Regulation of Receptor Fate by Ubiquitination of Activated β_2-Adrenergic Receptor and β-Arrestin

Sudha K. Shenoy, Patricia H. McDonald, Trudy A. Kohout, Robert J. Lefkowitz*

Although trafficking and degradation of several membrane proteins are regulated by ubiquitination catalyzed by E3 ubiquitin ligases, there has been little evidence connecting ubiquitination with regulation of mammalian G protein (heterotrimeric guanine nucleotide–binding protein)–coupled receptor (GPCR) function. Agonist stimulation of endogenous or transfected β_2-adrenergic receptors (β_2ARs) led to rapid ubiquitination of both the receptors and the receptor regulatory protein, β-arrestin. Moreover, proteasome inhibitors reduced receptor internalization and degradation, thus implicating a role for the ubiquitination machinery in the trafficking of the β_2AR. Receptor ubiquitination required β-arrestin, which bound to the E3 ubiquitin ligase Mdm2. Abrogation of β-arrestin 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. However, a β_2AR mutant lacking lysine residues, which was not ubiquitinated, was internalized normally but was degraded ineffectively. These findings delineate an adapter role of β-arrestin in mediating the ubiquitination of the β_2AR and indicate that ubiquitination of the receptor and of β-arrestin have distinct and obligatory roles in the trafficking and degradation of this prototypic GPCR.

Agonist binding to GPCRs results in conformational changes in the receptor that not only initiate cellular signaling events but also lead to receptor phosphorylation by G protein–coupled receptor kinases (GRKs). β-Arrestins are proteins that bind to phosphorylated receptors and functionally uncouple the receptor from G-protein activation, a process referred to as desensitization. β-Arrestins also regulate the internalization of GPCR away from the cell membrane. Internalized receptors are either recycled back to the surface after dephosphorylation or are degraded. Although such regulation of GPCRs has generated intense interest, the molecular mechanisms regulating these processes are not fully understood.

Ubiquitination, originally identified as a process that marks proteins for degradation by the 26*S* proteasome, also regulates the internalization of several plasma membrane proteins. Ubiquitination of proteins is the result of sequential action of three enzymes (*1*). First, the COOH-terminal glycine residue of ubiquitin is activated by the formation of a high-energy thioester bond with a ubiquitin-activating enzyme, E1. Activated ubiquitin is then transferred to an active-site cysteine residue in an E2, or a ubiquitin-carrying enzyme. The final step is catalyzed by a ubiquitin protein ligase (E3), which links the COOH-terminus of ubiquitin to the ε-amino group of a lysine residue of the substrate protein. The E3 ligases either interact directly with their substrates or do so through ancillary proteins that serve as adapters. Multiple attachments of ubiquitin molecules may occur by further addition on the first ubiquitin moiety or on other lysine residues in the protein. The ubiquitinated protein is then targeted to the proteasome or, if it is a membrane protein, may be targeted to the lysosomes (*2*).

Ligand-stimulated ubiquitination of several mammalian cell surface receptors leads to internalization, followed by degradation in lysosomes (*2*). In the case of the growth hormone receptor (GHR), polyubiquitination occurs at the plasma membrane before its recruitment to clathrin-coated pits (*3*). c-Cbl, a RING domain–containing E3-ligase, mediates ubiquitination and degradation of receptor protein-tyrosine kinases (*4*). For members of the large superfamily of seven-membrane–spanning receptors, ubiquitination of rhodopsin, and the presence of active ubiquitination machinery in preparations of rod outer segments, have been reported (*5*). The ubiquitin-proteasome pathway may influence agonist-induced degradation of opioid receptors (*6*). The yeast pheromone receptors Ste2p and Ste3p are internalized after ligand-induced ubiquitination (*7*, *8*).

We report the agonist-stimulated ubiquitination of the β_2AR, and of the adapter protein β-arrestin2, and demonstrate that the ubiquitination of the receptor is a requirement for its degradation, whereas that of β-arrestin2 is essential for rapid receptor internalization.

Isoproterenol-stimulated ubiquitination of the β_2AR and β-arrestin2. To evaluate possible ubiquitin modification of the β_2AR, we stimulated Chinese hamster fibroblast cells (CHW-1102) stably expressing β_2AR (CHW-β2) with the β-adrenergic agonist isoproterenol for different times, isolated the receptor on Sepharose 4B–alprenolol beads (*9*), and immunoblotted (*10*) the samples with a ubiquitin-specific antibody or antibody specific to the receptor. Isoproterenol-stimulated ubiquitination of β_2AR is observed as broad bands of large size ranging from 70 to >200 kD (Fig. 1A). No signal was detected with ubiquitin or β_2AR antibodies in untransfected control CHW cells, which contain very small amounts of endogenous β_2ARs (*11*). Enhanced ubiquitination of the receptor occurred within 15 min of agonist exposure, was sustained up to 1 hour, and then gradually declined.

Proteasomal inhibitors such as MG132 (*N*-carbobenzyloxy-L-leucyl-L-leucyl-L-leucinal) and lactacystin are cell-permeable compounds that specifically block the activity of the 26*S* proteasome. Thus, they cause accumulation of those ubiquitinated proteins that are degraded by proteasomes. However, ubiquitinated β_2AR was detectable even when cells were not pretreated with proteasomal inhibitors, suggesting that most of the ubiquitinated β_2AR is not an immediate target for the proteasomal proteases. Although 26*S* proteasomes often degrade ubiquitinated substrates, it has recently become clear that they also play roles in targeting ubiquitinated membrane proteins to lysosomes (*12*).

Isoproterenol-stimulated ubiquitination of β-arrestin2 (*13*) was also observed (Fig. 1B) and was even more rapid than that of the receptor, occurring within 1 min of agonist exposure. Moreover, the signal was transient and diminished almost completely within 10 min of stimulation (Fig. 1B). This is presumably due to rapid deubiquitination rather than to degradation of β-arrestin2 because the ubiquitinated signal was observed only when 5 mM *N*-ethylmaleimide (NEM, an inhibitor of deubiquitinating enzymes), but not MG132, was included in the lysis and immunoprecipitation (IP) buffers. Green fluorescent protein (GFP)–β-arrestin2 is recruited to the membrane-bound β_2AR within seconds of isopro-

Howard Hughes Medical Institute and Departments of Medicine, Cardiology and Biochemistry, Duke University Medical Center, Box 3821, Durham, NC 27710, USA.

