Intact MDM2 E3 ligase activity is required for the cytosolic localization and function of β-arrestin2

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ABSTRACT **β-arrestins are well known for their roles in desensitization and sequestration of G protein–coupled receptors. Unlike β-arrestin1, β-arrestin2 exhibits a predominant cytoplasmic distribution at steady state. However, the mechanism and functional significance underlying the regulation of β-arrestin2 subcellular localization remains undefined. Here we report that the subcellular localization and function of β-arrestin2 is tightly regulated by Mdm2 E3 ligase activity. Inhibition of Mdm2 E3 ligase activity either by expressing Mdm2 RING finger mutants or using specific Mdm2 E3 ligase inhibitor is sufficient to stabilize the Mdm2/β-arrestin2 complex and cause abnormal nuclear localization of β-arrestin2. Next we demonstrate that lysine residues at position 11 and 12 of β-arrestin2 are required for the interaction between Mdm2 RING finger mutant H457S ($Mdm2^{H457S}$) and β-arrestin2, mutation of which prevents $Mdm2^{H457S}$/β-arrestin2 interaction and subsequent nuclear localization of β-arrestin2. Finally, β-arrestin2–dependent signalings, such as receptor internalization and extracellular signal–regulated protein kinase activation, are found to be impaired once the β-arrestin2 is sequestered in the nuclei by $Mdm2^{H457S}$. Our findings depict the essential role of Mdm2 E3 ligase activity in determining β-arrestin2 subcellular localization and corresponding signaling.**

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INTRODUCTION

The investigation of G protein–coupled receptor (GPCR) signaling reveals a highly conserved "switch-off" model characterized by the involvement of GPCR kinases (GRK) and arrestins (Ferguson *et al.*, 1996; Ferguson, 2001). β-arrestin1 and β-arrestin2 (also known as arrestins 2 and 3) are well known for their roles in desensitization and internalization of most GPCRs (Laporte *et al.*, 1999; Shenoy and Lefkowitz, 2003). On agonist stimulation, the receptor is rapidly phosphorylated by GRKs, which promotes the recruitment and association of β-arrestins to the receptor. The binding of β-arrestins prevents the receptor–G protein interaction, leading to the inhibition of the G protein signaling. The association of β-arrestins also facilitates the sequestration of the receptors in the clathrin-coated pits, which leads to the endocytosis and sequestration of the receptors in various intracellular compartments.

During the past decade, novel adaptor and scaffold functions of β-arrestins have been discovered. Thus, β-arrestins can initiate new signaling from GPCRs while terminating G protein signals. β-arrestins interact with many important signaling molecules (Lefkowitz and Shenoy, 2005), such as c-Src (Luttrell *et al.*, 1999), extracellular signal–regulated protein kinase (ERK)1/2 (Tohgo *et al.*, 2002), JNK3 (McDonald *et al.*, 2000), etc., in response to various stimulations and modulate their functions. And more recently, β-arrestin1 was found to interact with a number of nuclear proteins, including cAMP responsive element binding protein, histone acetyltransferase p300, and transcription factor YY1 and thus modified gene transcription (Kang *et al.*, 2005; Yue *et al.*, 2009).

The oncoprotein Mdm2, an E3 ubiquitin ligase known for its prominent role in regulating p53 degradation (Momand *et al.*, 1992; Honda *et al.*, 1997), is one of the well-characterized binding partners

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Abbreviations used: β2AR, β2 adrenergic receptor; EGFP, enhanced green fluorescent protein; ERK, extracellular signal-regulated protein kinase; GPCR, G protein–coupled receptor; GRK, G protein–coupled receptor kinase; HA, hemagglutinin; Mdm2, murine double minute oncogene 2; MEF, mouse embryonic fibroblast.

