whole cell extracts (rat VSMCs), and purified recombinant β_2AR protein (3 ng) were probed with β_2AR–M-20 antibody as such (top) or after preblocking (1 ng of protein and 1 µg of antibody) with purified β_2AR protein (bottom). (B) Rat VSMCs were transfected with siRNA targeting no mRNA or β_2AR mRNA and the indicated amounts of lysate protein were analyzed for the specific β_2AR band as detected by anti-β_2AR IgG (M-20). β_2AR knockdown led to >50% decrease of this band. Mouse VSMCs were incubated at room temperature for antibody surface labeling with normal mouse IgG (C) and I3D6-β_2AR (D) along with LysoTracker red. After 1-h uptake, the antibody was removed and cells were stimulated with Iso for 20 min, fixed, and labeled with anti–mouse IgG conjugated to Alexa Flour 488. Images were obtained with a confocal microscope and a 100× oil objective. Bars, 10 µm.

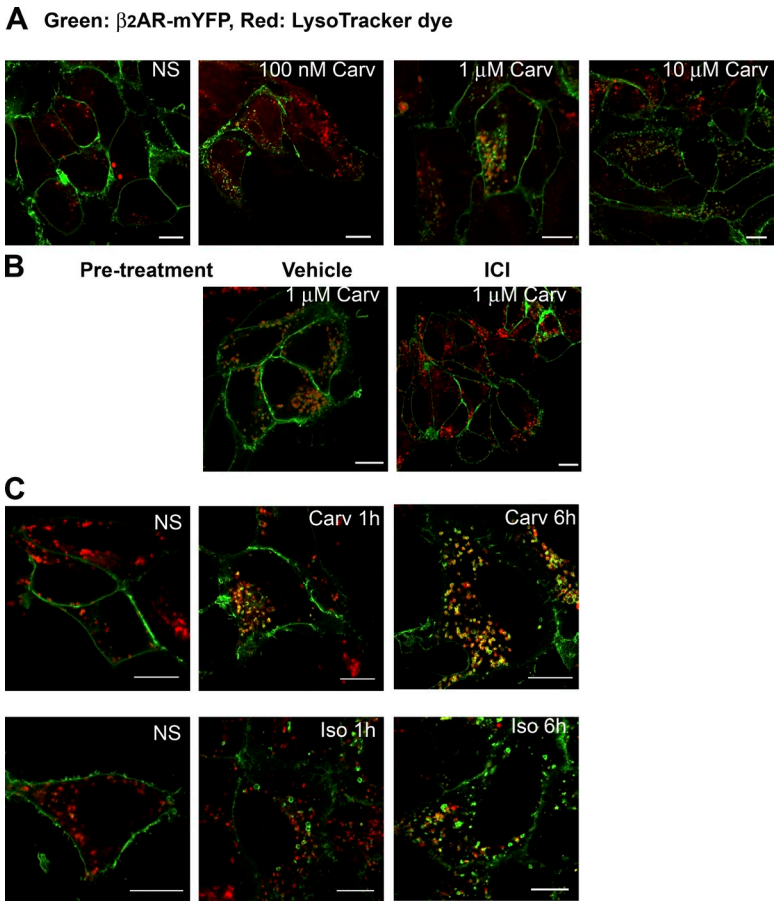
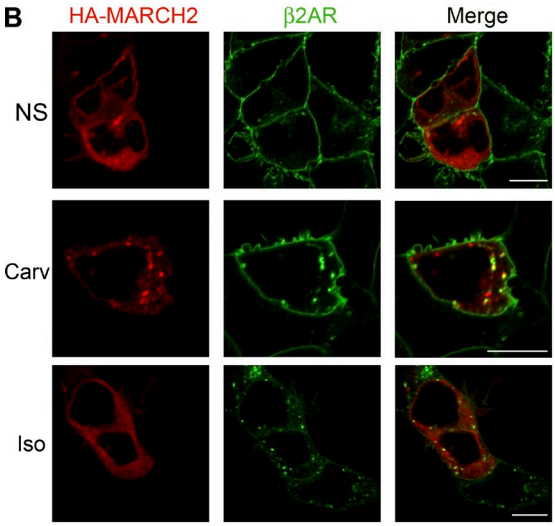