*To whom correspondence should be addressed: E-mail: lefko001@receptor-biol.duke.edu

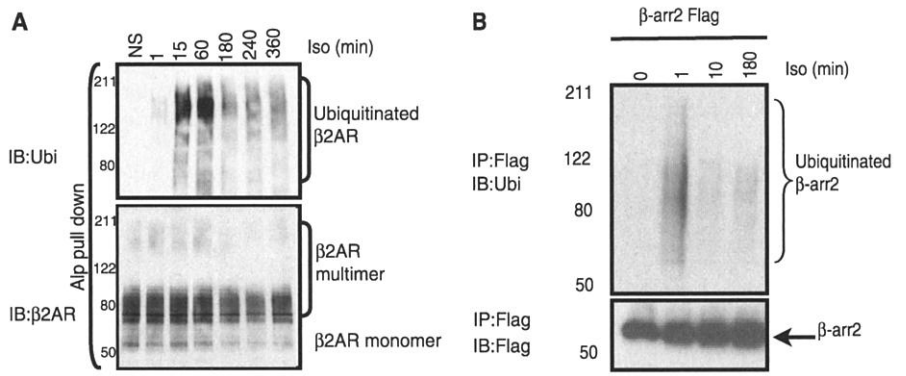

Fig. 1. Agonist-stimulated ubiquitination of β_2AR and β-arrestin. **(A)** β_2AR was isolated on Sepharose 4B–alprenolol (Alp) beads from CHW-β2 cells after incubation with or without (NS) isoproterenol at 37°C for the indicated times. The bound protein sample was separated by SDS-PAGE and immunoblotted with antibodies specific to ubiquitin and β_2AR (upper and lower panels, respectively). The blot shown is representative of similar blots from three separate experiments. β_2AR migrates as a broad set of bands because of its characteristic glycosylation pattern in CHW cells (*37*). The amount of β_2AR in untreated cells as determined by radioactive ligand binding was 600 fmol/mg total cellular protein. **(B)** COS-7 cells were transfected with β-arrestin2–Flag and the endogenous β_2ARs were stimulated for the indicated times. β-Arrestin was immunoprecipitated with Flag affinity beads, and the presence of ubiquitinated forms in the immunoprecipitates (IP) was detected with a ubiquitin-specific antibody (Ub P4D1 SantaCruz). Data are representative of five independent experiments. IB: immunoblot.

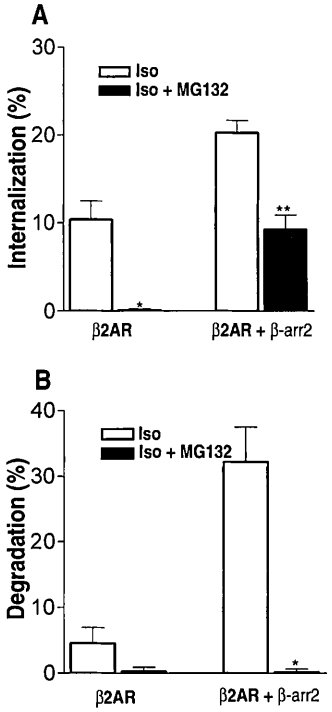

Fig. 2. Effect of proteasome inhibitors on internalization and degradation of the β_2AR. **(A)** COS-7 cells were transiently transfected with β_2AR alone or together with β-arrestin2. After incubation for 1 hour with or without MG132, cells were incubated with or without 10 μM isoproterenol for 30 min at 37°C. The agonist-promoted receptor internalization was determined by radioligand binding and is defined as the percentage of total specific radioligand binding sites not displaced by hydrophilic antagonist CGP12177 (*15*). The result shown is the mean ± SEM of five experiments. An unpaired *t* test was used to determine the statistical significance: $^*P < 0.001$; $^{**}P < 0.0001$. **(B)** COS-7 cells overexpressing β_2AR alone or with β-arrestin2 were treated or not with isoproterenol alone or with MG132 for 24 hours. Receptor degradation was determined by ^{125}I-CYP binding assays (*15*). The results are shown as the mean ± SEM of five independent experiments. $^*P < 0.001$ (unpaired *t* test).

terenol treatment, and the receptor–β-arrestin complex accumulates in coated pits. β-Arrestin2 then dissociates such that the β_2AR alone is internalized within 10 min into vesicles (*14*). Thus, temporally, the association and dissociation of β-arrestin2 from the receptor appear to parallel its ubiquitination and deubiquitination. These findings raise the possibility that the ubiquitinated form of β-arrestin corresponds to the receptor-bound β-arrestin complex.

Proteasomal involvement in β_2AR internalization and degradation. The trafficking of β_2AR is dictated by the duration of agonist treatment. A short-term exposure (up to 1 hour) leads to internalization or redistribution of the phosphorylated receptors away from the cell membrane into endocytic compartments in which they are dephosphorylated and then recycled to the cell membrane. More prolonged exposure (hours to days) leads to receptor degradation or down-regulation, an actual decline in the total cellular receptor number. To evaluate the involvement of proteasomes in regulating β_2AR trafficking, we tested the effects of proteasome inhibitors on β_2AR internalization and degradation. In the presence of MG132, internalization of β_2AR in COS-7 cells, as determined by ligand binding (*15*), was completely inhibited (Fig. 2A). In cells overexpressing β-arrestin2, β_2AR internalization was inhibited by about 50% upon MG132 treatment (Fig. 2A). Proteasome inhibitors did not affect transferrin receptor internalization under the same experimental conditions (*11*, *16*). To ascertain whether proteasome inhibitors affected the receptor degradation, we determined receptor numbers after a 24-hour treatment of cells with isoproterenol, in the presence or absence of MG132. In COS-7 cells, only a small percentage of receptors (~5%) is lost after 24 hours of isoproterenol treatment, and this effect was suppressed by MG132. However, after overexpression of β-arrestin2, ~30% of the receptors were degraded after prolonged treatment with isoproterenol. This too was completely blocked by MG132 (Fig. 2B) and lactacystin (*11*). These findings suggest that the ubiquitination machinery and 26*S* proteasome are involved in both endocytosis and degradation of β_2ARs but do not necessarily implicate the proteasomes as the proximate machinery of receptor degradation.

Requirement of receptor phosphorylation and subsequent β-arrestin binding for β_2AR ubiquitination. In cells stimulated with isoproterenol, β_2ARs are rapidly phosphorylated by the GRKs, leading to β-arrestin binding and rapid desensitization and internalization of the receptor. To test whether these events are required for receptor ubiquitination, we compared the ubiquitination of wild-type receptor (WT-β_2AR) with that of a phosphorylation-defective β_2AR, in which all the GRK and protein kinase A (PKA or cyclic adenosine 5′-monophosphate–dependent protein kinase) sites were mutated to alanines or glycines (β_2AR^{2-}) (*17*). In COS-7 cells, isoproterenol treatment led to ubiquitination of the WT-β_2AR but not the mutant receptor (Fig. 3A). The β_2AR^{2-} mutant also did not undergo agonist-stimulated internalization or β-arrestin binding, as determined by coimmunoprecipitation assays (*11*, *18*).