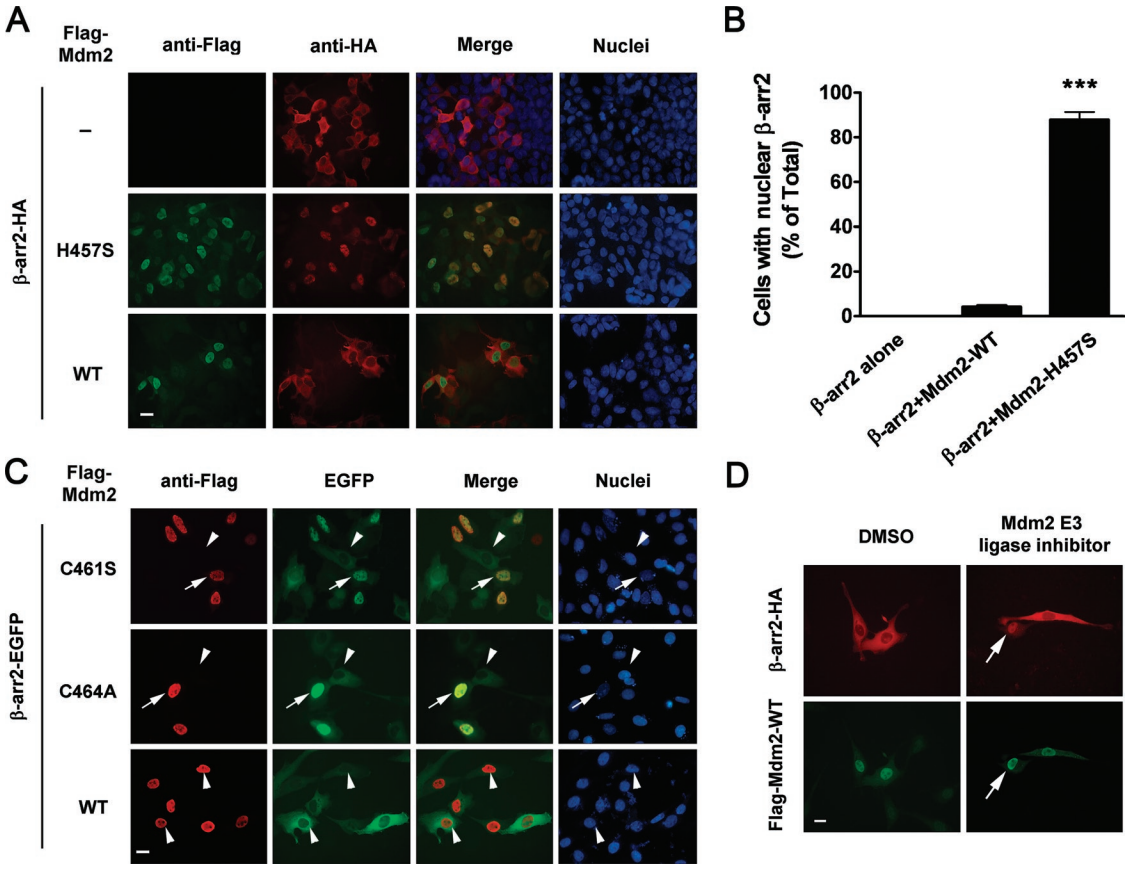

FIGURE 1: Mdm2 RING finger mutants cause dominant nuclear localization of β-arrestin2. (A) Mdm2^{H457S} causes nuclear localization of β-arrestin2. HEK-293 cells transfected with HA-tagged β-arrestin2 alone or in combination with Flag-tagged Mdm2^{wt} or Mdm2^{H457S} were analyzed by immunofluorescence microscopy. β-arrestin2 was stained with anti–HA antibody (red), and Mdm2 was detected with anti–Flag antibody (green). Nuclei were visualized by Hoechst 33342 staining (blue). (B) Statistical analysis of the fluorescent images in (A). Cells with nuclear β-arrestin2 were counted and presented as a percentage of the total cell number. More than 350 cells from 10 randomly selected fields were analyzed. ***, $P < 0.001$. (C) Other Mdm2 RING finger mutants also lead to the nuclear localization of β-arrestin2. U2OS cells were transfected with EGFP-tagged β-arrestin2 (green) together with Flag-tagged Mdm2 mutants (C461S or C464A, red). (D) Mdm2 E3 ligase inhibitor treatment leads to nuclear localization of β-arrestin2. U2OS cells transfected with β-arrestin2 and wild-type Mdm2 were treated with either 50 μM Mdm2-specific E3 ligase inhibitor (N-((3,3,3-trifluoro-2-trifluromethyl)propionyl)sulfanilamide) or vehicle control (0.5% dimethyl sulfoxide). Representative images are shown. Scale bar, 20 μm.

of β-arrestin2. Agonist stimulation of β2-adrenergic receptor (β2AR) led to rapid ubiquitination of both the receptors and β-arrestin2. Although β2AR ubiquitination is not directly dependent on Mdm2, it does require ubiquitination of β-arrestin2, which is mediated by Mdm2 (Shenoy *et al.*, 2001). Mdm2-catalyzed ubiquitination of β-arrestin2 is not only important for agonist-induced β2AR endocytosis (Shenoy *et al.*, 2001), but it also affects other downstream signaling pathways (Shenoy *et al.*, 2009). After insulin-like growth factor (IGF) receptor activation, β-arrestin2 also serves as a crucial adaptor for bringing Mdm2 to the receptor and eventually affects receptor trafficking and ERK activation (Girnita *et al.*, 2005, 2007). β-arrestin2 oligomers can also sequestrate Mdm2 in cytoplasm and thus modulate p53 activity and cell survival (Boularan *et al.*, 2007).

In this study, we found that the normal subcellular localization of β-arrestin2 is also tightly regulated by the E3 ligase activity of Mdm2. Mdm2 RING finger mutants (Mdm2^{H457S}) with the loss of ligase activity displayed a strong association with β-arrestin2 and caused abnormal dominant nuclear localization of the latter. With truncation and point mutation of β-arrestin2, we identified that the lysine residues at positions 11 and 12 are critically involved in the β-arrestin2/Mdm2 interaction. With K11,12R mutation, the β-arrestin2 localization was not affected by the Mdm2 mutants. When coexpressed with β2AR, the Mdm2^{H457S} mutant also prevented the agonist-induced β2AR internalization and β-arrestin2–mediated ERK phosphorylation. Our data strongly suggest that the intact E3 ligase activity of Mdm2 is critical for normal subcellular distribution and functions of β-arrestin2.

RESULTS

Loss of Mdm2 E3 ligase activity causes dominant nuclear localization of β-arrestin2

Mdm2 is a RING finger–dependent ubiquitin protein ligase for itself and p53 (Fang *et al.*, 2000). To investigate Mdm2 E3 ligase activity in regulating β-arrestin2 subcellular localization, a RING finger mutant Mdm2^{H457S} that lacks ubiquitin ligase activity was used. HEK-293 cells were transfected with plasmids encoding Mdm2^{wt} or Mdm2^{H457S} together with β-arrestin2, and the subcellular localization of β-arrestin2 was analyzed by immunofluorescence microscopy. In agreement with previous studies (Scott *et al.*, 2002; Wang *et al.*, 2003b), when expressed alone, β-arrestin2 localized mainly in

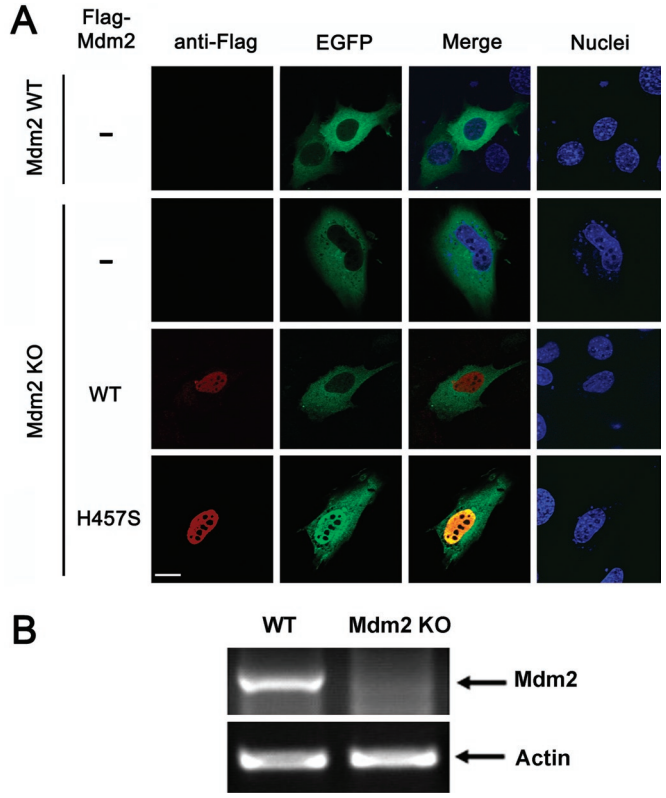

FIGURE 2: Mdm2^{H457S} causes nuclear localization of β-arrestin2 in Mdm2-knockout MEFs. (A) Mdm2-knockout MEFs and wild-type MEFs were transfected with β-arrestin2–EGFP (green) alone or together with Flag-tagged Mdm2^{wt} or Mdm2^{H457S} (red), and representative images are shown. Scale bar, 20 μm. (B) RT-PCR validation of Mdm2 expression in wild-type or Mdm2-knockout MEFs. Total RNA was extracted, and Mdm2 transcripts were examined by RT-PCR analysis using gene-specific primers.