Figure S2. **Carvedilol-stimulated trafficking of YFP-tagged β_2ARs.** (A) HEK-293 cells stably transfected with Flag-β_2AR-YFP and labeled with LysoTracker red were stimulated with the indicated concentrations of carvedilol for 1 h and then imaged by confocal microscopy. Yellow pixels indicate colocalization of the β_2AR-YFP with LysoTracker red. (B) Cells identical to those in A were pretreated ±20 μM ICI 118,551and then imaged as in A. (C) Cells identical to those in A were stimulated with 1 μM carvedilol or 1 μM Iso along with LysoTracker red for the indicated times, fixed, and analyzed by confocal microscopy. At 1 h, β_2AR-LysoTracker colocalization is visualized (yellow) in carvedilol-treated samples but no significant lysosomal trafficking is detected in Iso-treated samples. At 6 h, carvedilol-treated samples show more β_2ARs in the lysosomes than Iso-treated samples. Green, β_2AR-mYFP; red, LysoTracker; yellow, colocalization. Bars, 10 μm.

A

Accession	Protein Description
BUB1B	Mitotic checkpoint serine/threonine protein Kinase BUB1 beta
DOK3	Docking protein 3
FGD6	FYVE, RhoGEF and PH domain-containing protein6
KI16B	Kinesin-like protein KIF16B
MACF1	Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5
MARH2	**E3 ubiquitin protein ligase MARCH2**
MEP50	Methylosome protein 50
MYL6B	Myosin light chain 6B
TTYH2	Protein tweety homolog 2
ZEP1	Zinc finger protein 40



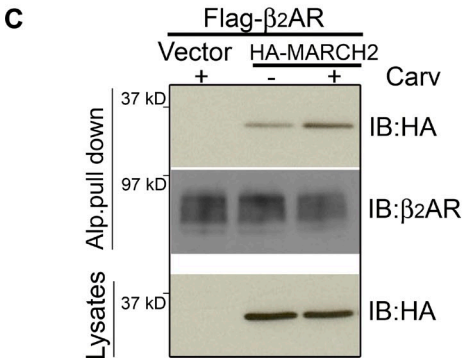


Figure S3. **Identification of novel regulators of β_2AR trafficking using proteomics.** (A) List of proteins that are unique to carvedilol-treated samples, which were not detected in repeated MS runs of NS or Iso-stimulated samples. (B) HEK-293 cells stably overexpressing β_2ARs were transfected with a plasmid encoding HA-MARCH2. Cells were then stimulated with carvedilol or Iso, fixed, permeabilized, and immunostained to detect subcellular distributions of the β_2AR and HA-MARCH2. Representative confocal images are shown for NS, carvedilol-, and Iso-stimulated localization. Bars, 10 μm. (C) β_2AR complexes were isolated with alprenolol-agarose affinity beads and HA-MARCH2 associated with the β_2AR was detected by immunoblotting with the monoclonal anti-HA antibody 12CA5. This method of pull-down helps to avoid interference from the IgG light chain, the molecular mass of which is close to that of HA-MARCH2 (~27 kD).