The role of β-arrestins in ubiquitination of the receptor was further corroborated by observations made in murine embryonic fibroblasts (MEFs) lacking β-arrestins. Several of the MEF cell lines had sufficient endogenous β_2AR expression to allow us to characterize ubiquitin modification without the need for transfection (~100 fmol/mg protein). Immunoprecipitates of endogenous β_2AR from such wild-type, β-arrestin1 knockout, and β-arrestin2 knockout MEFs were immunoblotted with antibodies to ubiquitin (*19*). Isoproterenol-dependent ubiquitination of β_2AR was observed in wild-type cells, and to a lesser extent in β-arrestin1 knockout cells, but not in β-arrestin2 knockout cells (Fig. 3B), suggesting an obligatory role of the β-arrestin2 isoform in receptor ubiquitination. We also used β-arrestin1 and -2 double knockout MEFs to test the ubiquitination of overexpressed β_2AR (*19*). Isoproterenol-stimulated ubiquitination was observed in wild-type

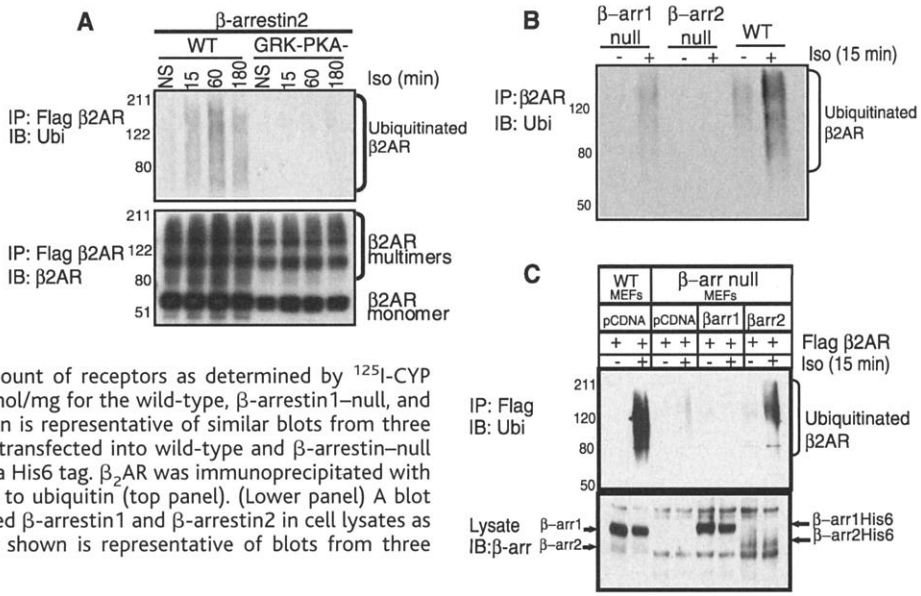

Fig. 3. Requirement of β-arrestin for β_2AR ubiquitination. (**A**) Western blot of immunoprecipitates of wild-type β_2AR and a mutant lacking GRK2 and PKA phosphorylation sites (β_2AR^{2-}) transiently expressed in COS-7 cells. (Upper panel) Ubiquitinated proteins. (Lower panel) Amount of receptor protein. The blot shown is representative of similar blots from three independent experiments. (**B**) Western blot of immunoprecipitates of endogenously expressed β_2AR from wild-type, β-arrestin1–, and β-arrestin2–knock out MEFs. After 15 min of isoproterenol stimulation (10 μM at 37°C), β_2AR was immunoprecipitated with a β_2AR-specific antibody (SantaCruz), and the samples were probed with antibody to ubiquitin. The amount of receptors as determined by ^{125}I-CYP binding was 154 ± 15, 72 ± 25, and 128 ± 12 fmol/mg for the wild-type, β-arrestin1–null, and β-arrestin2–null cells, respectively. The blot shown is representative of similar blots from three separate experiments. (**C**) Flag-tagged β_2AR was transfected into wild-type and β-arrestin–null cells without or with β-arrestin isoforms carrying a His6 tag. β_2AR was immunoprecipitated with M2 Flag affinity beads and blotted with antibody to ubiquitin (top panel). (Lower panel) A blot showing amounts of endogenous and overexpressed β-arrestin1 and β-arrestin2 in cell lysates as detected by an antibody to β-arrestin. The blot shown is representative of blots from three independent experiments.

MEFs but not in the cell line that lacked both endogenous β-arrestins (Fig. 3C). In addition, the isoproterenol-stimulated ubiquitination of β_2AR was reestablished when β-arrestin2, but not β-arrestin1, expression was restored in these cells by transfection. The amount of β_2AR in the immunoprecipitates was fairly uniform as visualized by immunoblotting with an antibody to β_2AR. These findings in MEFs further confirm the requirement for β-arrestin2 in β_2AR ubiquitination.

Interaction of the E3 ubiquitin ligase Mdm2 with β-arrestin. The finding that β-arrestin2 is required for β_2AR ubiquitination suggests that β-arrestin2 may serve as an adapter that links the receptor to the ubiquitination machinery. Accordingly, we sought to identify ubiquitin ligases that might interact with β-arrestin2 in a yeast two-hybrid screen with β-arrestin2 as the bait (*20*). Screening of a rat brain cDNA library with GAL4 fusions of full-length β-arrestin2 cDNA as bait yielded, among other clones, five independent interacting clones encoding fusion proteins of the GAL4 activation domain with an NH_2-terminally truncated rat homolog of Mdm2. Mdm2 is an oncoprotein that acts as a negative regulator of p53 and functions as an E3 ubiquitin ligase for p53 (*21*, *22*).