the cytoplasm (Figure 1A, top row). Surprisingly, coexpression of Mdm2^{H457S} caused a dominant nuclear colocalization of β-arrestin2 and Mdm2^{H457S} (Figure 1A, middle row). Approximately 90% of the Mdm2^{H457S}-positive cells showed nuclear localization of β-arrestin2 (Figure 1B). However, no obvious nuclear β-arrestin2 was found when wild-type Mdm2 was coexpressed (Figure 1, A, bottom row, and B). To ensure that the phenomena we observed was not due to one specific mutation, two other Mdm2 RING finger mutants, C461S and C464A (Fang *et al.*, 2000), were introduced. As expected, β-arrestin2 displayed a similar nuclear localization when coexpressed with these two Mdm2 mutants (Figure 1C, top and middle rows, arrows). Cells in the same field but only expressing β-arrestin2 served as internal controls, where the β-arrestin2 mainly localized in the cytoplasm (Figure 1C, top and middle rows, arrowheads). To further confirm that the nuclear localization of β-arrestin2 was due to the loss of Mdm2 E3 ligase activity, an Mdm2 E3 ligase inhibitor, N-((3,3,3-trifluoro-2-trifluromethyl)propionyl) sulfanilamide (50 μM) (Lai *et al.*, 2002), was added after cotransfection of β-arrestin2 and Mdm2^{wt}, and the nuclear localization of β-arrestin2 was observed (Figure 1D, arrow). In contrast, vehicle control (0.5% dimethyl sulfoxide) did not affect the normal cytoplasmic distribution of β-arresint2 (Figure 1D). To test the influence of endogenous Mdm2 on β-arrestin2 subcellular localization, an Mdm2-knockout mouse embryonic fibroblast (MEF) cell line was used. Mdm2 expression in MEFs was validated by reverse transcriptase (RT)-PCR. Compared with the control MEFs, the mRNA transcripts of Mdm2 were absent in the Mdm2-knockout MEFs (Figure 2B). In control MEFs, the majority of cells showed cytoplasmic localization of β-arrestin2, and no obvious changes were observed in Mdm2-knockout MEFs (Figure 2A, top and second rows). When coexpressed with Mdm2^{H457S} in Mdm2-knockout MEFs, β-arrestin2 was found to localize mainly in the nuclei (Figure 2A, bottom row). However, expression of Mdm2^{wt} did not affect the cytoplasmic localization pattern of β-arrestin2 (Figure 2A, third row). These results indicate that the subcellular localization of β-arrestin2 is affected by the Mdm2 E3 ligase activity. But the dominant nuclear localization of β-arrestin2 is not likely due to the lack of ubiquitination by Mdm2 because Mdm2 knockout did not change the cytosolic distribution of β-arrestin2. It has been reported that Mdm2 interacts with β-arrestin2 (Shenoy *et al.*, 2001; Wang *et al.*, 2003a), so we wonder whether the stability of the interaction is regulated by the ligase activity of Mdm2 and eventually affects the localization of β-arrestin2.

Loss of Mdm2 E3 ligase activity strengthens the interaction between β-arrestin2 and Mdm2

It has been reported that mutation of any of the eight potential zinc binding residues within the RING finger domain results in a complete loss of Mdm2 ligase activity (Fang *et al.*, 2000). Using p53 as a substrate, the ubiquitin ligase activity of the RING finger mutant Mdm2^{H457S} was tested. The p53-null SaoS-2 cells were transfected with p53 and Mdm2^{wt} or Mdm2^{H457S}, and the ubiquitination status of p53 was detected by Western blotting with an anti–p53 antibody. As expected, the ability of Mdm2^{H457S} in promoting p53 ubiquitination was significantly impaired (Figure 3A). Next we investigated whether this RING finger mutant was also deficient in mediating β-arrestin2 ubiquitination. Mdm2^{wt} or Mdm2^{H457S}, β-arrestin2, and Myc-tagged ubiquitin (Myc-Ub) were coexpressed in HEK-293 cells. β-arrestin2 was immunoprecipitated with anti-hemagglutinin (HA) affinity gel and subjected to Western blot analysis. Indeed, the ubiquitination of β-arrestin2 by Mdm2^{H457S} was severely reduced in comparison to Mdm2^{wt} (Figure 3B). Additionally, we found that, without Mdm2, β-arrestin 2-HA was also ubiquitinated, indicating that Mdm2 is not the only E3 ligase that ubiquitinates β-arrestin2. This has also been speculated by other researchers (Boularan *et al.*, 2007; Shenoy *et al.*, 2009). After wt-Mdm2 transfection, the ubiquitination pattern of β-arrestin2 changed dramatically. The multiple-ubiquinated bands above 95 kDa were reduced, and one major band at ~70 kDa appeared. In contrast, Mdm2^{H457S} is unable to ubiquitinate β-arrestin2. In fact, it even prevented β-arrestin2 being ubiquitinated by other ligases.

Next we investigated the interaction between Mdm2 and β-arrestin2 by coimmunoprecipitation (coIP) assay. In agreement with previous reports (Shenoy *et al.*, 2001; Wang *et al.*, 2003a), β-arrestin2 coimmunoprecipitates with Mdm2^{wt} (Figure 3C, left panel, lane 2). More interestingly, Mdm2^{H457S}, which lacks E3 ligase activity, displayed a much stronger interaction with β-arrestin2 compared with Mdm2^{wt} (Figure 3C, left panel, lane 4). Furthermore, treatment with Mdm2 E3 ligase inhibitor (50 μM) also strengthens the interaction between β-arrestin2 and Mdm2^{wt} (Figure 3C, left panel, lane 3). Similar results were obtained when reciprocal immunoprecipitation was performed (Figure 3C, right panel). We also constructed Mdm2 RING domain deletion mutants to further confirm our finding that loss of ligase activity leads to strengthened interaction between Mdm2 and β-arrestin2. By using coIP assay, we found that, compared with Mdm2^{wt}, the Mdm2 RING domain deletion mutant Mdm2$^{1–432}$ displayed a similar stronger interaction with β-arrestin2, just like Mdm2^{H457S} (Figure 3D, left panel, lanes 4 and 5). On the other hand, Mdm2$^{1–400}$ did not interact with β-arrestin2