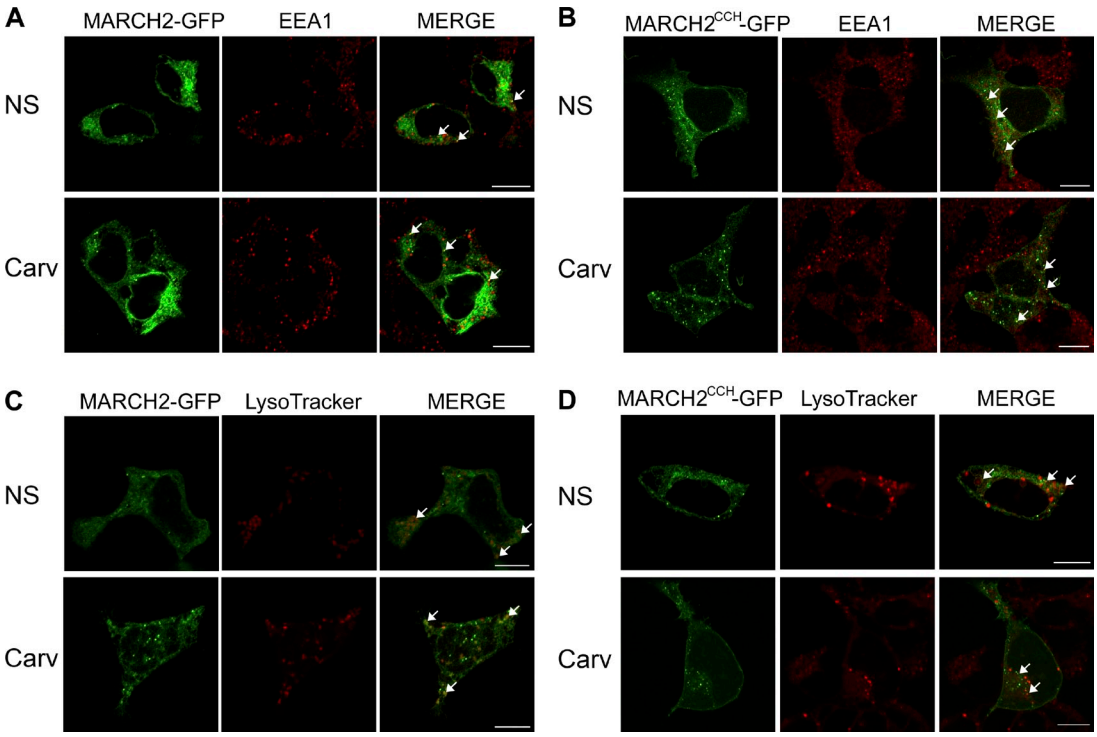


Figure S4. **Subcellular distribution of MARCH2 and MARCH2^{CCH}.** MARCH2-GFP (A and C, green) or MARCH2^{CCH}-GFP (B and D, green) was transiently expressed in HEK-293 cells that were stably transfected with the Flag-β_2AR. Quiescent or carvedilol-treated cells (1 μM carvedilol, 1 h, 37°C) were fixed, permeabilized, and immunostained for early endosomal antigen 1 (EEA1), a marker for early endosomes (A and B) or LysoTracker red, a late endosomal/lysosomal marker dye (C and D, red). Merged images show colocalization of MARCH2-GFP or MARCH2^{CCH}-GFP with these markers. Arrows in the merged images indicate some of the vesicles positive for colocalization. The images are from one of three independent experiments with similar results. Bars, 10 μm.