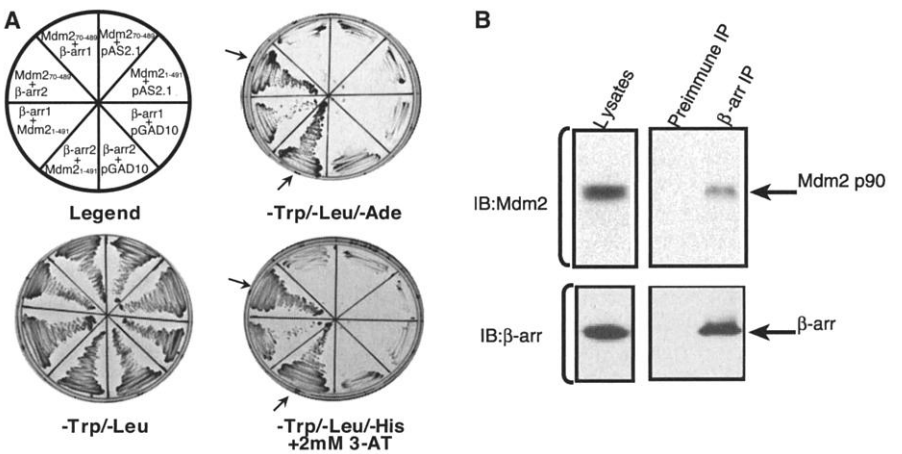

Fig. 4. Interaction of E3 ubiquitin ligase Mdm2 with β-arrestin. (**A**) Yeast two-hybrid interaction of Mdm2 and β-arrestin. Mdm2 plasmids were cotransformed with pAS2-1 or pAS2-1vectors containing β-arrestin1 or β-arrestin2 (top left). All the yeast transformants grew on tryptophan- and leucine-deficient plates (bottom left). However, only when both Mdm2 and β-arrestin were cotransformed was growth observed on the adenine-deficient (top right) or histidine-deficient (bottom right) plates. As seen in the right panels, β-arrestin2 shows a stronger interaction than does β-arrestin1 with Mdm2 homologs. (**B**) Coimmunoprecipitation of endogenous Mdm2 and β-arrestin2 from mouse brain lysates. β-Arrestin was immunoprecipitated from mouse brain lysates with A1CT, an antibody specific to β-arrestin covalently cross-linked to Reactigel beads (Pierce), and resolved by SDS-PAGE. The amount of β-arrestin and Mdm2 in the lysates shown represents 5% of the input amount. Mdm2 was detected (upper panels) with an Mdm2-specific antibody (2A10). The lower panels show the amount of β-arrestin in the lysates and immunoprecipitates (IP) as detected by A1CT antibody.

The β-arrestin–Mdm2 interaction was further confirmed by transforming yeast with pAS2.1–β-arrestin2 and pGAD10-$Mdm2_{70\text{-}489}$ (yeast two-hybrid clone, rat homolog), or pGAD10-$Mdm2_{1\text{-}491}$ (full-length human homolog of Mdm2) and testing for growth on selective plates (Fig. 4A). β-Arrestin2 interacted strongly with both $Mdm2_{70\text{-}489}$ and $Mdm2_{1\text{-}491}$. β-Arrestin1 also interacted with $Mdm2_{1\text{-}491}$ (Fig. 4A).

To assess whether the β-arrestin–Mdm2 interaction occurs in cells expressing endogenous amounts of the two proteins, we prepared extracts from mouse brain and tested the interaction by immunoprecipitation and immunoblotting (*23*). Mdm2 was detected in β-arrestin IPs but not in IPs with control preimmune antibody (Fig. 4B).

The β-arrestin–Mdm2 interaction was further confirmed by cellular coimmunoprecipitation assays in COS-7 cells and by in vitro glutathione *S*-transferase (GST)–pull-down experiments (*24*). Mdm2 showed a slightly higher affinity for β-arrestin2 in these assays. Moreover, β-arrestin2 preferentially bound to p90Mdm2, which has been reported to be a sumoylated form of Mdm2 (*25*). The failure to observe binding to p75 Mdm2 or the nonsumoylated Mdm2 could be either due to lower affinity or lower levels of expression of the unmodified Mdm2.

In vitro ubiquitination of β_2AR and β-arrestin2 catalyzed by Mdm2. We assessed the role of Mdm2 in the ubiquitination of the β_2AR in an in vitro cell-free assay with purified recombinant β_2AR (overexpressed in Sf9 insect cells) reconstituted in phospholipid vesicles as the substrate (*26*). As assessed by immunoblot with an antibody specific for the

β_2AR, unmodified receptor from Sf9 cells migrated as a major band of 55 kD in SDS gels (Fig. 5A). In this system, ubiquitinated receptor is apparent as broad bands of large molecular size. When GST–β-arrestin2 was included in the reaction with or without GRK2, no ubiquitination of β_2AR was detected. A very small amount of ubiquitinated β_2AR was detected when Mdm2 was added with or without β-arrestin2 in the absence of GRK2. However, when recombinant β-arrestin2, Mdm2, and GRK2 were all present in the reaction simultaneously, a large amount of agonist-dependent ubiquitination of β_2AR was detected. Thus, β_2AR ubiquitination in this system appears to require agonist occupancy of receptors, GRK-mediated receptor phosphorylation, β-arrestin binding, and Mdm2-catalyzed ubiquitination.

We used β-arrestin2His6 isolated from *Escherichia coli* cells (*27*) to test the ability of Mdm2 to ubiquitinate β-arrestin in vitro. β-Arrestin2, when added to COS-7 lysates, was ubiquitinated. Ubiquitination was increased considerably when extracts of cells overexpressing Mdm2 were added (Fig. 5B). Thus, at least in vitro, Mdm2 appears to serve as a ubiquitin ligase for both the β_2AR and β-arrestin.

Mdm2-catalyzed ubiquitination of β-arrestin is required for β_2AR internalization. To assess the functional relevance of Mdm2-catalyzed β-arrestin2 and β_2AR ubiquitination, we performed ubiquitination experiments in cells that express no Mdm2 without or with transfected Mdm2. Stimulation of endogenous β_2AR in Mdm2-null MEFs did not result in ubiquitination of β-arrestin2 (Fig. 6A). However, when these cells were transfected with Mdm2, isoproterenol-stimulated β-arrestin2 ubiquitination was observed. Thus, ubiquitination of β-arrestin requires the expression of the RING domain–containing E3 ubiquitin ligase Mdm2. In contrast, ubiquitination of β_2AR in Mdm2-null cells was not impaired (*11*). Thus, it appears that although β-arrestin ubiquitination requires Mdm2, β_2AR ubiquitination can be catalyzed by alternative, as yet unidentified E3 ligases, but this still requires β-arrestin2.

To analyze the functional consequences of β-arrestin ubiquitination, we tested the internalization and degradation of the β_2AR in Mdm2-null MEFs in which the β_2ARs, but not β-arrestin, can be ubiquitinated. Little internalization of β_2AR occurred in Mdm2-null cells (3.6 ± 1.2%) (Fig. 6B), but internalization was restored to amounts comparable to those in wild-type MEFs (20.6 ± 2.25%) when Mdm2 was transfected into these cells. Such overexpression of Mdm2 also restored the ubiquitination of β-arrestin in these cells (Fig. 6A). On the other hand, the degradation of the β_2AR was not impaired in Mdm-null cells (Fig. 6C). These data indicate that β-arrestin ubiquitination is required for internalization of the receptor but not for the degradation of receptors. Either the small percentage of receptor internalization occurring in Mdm-null cells is sufficient to cause a significant decline in the receptor numbers over the course of 24 hours of isoproterenol treatment, or alternative degradation pathways that do not require internalization could exist in these cell types. A normal rate of degradation of lutropin/choriogonadotropin receptor mutants that are impaired in internalization has been reported (*28*). Furthermore, different subtypes of α2 adrenergic receptors degrade to the same extent in cells, although their internalization rates differ (*29*).