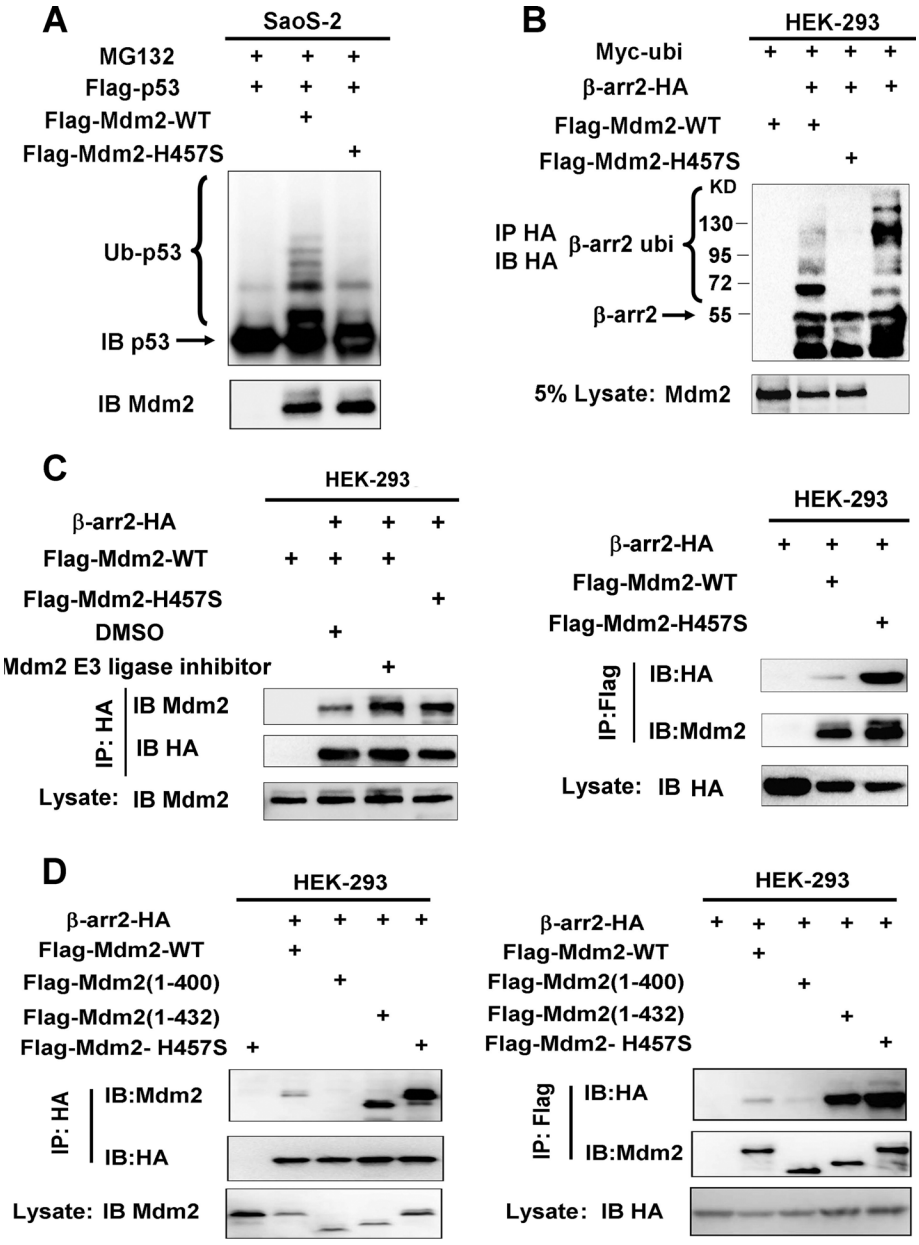

FIGURE 3: Loss of Mdm2 E3 ligase activity strengthens the interaction between β-arrestin2 and Mdm2. (A) $Mdm2^{H457S}$ loses the ability to promote p53 ubiquitination. SaoS-2 cells were transfected with plasmids encoding p53, wild-type $Mdm2^{wt}$, or $Mdm2^{H457S}$. Cells were treated with 10 μM MG132 for 4 h, and Western blotting was carried out with antibodies against p53 (DO-1) and Mdm2 (SMP14). (B) $Mdm2^{H457S}$ is unable to ubiquitinate β-arrestin2. HEK-293 cells were transfected with Flag-$Mdm2^{wt\ or\ H457S}$, β-arrestin2–HA, and Myc-Ubiquitin. Cell lysates were immunoprecipitated with affinity beads against HA-tag and analyzed by immunoblotting with anti–HA and anti–Mdm2 (SMP14) antibodies. (C) The interaction between Mdm2 and β-arrestin2 is strengthened when Mdm2 loses its E3 ligase activity. HEK-293 cells were cotransfected with β-arrestin2 and $Mdm2^{wt\ or\ H457S}$. Mdm2-specific E3 ligase inhibitor (50 μM) was added 8 h after transfection. Forty-eight hours later, cell lysates were immunoprecipitated with anti–HA (left) or anti–Flag antibody (right) and analyzed by immunoblotting. (D) Identification of the interaction domain of Mdm2 with β-arrestin2. Cells were transfected with β-arrestin2 and Mdm2 deletions. Cells were harvested and immunoprecipitated with anti–HA (left) or anti–Flag antibody (right) and analyzed by immunoblotting.

(Figure 3D, left panel, lane 3), which was in agreement with the previous report (Wang *et al.*, 2003a). Similar phenomena were observed in a reciprocal immunoprecipitation experiment (Figure 3D, right panel). The results suggested that the region from 400–432 of Mdm2 was critical for β-arrestin2 binding. Taken together, these data suggest that β-arrestin2 interacts transiently with wild-type Mdm2, and subsequent ubiquitination by Mdm2 may change the binding interface of β-arrestin2, thus leading to the dissociation of these two proteins. If Mdm2 loses its E3 ligase activity due to mutation, truncation, or drug treatment, β-arrestin2 cannot be ubiquitinated and thus cannot dissociate from Mdm2. Therefore the Mdm2 E3 ligase activity is important in regulating the stability of the β-arrestin2/Mdm2 protein complex.

The N terminus of β-arrestin2 is required for the interaction and colocalization with Mdm2 RING finger mutant in the nucleus

Previous studies have demonstrated that N-terminal amino acids 1–185 of β-arrestin2 interacts with the central region of wild-type Mdm2 (Shenoy *et al.*, 2001; Wang *et al.*, 2003a). To further delineate the regions of β-arrestin2 that are required for binding to Mdm2 RING finger mutant, a series of β-arrestin2 truncation mutants (Figure 4A) were used in coIP assay. The results showed that β-arrestin2 N-terminal fragment containing amino acids 1–185 interacted with $Mdm2^{H457S}$ even more efficiently than full-length β-arrestin2. In contrast, deletion of amino acids 1–60, 1–185, or 1–240 from the N terminus of β-arrestin2 led to nearly complete loss of its ability to bind to $Mdm2^{H457S}$ (Figure 4B). These results indicate that the N terminus of β-arrestin2 is the $Mdm2^{H457S}$ binding region, and amino acids 1–60 are indispensable for the interaction. In parallel, the colocalization of full-length β-arrestin2 or its truncation mutants (1–185 or 186–409) with $Mdm2^{H457S}$ was examined (Figure 4C). Consistent with the physical association data, we found that the C terminus of β-arrestin2 (186–409), which did not interact with $Mdm2^{H457S}$, was retained in the cytoplasm (Figure 4C, lane 3, arrowheads), whereas the full-length and the N terminus (1–185) of β-arrestin2 displayed dominant nuclear colocalization with $Mdm2^{H457S}$ (Figure 4C, lanes 1 and 2, arrows). These results suggest that the amino acids at the far N-terminal end of β-arrestin2, especially amino acids 1–60, are critical for its interaction with Mdm2 RING finger mutant and subsequent nuclear localization.