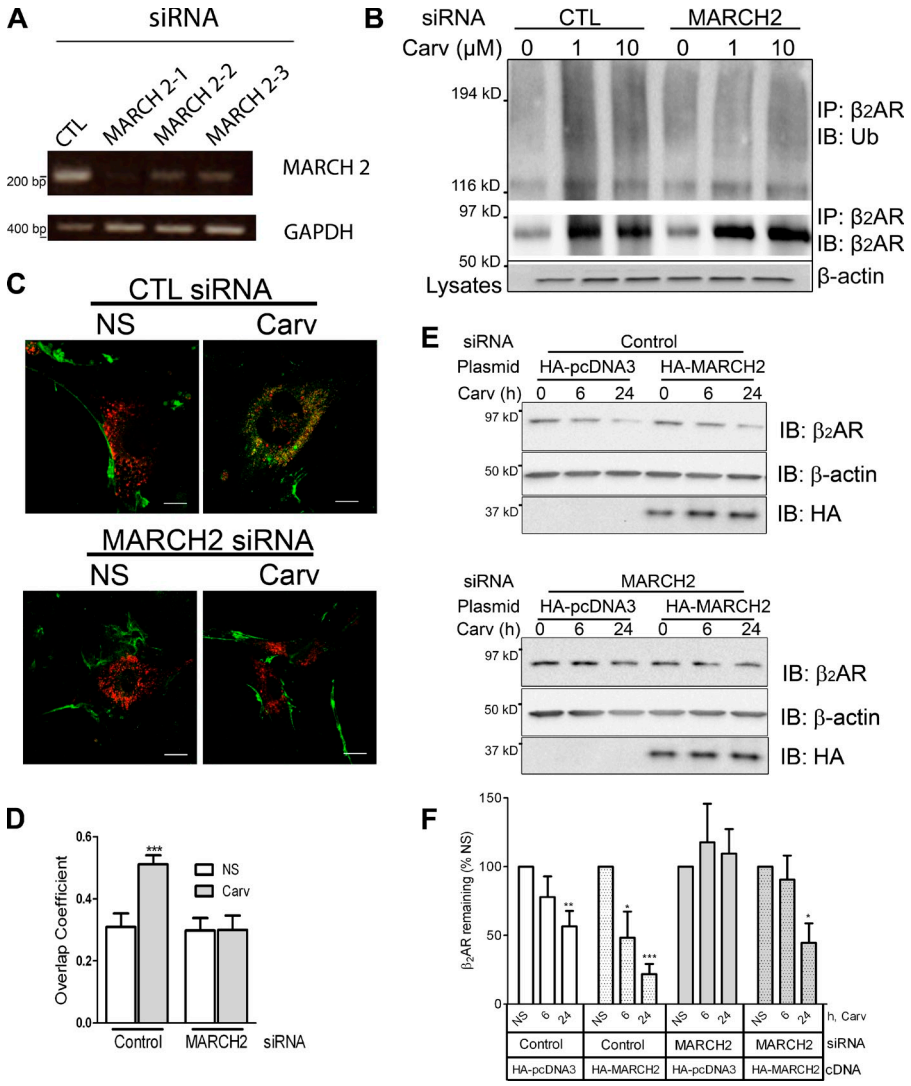


Figure S5. **MARCH2 regulates carvedilol-induced ubiquitination and degradation of endogenous β_2ARs in mouse VSMCs.** (A) Mouse VSMCs were transfected with siRNA oligonucleotides targeting no known protein (CTL) or MARCH2 mRNA; 3 d later, total RNA was extracted and RT-PCR was performed for MARCH2 or GAPDH. (B) Mouse VSMCs were transiently transfected with siRNA targeting no mRNA (CTL), or MARCH2. Cells were then stimulated with carvedilol for 1 h and β_2AR immunoprecipitates were probed with anti-Ub or anti-β_2AR IgG. (C) Trafficking of endogenous β_2ARs in NS and carvedilol-stimulated cells was assessed by cell surface antibody labeling as in Fig. 2 in cells with normal or depleted levels of MARCH2. Bars, 20 μm. (D) The bar graphs (mean ± SEM) represent Pearson's correlation coefficients that were calculated for β_2AR and LysoTracker red colocalization for NS and carvedilol-stimulated conditions. ***, $P < 0.001$; one-way ANOVA; $n > 20$ cells for all conditions. (E) Mouse VSMCs were transiently transfected with CTL or MARCH2 siRNA along with either vector (HA-pCDNA3) or HA-MARCH2 (human MARCH2 cDNA). Cells were then stimulated with 1 μM carvedilol for the indicated times in the presence of 20 μM cycloheximide, and then solubilized. Whole cell extracts were immunoblotted successively for β_2AR, HA-MARCH2, and β-actin. (F) The signals for the β_2AR band were quantified from six independent 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: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus respective NS samples.