To better define the role of Mdm2 in the ubiquitination of β-arrestin and to identify a putative dominant-negative Mdm2 mutant, we constructed deletion mutants of Mdm2 (*30*) (Fig. 7A) and mapped the region of β-arrestin interaction by analyzing their binding to full-length β-arrestin2 in coimmunoprecipitation experiments (Fig. 7B). The NH_2-terminal region of Mdm2, $Mdm2_{1\text{-}161}$, which includes the p53 binding sites (*31*), did not bind to β-arrestin

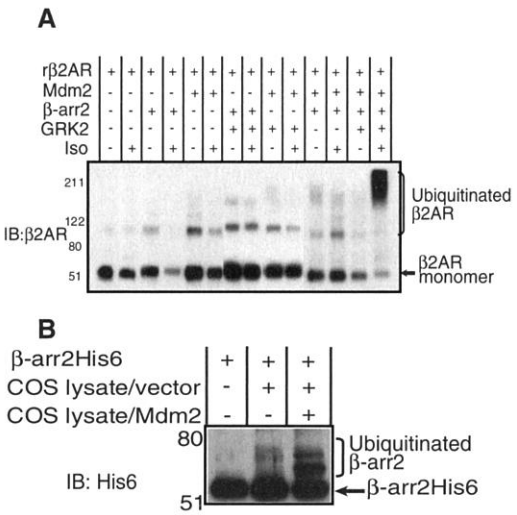

Fig. 5. Ubiquitin ligase activity of Mdm2 for β_2AR and β-arrestin2 in vitro. (**A**) Phospholipid vesicles containing purified β_2AR were incubated alone or with the indicated protein(s) in a reaction mixture supplemented with RRL (*26*). β_2AR protein was detected by immunoblotting with β_2AR-specific antibody (M-20, SantaCruz). The data are representative of four similar blots. (**B**) β-Arrestin2His6 purified from *E. coli* was used in a reaction mixture (*27*) containing RRL only, COS-7 lysate, or lysate of COS-7 cells overexpressing Mdm2, as indicated. β-Arrestin was detected with an antibody to the His6 tag. The data are representative of three similar experiments.

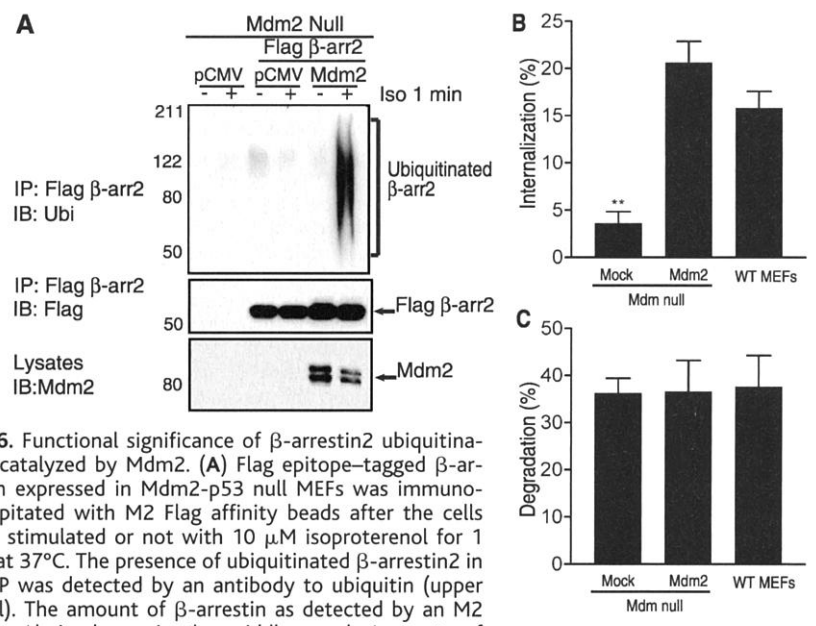

Fig. 6. Functional significance of β-arrestin2 ubiquitination catalyzed by Mdm2. (**A**) Flag epitope–tagged β-arrestin expressed in Mdm2-p53 null MEFs was immunoprecipitated with M2 Flag affinity beads after the cells were stimulated or not with 10 μM isoproterenol for 1 min at 37°C. The presence of ubiquitinated β-arrestin2 in the IP was detected by an antibody to ubiquitin (upper panel). The amount of β-arrestin as detected by an M2 Flag mAb is shown in the middle panel. Amounts of Mdm2 detected by the Mdm2 antibody 2A10 are shown in the lower panel. (**B**) Mdm2-p53–null cells transiently expressing β_2AR (300 to 600 fmol per mg of total cellular protein), without or with Mdm2, were stimulated with 10 μM isoproterenol for 25 min at 37°C. Internalization of β_2AR was determined by radioligand binding (*15*). The data are the mean ± SEM of four independent experiments. The statistical analyses were performed with Tukey repeated measures ANOVA; $^{**}P < 0.01$ for Mock versus Mdm2 as well as Mock versus WT samples. (**C**) Mdm2-p53 null cells transiently expressing β_2AR (300 to 600 fmol per mg of total cellular protein), without and with Mdm2, were stimulated with 10 μM isoproterenol for 24 hours at 37°C. Receptor degradation was determined by ^{125}I-CYP binding (*15*). The results are the mean ± SEM of five independent experiments.

(Fig. 7B). However, the deletion mutants $Mdm2_{1-321}$ and $Mdm2_{1-400}$ bound to β-arrestin to an extent comparable to binding of the full-length Mdm2. Thus, the region between amino acid residues 161 and 321 contains the β-arrestin–binding site (Fig. 7B).

$Mdm2_{1-321}$ containing the β-arrestin binding domain lacks the catalytic RING domain and could potentially act as a dominant-negative mutant with respect to Mdm2-catalyzed ubiquitination of β-arrestin or the β_2AR. Agonist-stimulated ubiquitination of β-arrestin2 was completely blocked when $Mdm2_{1-321}$ was coexpressed in this system (Fig. 7C). On the other hand, the same Mdm2 mutant did not decrease ubiquitination of the β_2AR (*11*).