Lysines 11 and 12 of β-arrestin2 are the major interaction sites that regulate the nuclear sequestration of β-arrestin2 by $Mdm2^{H457S}$

With the truncation mutations, we had mapped amino acids 1–60 of β-arrestin2 as the interaction region for $Mdm2^{H457S}$ binding. There are several lysine residues within this region that can serve as

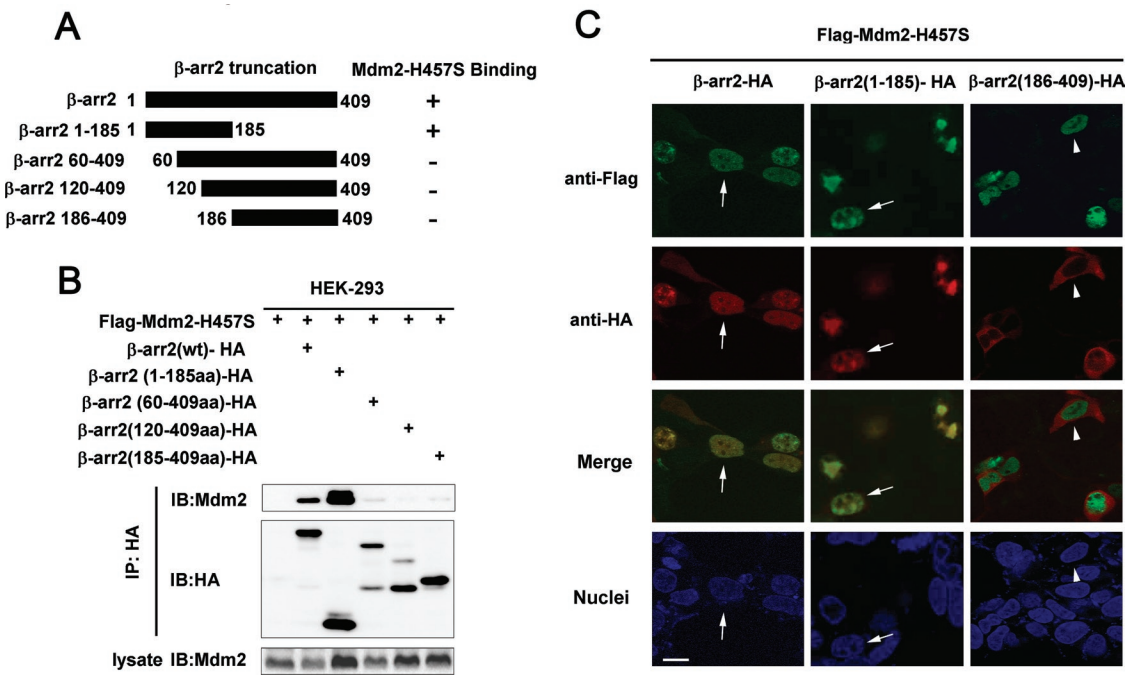

FIGURE 4: Delineation of the critical region of β-arrestin2 for Mdm2^{H457S}/β-arrestin2 interaction. (A) Schematic representation of the full-length and truncation mutants of β-arrestin2. Their binding ability with Mdm2 was summarized. "+" indicates clearly positive association, whereas "–" indicates no interaction. (B) N terminus of β-arrestin2 interacts with Mdm2^{H457S}. HEK-293 cells were transfected with HA-tagged β-arrestin2 constructs together with Flag-tagged Mdm2^{H457S}. β-arrestin2 was immunoprecipitated with anti–HA affinity beads, and the presence of Mdm2^{H457S} in the immunoprecipitates was detected using anti–Mdm2 antibody (SMP14). (C) N terminus of β-arrestin2 colocalized with Mdm2^{H457S}. HEK-293 cells were transfected with Flag-tagged Mdm2^{H457S} and HA-tagged β-arrestin2 and analyzed by immunofluorescence staining and confocal microscopy. Cells with nuclear localization of β-arrestin2 are indicated by arrows, whereas cell with cytosolic β-arrestin2 is pointed out with arrowhead. Scale bar, 10 μm.

potential ubiquitination and interaction sites. Lysines at positions 11 and 12 (K11,12) were reported to be specifically required for sustained ubiquitination of β-arrestin2 upon Angiotensin II (AngII) stimulation (Shenoy and Lefkowitz, 2005). Therefore we tested whether K11,12 are also important for the binding of β-arrestin2 to Mdm2 RING finger mutant. Indeed, replacement of K11,12 by alanine (A) or arginine (R) resulted in nearly complete loss of their ability to bind to Mdm2^{H457S} (Figure 5, A and B, lanes 3 and 4). Arginine is a positively charged amino acid commonly used in mutagenesis studies to replace lysine. The lack of interaction between β-arrestin2^{K11,12R} and Mdm2^{H457S} indicated that the structure of the lysine side chain may play a more important role than the electrical charge. In agreement with the interaction data, β-arrestin2^{K11,12R} retained the cytosolic distribution even when coexpressed with Mdm2^{H457S}, whereas β-arrestin2^{wt} displayed a strong nuclear localization in the presence of Mdm2^{H457S} in U2OS cells (Figure 5C). Similar localization results were obtained when K11,12 were substituted with alanines (data not shown). Taken together, these results demonstrated that K11,12 within the N terminus of β-arrestin2 are indispensable for their interaction with Mdm2^{H457S} and nuclear sequestration by the latter.

Cellular function of β-arrestin2 is impaired when sequestered in the nucleus by Mdm2 RING finger mutant

GPCR internalization is a β-arrestin–mediated process. To analyze the functional consequences of the entrapment of β-arrestin2 in the nucleus by Mdm2 E3 ligase mutants, the internalization of β2AR upon isoproterenol stimulation was tested. SaoS-2 cells expressing β-arrestin2–enhanced green fluorescent protein (EGFP) and HA–β2AR with or without Mdm2 ^{H457S} were starved for 2 h in serum-free medium. After treatment with 10 μM isoproterenol for 15 min, cells expressing only β-arrestin2 and β2AR displayed robust internalization of β2AR into the endocytic vesicles (Figure 6A, upper row, arrowheads). Coexpression of Mdm2^{H457S} leads to the sequestration of β-arrestin2 in the nuclei, and the isoproterenol-induced internalization of β2AR was severely impaired (Figure 6A, bottom row, cells denoted with arrow). The cells in the same field but not expressing Mdm2^{H457S} served as internal controls, which displayed normal receptor internalization after stimulation (Figure 6A, bottom row, cells denoted with arrowhead). Statistical analysis of the fluorescent images revealed that isoproterenol stimulation led to significant β2AR internalization in ~75% of the cells with normal cytosolic distribution of β-arrestin2. In contrast, in cells in which the β-arrestin2 was sequestered in the nuclei, only ~15% of the cells displayed normal receptor internalization (Figure 6B).