Coexpression of the $Mdm2_{1-321}$ mutant decreased internalization of β_2AR by 72% compared with that in mock-transfected cells (Fig. 7D). A similar inhibition of internalization was seen when a mutant dynamin (K44A), a classical inhibitor of clathrin-mediated endocytosis, was used. The $Mdm2_{1-321}$ mutant did not reduce degradation of the receptors (Fig. 7E). Thus, when β-arrestin ubiquitination is eliminated, either in the Mdm2-null cells or by coexpression of a dominant-negative Mdm2 mutant, the internalization of the β_2AR is impaired, whereas receptor degradation is not. Therefore, rapid agonist-stimulated transient ubiquitination of β-arrestin appears to be a prerequisite for rapid receptor internalization, but not for receptor degradation.

β_2AR ubiquitination is essential for its degradation. To define the functional significance of the ubiquitin modification of the β_2AR, we used a mutant β_2AR (0-Kβ_2AR) in which all 16 of the lysine residues are replaced with arginine residues (*32*) and compared it with the wild-type β_2AR (WT-β_2AR). This mutant binds ligands and activates adenylyl cyclase, as the wild-type receptor does (*32*). The 0-Kβ_2AR was not ubiquitinated in COS-7 cells stimulated with isoproterenol (Fig. 8A). β-Arrestin transfection was required to observe receptor ubiquitination in COS-7 cells because of the low expression of β-arrestin in these cells. The ability of the 0-Kβ_2AR to interact with β-arrestin is, however, unaffected as seen in the receptor immunoprecipitation assays (Fig. 8A) and in confocal microscopy experiments done with GFP–β-arrestin2 in both COS-7 and human embryonic kidney 293 (HEK293) cells (*11*). Further, the 0-Kβ_2AR internalized normally as compared with the WT-β_2AR in HEK293 cells (*33*) (Fig. 8B). These data indicate that ubiquitin modification of the β_2AR is not required for receptor internalization.

Although internalization of 0-Kβ_2AR remained normal, its agonist-promoted degradation, measured after 24 hours of isoproterenol treatment in HEK293 cells, was impaired (Fig. 8C). Indeed, the small amount of residual degradation (~5%) observed for the 0-Kβ_2AR could represent degradation of endogenous wild-type β_2AR present in these cells. Therefore, unlike internalization, the degradation of the β_2AR does require ubiquitination of the receptor in these cells. Although the results obtained with 0-Kβ_2AR experiments indicate that the ubiquitination on the cytosolic lysines is a prerequisite for trafficking to lysosomes, the possibility that the lysine residue(s) on the cytoplasmic regions of the β_2AR instead form part of a motif necessary for trafficking to lysosomal compartments cannot be ruled out.

Distinct roles of receptor and β-arrestin ubiquitination. Agonist-dependent trafficking of GPCRs, including internalization, recycling, and degradation, is a general feature of their biology. Although the yeast pheromone receptors Ste2p and Ste3p (which are heptahelical receptors) are ubiquitinated as a signal for internalization (*7*, *8*) and rout-

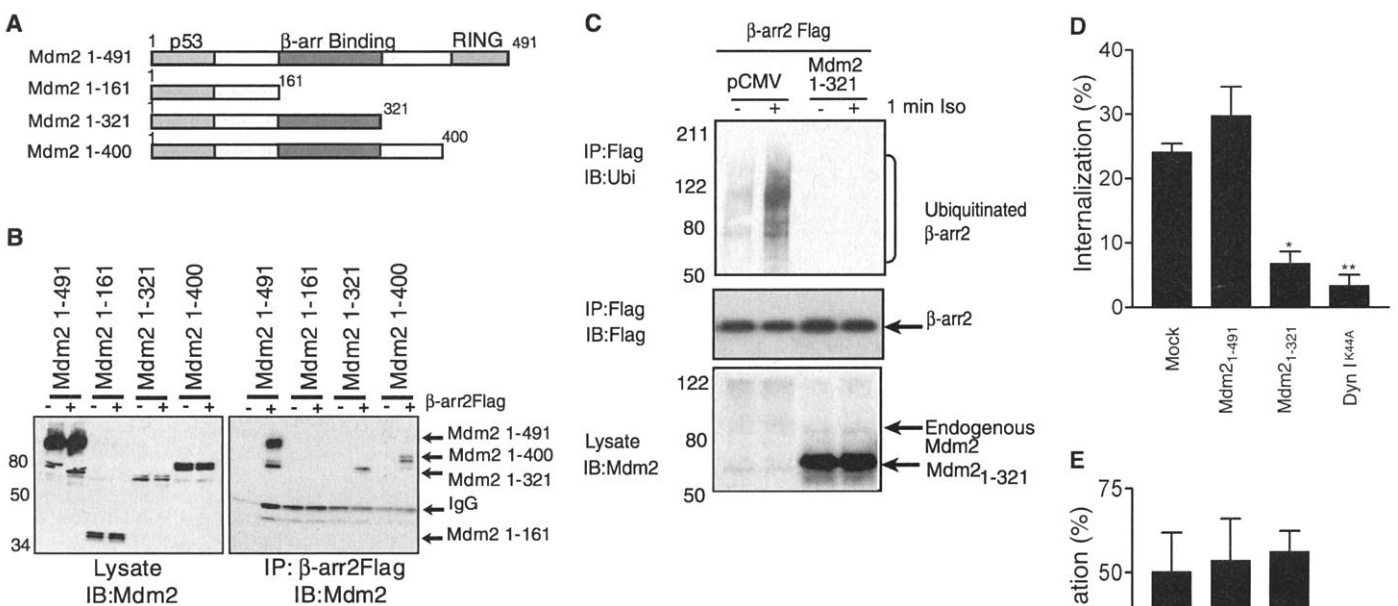

Fig. 7. Identification of a dominant-negative Mdm2 that affects β-arrestin ubiquitination and β_2AR internalization. (**A**) Schematic representation of wild-type and COOH-terminal truncation mutants of $Mdm2_{1-491}$. (**B**) COS-7 cells were transiently transfected with full-length or truncated Mdm2 alone or with β-arrestin2–Flag. β-Arrestin was immunoprecipitated with M2 Flag affinity beads, and the presence of Mdm2 in the lysates and IP was detected with an antibody to Mdm2 (Ab-1, Oncogene Research Products). (**C**) COS-7 cells were transfected with β-arrestin2–Flag alone or with $Mdm2_{1-321}$. The endogenous β_2ARs were stimulated for 1 min with 10 μM isoproterenol. β-Arrestin was precipitated with M2 FLAG beads, and the presence of ubiquitin was detected with antibody UbP4D1. The expression of Mdm2 mutant and the amount of endogenous Mdm2 are shown in the bottom panel. Data are representative of four similar experiments. (**D**) CHW-β2 cells were transfected with pcDNA3, $Mdm2_{1-491}$ (WT), $Mdm2_{1-321}$, or dynamin K44A. Internalization of β_2AR was determined by radioligand binding after 20 min of stimulation at 37°C with 10 μM isoproterenol (*15*). The data are the mean ± SEM of five independent experiments. Statistical analyses were performed by one-way ANOVA; $^*P < 0.05$, $^{**}P < 0.01$ versus Mock. (**E**) CHW-β2 cells were transfected with pcDNA3, $Mdm2_{1-491}$, $Mdm2_{1-321}$, or dynamin K44A. Degradation of β_2AR was determined by radioligand binding after 24 hours of stimulation at 37°C with 10 μM isoproterenol (*15*). The results are the mean ± SEM of three independent experiments. $^*P < 0.05$, ANOVA.

ing to lysosomes, there is little evidence linking ubiquitination to the trafficking of mammalian GPCRs. Many mammalian GPCRs are internalized by a clathrin-mediated process that has an absolute requirement for the participation of β-arrestin, which appears to function as an adapter binding to both clathrin (*34*) and adapter protein2 (AP2) (*35*). Our data show that β_2AR as well as β-arrestin2 undergo rapid, agonist-stimulated ubiquitination. Moreover, the ubiquitination of the receptor requires its agonist-dependent interaction with β-arrestin. β-Arrestin acts as an adapter protein to bring an E3 ligase to the activated receptor. The role of Mdm2 is to function as an obligatory ligase to ubiquitinate β-arrestin upon agonist stimulation. Mdm2-catalyzed ubiquitination of β-arrestin is required for β_2AR internalization. β_2AR ubiquitination, however, is unaffected in Mdm2-null cells, and a dominant-negative Mdm2 (which hinders both β-arrestin ubiquitination and β_2AR internalization) is unable to inhibit β_2AR ubiquitination, allowing the receptor degradation to proceed normally. Thus, β-arrestin may bind and recruit other E3 ligases that can act on the receptor, although their identity is currently unknown.

Ubiquitination of β-arrestin and of the receptor appear to subserve different functions in regulating the life cycle of the receptor. β-Arrestin ubiquitination is required for receptor internalization, whereas receptor ubiquitination is required for receptor degradation. Ubiquitination of a number of membrane proteins is now known to regulate their internalization, although the molecular mechanisms involved remain obscure. Currently it is unknown whether the ubiquitin moieties remain attached during lysosomal transport of the membrane proteins. Perhaps the ubiquitin moiety serves as a link between receptor and/or adapter proteins and the components of the endocytic machinery, or regulates these interactions in alternative ways.

Stimulation of GHR leads to receptor ubiquitination followed by its internalization by way of a clathrin-dependent process (*3*). Moreover, proteasomal inhibitors block the internalization of GHR, just as they do for the β_2AR (*16*). Nevertheless, mutants of GHR that cannot be ubiquitinated are still internalized normally, and this too is blocked by proteasome inhibitors (*16*). The authors speculate that some GHR-associated protein must be ubiquitinated for receptor internalization to proceed. The identity of this putative protein, the postulated role of which is analogous to that of β-arrestin, is currently unknown. Also unclear is how proteasomes might function to promote internalization and ultimately degradation of molecules such as GHR, β_2AR, and others, which appear to be degraded in lysosomes rather than by proteasomes. Proteasomes might function in targeting the internalized receptors to lysosomes for degradation.

The kinetics of receptor and β-arrestin ubiquitination differ in several respects. After receptor stimulation, ubiquitination of β-arrestin is both more rapid and more transient than that of the receptor. The time frame of ubiquitination and deubiquitination of β-arrestin appears to correspond to the time frame of its binding to and dissociation from the receptor. Whether its deubiquitination is a cause or consequence of its dissociation from the receptor, however, is not known. Our findings thus reveal mechanisms by which the ubiquitination machinery and β-arrestin interact to regulate the internalization and degradation of GPCRs.

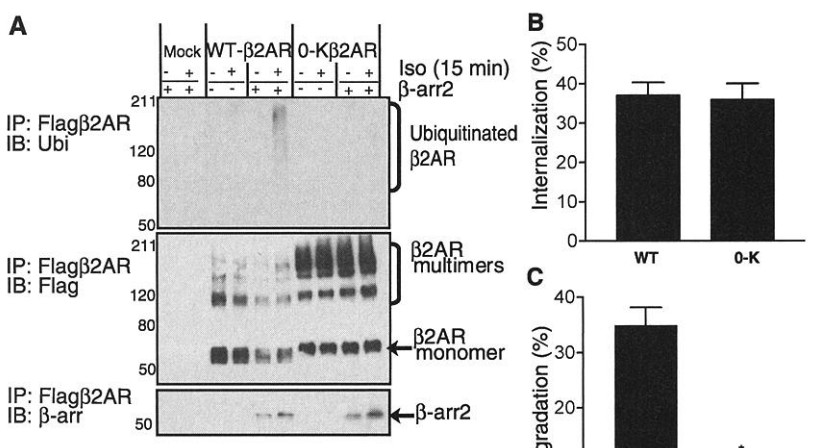

Fig. 8. Functional significance of β_2AR ubiquitination. (**A**) Western blot of immunoprecipitates of WT-β_2AR and a mutant receptor with no lysine residues (0-Kβ_2AR) transiently expressed in COS-7 cells. (Upper panel) Detection of ubiquitinated proteins after 15 min of isoproterenol stimulation (10 μM); (middle panel) amount of receptor protein in each sample lane; (bottom panel) amount of β-arrestin in the receptor IPs as detected by antibody to β-arrestin. (**B**) Receptor internalization induced by a 30-min exposure to isoproterenol was determined by flow cytometry (*33*) performed on HEK293 cells transiently overexpressing WT-β_2AR or 0-Kβ_2AR. Data represent the mean ± SEM of eight independent experiments. (**C**) The degradation of WT-β_2AR (500 fmol/mg), and of 0-Kβ_2AR (300 fmol/mg) transiently expressed in HEK293 cells, was determined by radioligand binding after 24 hours of stimulation at 37°C with 10 μM isoproterenol (*15*). The results are the mean ± SEM of eight independent experiments. The statistical significance was determined by a paired *t* test: $^*P < 0.001$ for the WT versus 0-K samples.