It is well documented that β-arrestins can function as mitogen-activated protein kinase scaffolds and mediate G protein–independent signaling (Ma and Pei, 2007). We wondered whether Mdm2^{H457S} could affect β-arrestin2–mediated ERK activation. HEK-293 cells stably expressing β2AR were transfected with vector (control), Mdm2^{wt}, or Mdm2^{H457S} and challenged with the β2AR inverse agonist propranolol, which was able to stimulate ERK activity in a G protein–independent, β-arrestin–dependent manner (Azzi *et al.*, 2003). As shown in Figure 6C, propranolol-induced ERK activation reached maximal activity at 2 min, which was consistent with the previous report (Azzi *et al.*, 2003). Expression of Mdm2^{H457S} significantly reduced propranolol-stimulated ERK activation (Figure 6, C and D). In contrast, expression of Mdm2^{wt} did not significantly affect ERK phosphorylation (Figure 6, C and D). These data support our

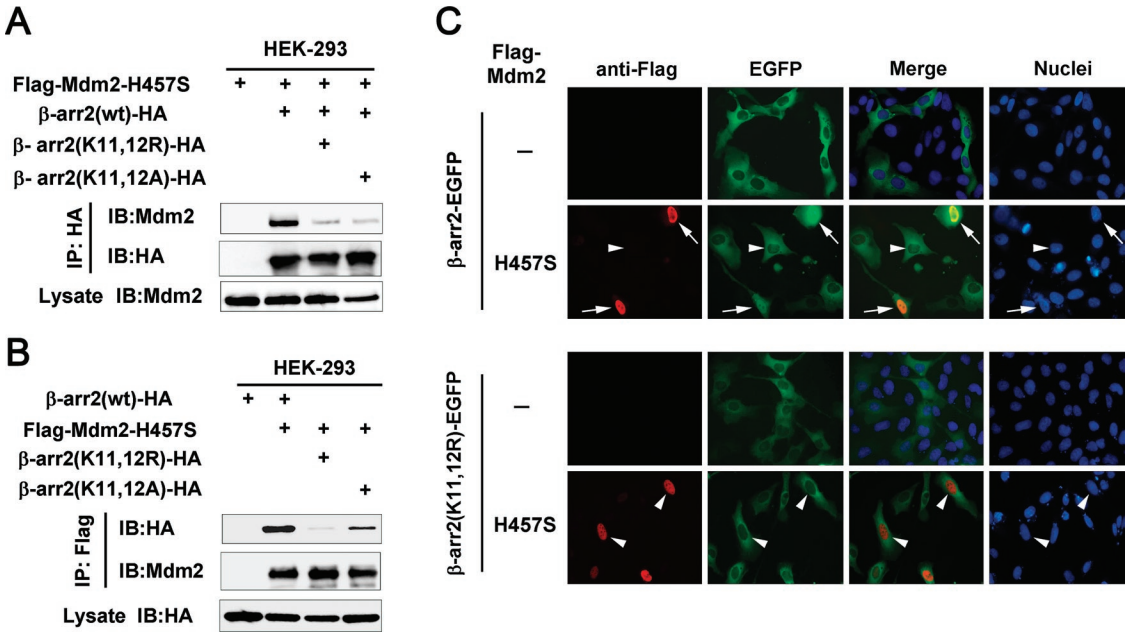

FIGURE 5: Lysine 11, 12 of β-arrestin2 are indispensable for its nuclear sequestration by Mdm2^{H457S} and their interaction. (A, B) β-arrestin2^{K11,12A} and β-arrestin2^{K11,12R} lose their binding ability to Mdm2^{H457S}. HEK-293 cells were transfected with Mdm2^{H457S} together with the β-arrestin2 mutants. Cell lysates were immunoprecipitated with affinity beads against HA-tag (A) or Flag-tag (B) and analyzed with immunoblotting. (C) β-arrestin2^{K11,12R} is not sequestered in the nuclei by Mdm2^{H457S}. HEK-293 cells were transfected with EGFP-tagged β-arrestin2^{wt} or β-arrestin2^{K11,12R}, alone or in combination with Mdm2^{H457S}. The subcellular localization of β-arrestin2 and Mdm2 was traced by EGFP tag (green) and anti–Flag antibody (red). Arrows indicate cells with nuclear β-arrestin2, whereas arrowheads indicate cells with cytosolic β-arrestin2 localization.

hypothesis that the nuclear sequestration of β-arrestin2 caused by the tightened interaction between β-arrestin2 and Mdm2 impairs the normal cytosolic signaling mediated by β-arrestin2.

DISCUSSION

β-arrestin1 and -2 are two highly homologous proteins sharing 78% identity in amino acid composition, and both of them participate in modulating GPCR signaling (Luttrell and Lefkowitz, 2002). The functions of β-arrestins are tightly regulated by posttranslational modifications, including phosphorylation and ubiquitination. β-arrestin1 is phosphorylated on S412 at resting state (Lin *et al.*, 1999). On translocation to the membrane, β-arrestin1 is rapidly dephosphorylated, which is necessary for the receptor/β-arrestin complex to engage the endocytic machinery. The kinases responsible for β-arrestin1 phosphorylation appear to be ERK1/2 (Lin *et al.*, 1999). Another study revealed that CK2 phosphorylation of β-arrestin2 at Ser-361 and Thr-383 blocks its interaction with IκBα and in turn abolishes its suppression of NF-κB activation (Luan *et al.*, 2005). Interestingly, the endocytic functions of β-arrestin2 are not regulated by phosphorylation at its C terminus but mainly by ubiquitination. It was reported that abrogation of β-arrestin2 ubiquitination, either by expression in Mdm2-null cells or by dominant-negative forms of Mdm2 lacking E3 ligase activity, inhibited receptor internalization with marginal effects on receptor degradation (Shenoy *et al.*, 2001). Therefore the E3 ubiquitin ligase Mdm2, a master negative regulator of the tumor suppressor p53, is a key factor that binds directly to β-arrestin2, catalyzes its ubiquitination, and regulates its function.

Another interesting difference between β-arrestin1 and -2 is their subcellular localization. When expressed in HEK-293 and HeLa cells, β-arrestin1 is localized in both cytoplasm and nucleus, but β-arrestin2 is predominantly distributed in the cytoplasm (Wang *et al.*, 2003b). A previous study also showed that the two β-arrestins shuttle differentially between the nucleus and cytoplasm due to the presence of a two-leucine nuclear export signal (NES) in the C terminus of β-arrestin2 that is absent in β-arrestin1 (Wang *et al.*, 2003b). However, other factors that might also participate in their normal subcellular localization remain elusive.

Here we reported an unexpected observation that Mdm2 RING finger mutants (Mdm2^{H457S}, Mdm2^{C461S}, and Mdm2^{C464A}) drive dominant nuclear localization of β-arrestin2 (Figure 1A–C). The RING finger domain of Mdm2 contains the active site for its E3 ubiquitin ligase activity, and mutation of any of the eight potential zinc coordination residues in RING domain results in a complete loss of Mdm2 ubiquitin ligase activity (Fang *et al.*, 2000) (Figure 3, A and B). To make sure the phenomena we observed were not due to possible structural change induced by mutations, an Mdm2-specific E3 ligase inhibitor (Lai *et al.*, 2002) was used to further confirm that the abnormal nuclear localization of β-arrestin2 was due to the loss of Mdm2 E3 ligase activity (Figure 1D). We also used Mdm2-deficient MEFs to evaluate the function of endogenous Mdm2 on β-arrestin2 subcellular localization. To our surprise, the absence of Mdm2 did not cause significant change in β-arrestin2 localization (Figure 2A). Only in the presence of mutant Mdm2, or when the Mdm2 ligase activity is blocked by drug, is β-arrestin2 localized in the nuclei (Figure 2A).

The change of distribution of β-arrestin2 from cytoplasm to nucleus may have two explanations: 1) The balance between nuclear import and export of β-arrestin2 is shifted due to the change of its ubiquitin status mediated by Mdm2. 2) β-arrestin2 is sequestered in the nuclei by its binding partners, possibly by Mdm2. Because the knockout of Mdm2 did not change the cytosolic distribution of β-arrestin2, it is unlikely that the dominant nuclear localization of β-arrestin2 is driven by the lack of ubiquitination by Mdm2 mutants. Therefore we tested the second possibility. With coIP assay, we

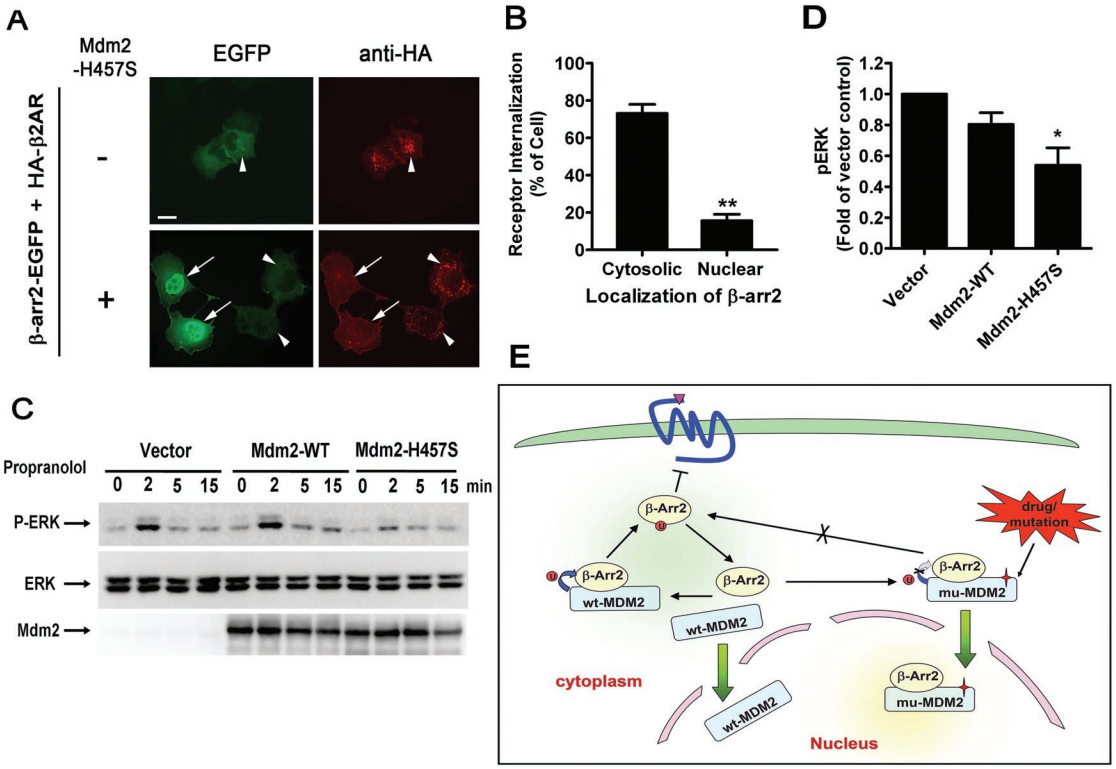

FIGURE 6: Cellular function of β-arrestin2 is impaired when trapped into the nucleus by Mdm2^{H457S}. (A) β-arrestin2–mediated β2AR trafficking was disturbed by Mdm2^{H457S} expression. SaoS-2 cells were transfected with β-arrestin2, β2AR in the presence or absence of Mdm2^{H457S}. After 2 h of starvation, cells were treated with 10 μM isoproterenol and analyzed by immunofluorescence staining. Cells with normal cytosolic β-arrestin2 distribution are indicated with arrowheads, and cells with nuclear β-arrestin2 localization are denoted with arrows. Scale bar, 20 μm. (B) Statistical analysis of the fluorescent images in (A). Isoproterenol-induced β2AR internalization was analyzed in cells with nuclear or cytosolic β-arrestin2 distribution. Data shown are mean ± SEM of 3 independent experiments; ~300 cells from each group were analyzed. **, $P < 0.01$. (C) Mdm2^{H457S} expression leads to impaired propranolol-stimulated ERK activation. HEK-293 cells stably expressing β2AR were transfected with control vector, Mdm2^{wt}, or Mdm2^{H457S}. After serum starvation, cells were stimulated with β2AR inverse agonist propranolol (10 μM) for various time periods as indicated. The whole cell lysates were analyzed by Western blotting. (D) Statistical analysis of ERK activation at 2 min after stimulation as shown in (C). The signal of pERK was first normalized to total ERK and then normalized to time 0. Data shown are mean ± SEM of 5 independent experiments. *, $P < 0.05$. (E) A model for Mdm2 ligase activity–dependent localization and function of β-arrestin2. Wild-type Mdm2 interacts with β-arrestin2 transiently, and subsequent ubiquitination of β-arrestin2 leads to dissociation of these two proteins. When Mdm2 E3 ligase activity is abolished by either mutation or drug treatment, the interaction between Mdm2 and β-arrestin2 is strengthened, and therefore β-arrestin2 is sequestrated into the nucleus and loses it normal function.

found that the interaction between β-arrestin2 and Mdm2^{H457S} is much stronger than Mdm2^{wt}, and application of Mdm2 E3 ligase inhibitor also enhances the interaction between β-arrestin2 and Mdm2^{wt} (Figure 3, C and D). This indicates that the abnormal nuclear localization of β-arrestin2 in the presence of ligase activity–null Mdm2 is likely due to the strengthened β-arrestin2–Mdm2^{H457S} interaction.

Next we sought to resolve the structural basis of this strong interaction between β-arrestin2 and Mdm2^{H457S}. It has been reported that the N-terminal 1–185 amino acids are involved in the interaction between β-arrestin2 and Mdm2^{wt} (Wang *et al.*, 2003b). We applied a series of truncation mutations of β-arrestin2 in the coIP assay and discovered that the N-terminal 1–60 amino acids are indispensable for the β-arrestin2/Mdm2^{H457S} interaction (Figure 4, A and B). Without the N-terminal binding sites, β-arrestin2 always localizes in the cytosol, even with the overexpression of Mdm2^{H457S} (Figure 4C). There are quite a few charged amino acid residues within this region that can serve as potential interaction sites. Lysines at positions 11 and 12 (K11,12) were reported to be specifically required for sustained ubiquitination of β-arrestin2 upon AngII stimulation (Shenoy and Lefkowitz, 2005), so we tested these sites first. Indeed, β-arrestin2^{K11,12A} and β-arrestin2^{K11,12R} almost completely lose their ability to bind to Mdm2^{H457S} (Figure 5, A and B). Arginine is also a positively charged amino acid similar to lysine. The lack of interaction between β-arrestin2^{K11,12R} and Mdm2^{H457S} indicated that the structure of the lysine side chain may play a more important role than the electrical charge in the interaction. With the loss of binding ability, β-arrestin2^{K11,12R} retained the cytosolic distribution even when coexpressed with Mdm2^{H457S} (Figure 5C). Interestingly, our results suggested that Mdm2 is likely to be responsible for the diubiquitination of β-arrestin2 (Figure 3B). Whether K11,12 of β-arrestin2 are the precise sites for such modulation remains to be elucidated.

Intact E3 ligase activity of Mdm2 has also been reported to be important in the nuclear exclusion of p53 (Boyd *et al.*, 2000). A mutant of the Hdm2 RING finger domain that fails to ubiquitinate p53 does not cause export of p53 from nuclei to the cytosol. Similar

functions in regulating protein subcellular distribution were also reported in other E3 ligases. For example, NEDD4–1 regulates the nuclear import of PTEN and subsequent tumor suppression function of the latter (Trotman *et al.*, 2007). Our results also indicate that the intact E3 ligase activity of Mdm2 is important in normal cytoplasmic β-arrestin2 distribution. We also examine the functional consequence of the abnormal nuclear sequestration of β-arrestin2 once the Mdm2 ligase activity is disrupted. In the presence of $Mdm2^{H457S}$, β-arrestin2–mediated β2AR internalization is significantly inhibited (Figure 6, A and B) and β-arrestin2–mediated ERK activation stimulated by a β-arrestin–biased ligand of β2AR (propranolol) is also impaired (Figure 6, C and D). These findings agree with the aforementioned studies that the correct cellular localization of a protein is important for its normal function.

As presented in Figure 6E, our study revealed that the intact E3 ligase activity is essential for the appropriate cytosolic distribution and function of β-arrestin2. Under normal conditions, β-arrestin2 interacts transiently with Mdm2, and subsequent ubiquitination by Mdm2 leads to the dissociation of these two proteins. Therefore β-arrestin2 is mainly located in the cytoplasm probably due to its NES, which is in agreement with the current knowledge. Once Mdm2 loses its E3 ligase activity due to mutation or drug treatment, Mdm2 and β-arrestin2 form a more stable complex that sequestrates β-arrestin2 in the nucleus and therefore separates β-arrestin2 from its partners and interferes with its normal functions.

MATERIALS AND METHODS

Reagents and plasmids

Propranolol and isoproterenol were purchased from Sigma (St. Louis, MO). MG132 and an Mdm2-specific E3 ligase inhibitor, N-((3,3,3-trifluoro-2- trifluromethyl) propionyl) sulfanilamide, were purchased from Calbiochem (San Diego, CA). Flag-Mdm2, β-arrestin2–EGFP, β-arrestin2–HA, and its truncation mutants were described previously (Wang *et al.*, 2003a, 2003b). Flag-$Mdm2^{H457S}$, Flag-$Mdm2^{C461S}$, Flag-$Mdm2^{C464A}$, β-$arrestin2^{K11,12A}$, and β-$arrestin2^{K11,12R}$ were created by PCR-mediated site-directed mutagenesis using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA). Flag-Mdm2 deletion constructs (1–432 and 1–400) were constructed by PCR cloning. The HA-β2AR plasmid was purchased from Missouri S&T cDNA Resource Center (Rolla, MO). The ubiquitin plasmid was kindly provided by Songcheng Zhu (Tongji University, Shanghai, China).

Cell culture and transfection

MEFs, human embryonic kidney cell HEK-293, human osteosarcoma cell SaoS-2, and U2OS were maintained in DMEM supplemented with 10% fetal bovine serum in an atmosphere containing 5% CO_2 at 37°C. Transient transfection of HEK-293 and SaoS-2 cells was performed using the calcium phosphate transfection method as described previously (Wang *et al.*, 2003a). MEF and U2OS cells were transfected with FuGENE HD Transfection Reagent from Roche (Indianapolis, IN), according to the manufacturer's instruction. p53/Mdm2 double-knockout MEF cell line was a gift from Karen Vousdens (Jones *et al.*, 1995; Lukashchuk and Vousden, 2007). The absence of Mdm2 mRNA was validated by RT-PCR analysis using following primer set: 5′-AATCCTCCCCTTCCATCAC-3′ and 5′-TTGGATTGGCTGTCTGCA-3′.

Coimmunoprecipitation and Western blotting

Cells were lysed in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Chaps, 1 mM EDTA containing 1 mM NaF, 1 mM Na_3VO_4, and protease inhibitors) by sonication for 30 s on ice. Crude lysate was centrifuged at 13,000 rpm for 15 min at 4°C, and the supernatant was incubated with anti-HA or anti-Flag affinity gel (Sigma) on a rotator at 4°C overnight. The affinity gel was then collected by centrifugation and washed with lysis buffer four times. The protein complex was eluted with SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 3% SDS, 15% glycerol, 2% β-mercaptoethanol) and resolved by SDS–PAGE. Western blotting was performed using the following primary antibodies: anti–HA Y-11 (Santa Cruz Biotechnology, Santa Cruz, CA), anti–Flag M2 (Sigma), anti–Mdm2 clone HDM2–323 (Sigma), anti–p53 DO-1 (Santa Cruz Biotechnology), and corresponding horseradish peroxidase–conjugated secondary antibodies (Promega, Madison, WI).

Immunofluorescence microscopy

Cells grown on glass coverslips were fixed with 4% paraformaldehyde for 20 min at room temperature and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min. Cells were then incubated with primary antibodies for 1 h at room temperature followed by appropriate fluorescence-conjugated secondary antibodies for 1 h in dark. The coverslips were then mounted onto the slides and sealed with nail polish. The following antibodies were used: TRITC-conjugated anti–HA clone HA-7 (Sigma), FITC-conjugated anti–Flag M2 (Sigma), rabbit anti–Flag antibody (Sigma), and Alexa Fluor 546 or -488–conjugated goat anti–rabbit immunoglobulin G (Invitrogen, Carlsbad, CA). Images were taken with an Olympus IX71 fluorescence microscope or FV10i confocal microscope.

Ubiquitination assay

To investigate p53 ubiquitination mediated by Mdm2, p53-null SaoS-2 cells were transfected with plasmids encoding wild-type or mutant Mdm2 together with p53. Twenty-four hours after transfection, cells were treated with 30 μM MG132 for 4 h. Cells were then lysed in hot SDS sample buffer, and Mdm2-mediated p53 ubiquitination was assessed by Western blotting with antibody against p53 (DO-1; Santa Cruz).

To analyze the ubiquitination status of β-arrestin2, cells were transfected with plasmids encoding β-arrestin2, wild-type or mutant Mdm2, and Myc-Ub. Thirty hours later, cells were treated with 30 μM MG132 for 4 h prior to harvesting in 1% SDS sample buffer by boiling for 10 min. β-arrestin2 was immunoprecipitated with anti–HA affinity gel (Sigma) and analyzed by immunoblotting with anti–HA antibody.

ERK activation assay

ERK activation assay was carried out on HEK-293 cells stably expressing β2AR. Twenty-four hours after transfection with indicated plasmids, cells were serum-starved for 2 h and then stimulated with 10 μM propranolol in PBS at 37°C. Cells were then scraped and subjected to Western blot analysis. Anti–phospho-Erk1/2 (Thr202/Tyr204) and anti–Erk1/2 antibodies (Cell Signaling Technology, Beverly, MA) were used.

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