References and Notes

1. A. Hershko, A. Ciechanover, *Annu. Rev. Biochem.* **67**, 425 (1998).
2. J. S. Bonifacino, A. M. Weissman, *Annu. Rev. Cell Dev. Biol.* **14**, 19 (1998).
3. P. van Kerkhof, M. Sachse, J. Klumperman, G. J. Strous, *J. Biol. Chem.* **276**, 3778 (2001).
4. C. A. Joazeiro *et al.*, *Science* **286**, 309 (1999).
5. M. S. Obin, J. Jahngen-Hodge, T. Nowell, A. Taylor, *J. Biol. Chem.* **271**, 14473 (1996).
6. K. Chaturvedi, P. Bandari, N. Chinen, R. D. Howells, *J. Biol. Chem.* **276**, 12345 (2001).
7. L. Hicke, H. Riezman, *Cell* **84**, 277 (1996).
8. A. F. Roth, N. G. Davis, *J. Biol. Chem.* **275**, 8143 (2000).
9. M. G. Caron, Y. Srinivasan, J. Pitha, K. Kociolek, R. J. Lefkowitz, *J. Biol. Chem.* **254**, 2923 (1979).
10. CHW1102 cells expressing untagged β_2AR were lysed in lysis buffer [LB; 50 mM Hepes (pH 7.5), 0.5% NP-40, 250 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, leupeptin (10 μg/ml)], mixed with affinity beads (Sepharose 4B–alprenolol), and rotated at 4°C for 4 hours. Nonspecific binding was eliminated by repeated washes with LB, and bound receptor protein was eluted with sample buffer containing SDS. The proteins were transferred to nitrocellulose membrane for Western blotting. Chemiluminiscent detection was performed with SuperSignal West Pico reagent (Pierce). Antibodies Ub P4D1 (SantaCruz) and β_2AR H-20 (SantaCruz) were used to detect ubiquitin and β_2AR, respectively.
11. S. K. Shenoy *et al.*, unpublished data.
12. A. Yu, T. R. Malek, *J. Biol. Chem.* **276**, 381 (2001).
13. β-Arrestin2 with a COOH-terminal Flag epitope (pcDNA3–β-arr2Flag) was transiently transfected with Lipofectamine (Life Technologies, Rockville, MD) in COS-7 cells, which were then stimulated by isoproterenol. Cells were solubilized in LB supplemented with 5 mM *N*-ethylmaleimide (NEM). β-Arrestin was immunoprecipitated with Flag affinity beads (Sigma), and the immunoprecipitates were resolved by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoreacted with Ub P4D1 to detect ubiquitinated β-arrestin2. Longer exposure of β-arrestin blots showed broad bands, as in the ubiquitin blots.
14. R. H. Oakley, S. A. Laporte, J. A. Holt, L. S. Barak, M. G. Caron, *J. Biol. Chem.* **274**, 32248 (1999).
15. Internalization and degradation assays were done with [^{125}I](−)iodocyanopindolol (^{125}I-CYP) radioligand binding (*18*) on whole cells gently resuspended in Dulbecco's modified Eagle's medium (Life Technologies) buffered with 10 mM Hepes (pH 7.5). Binding was performed in triplicates with 400 pM ^{125}I-CYP in the presence or absence of the hydrophobic antagonist propranolol (10 μM, to define nonspecific binding) and in the presence or absence of the hydrophilic antagonist CGP12177 (0.3 μM, to assess internalized receptors). Incubation for internalization assays was at 13°C for 3 hours. Binding was terminated by rapid dilution and filtration on Whatman GFC glass fiber filters. To obtain the number of internalized receptors, we determined the percentage of total specific ^{125}I-CYP binding sites that could not be displaced by CGP12177. The isoproterenol-stimulated internalization was determined as the difference between the percentage of total receptors internalized after stimulation and the percentage of receptors internalized

in untreated cells. For degradation assays, cells were incubated at 25°C for 1 hour, and receptor number (total specific ^{125}I-CYP binding sites) was determined after 24 hours of isoproterenol treatment and expressed as the percentage of receptor number assessed in nonstimulated cells. Where necessary, MG132 (20 μM) or lactacystin (20 μM) mixed in serum-free media was added to cells 1 hour before stimulation.

16. P. van Kerkhof, R. Govers, C. M. Alves dos Santos, G. J. Strous, *J. Biol. Chem.* **275**, 1575 (2000).
17. Flag epitope–tagged WT-β_2AR and β_2AR^{2-}, along with β-arrestin2, were transiently transfected in COS-7 cells with Lipofectamine. After agonist treatment, receptors were immmunoprecipitated with Flag affinity beads, and the immunoprecipitates were resolved on SDS-PAGE and probed with antibodies Ub P4D1 and β_2AR H-20.
18. W. P. Hausdorff *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2979 (1991).
19. Confluent cells on 150-mm dishes were treated or not for 15 min with isoproterenol, after which cells were lysed in LB. Endogenous β_2AR was immunoprecipitated with the antibody β_2AR M-20. In the case of overexpressed β_2AR, cells seeded at 400,000 per 100-mm dish were transfected with pcDNA3-Flag-β_2AR plasmid using Lipofectamine, and receptor was immunoprecipitated using FLAG affinity beads (Sigma). Ubiquitinated species were detected with antibody Ub P4D1.
20. C. T. Chien, P. L. Bartel, R. Sternglanz, S. Fields, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9578 (1991).
21. S. Fang, J. P. Jensen, R. L. Ludwig, K. H. Vousden, A. M. Weissman, *J. Biol. Chem.* **275**, 8945 (2000).
22. R. Honda, H. Tanaka, H. Yasuda, *FEBS Lett.* **420**, 25 (1997).
23. For coimmunoprecipitation of endogenous proteins from whole-brain extract, brain tissue was solubilized in LB (*10*). β-Arrestin was immunoprecipitated from extracts by incubation for 4 hours at 4°C with antibody specific for β-arrestin, covalently cross-linked to Reactigel beads (Pierce). Immunoprecipitated proteins were analyzed by SDS-PAGE and detected by immunoblot analysis. For cellular experiments, COS-7 cells were transfected with COOH-terminal Flag-tagged β-arrestin and untagged Mdm2 plasmids. Flag–β-arrestins were immunoprecipitated with M2 Flag affinity beads (Sigma); the immunoprecipitates were separated by SDS-PAGE, and the presence of Mdm2 was demonstrated by immunoblotting with monoclonal antibodies (mAbs) 2A10 or AB1.
24. Supplementary Web material is available on *Science* Online at www.sciencemag.org/cgi/content/full/1063866/DC1.
25. T. Buschmann, S. Y. Fuchs, C.-G. Lee, Z-Q. Pan, Z. Ronai, *Cell* **101**, 753 (2000).
26. Recombinant β_2AR reconstituted in vesicles was used as the substrate in a ubiquitination reaction containing 20 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 2 mM adenosine 5′-triphosphate (ATP), 5 μg of ubiquitin, 20 μM MG132, and crude rabbit reticulocyte lysate (RRL, Promega), a source for the ubiquitination machinery (*36*) (5 μl/reaction). The reactions were supplemented with either His–β-arrestin2 (7 μg) or GST–β-arrestin2 (5 μg), GRK2, (1 μg), or Mdm2 [either GST-Mdm2$_{1-491}$ (8 μg) or COS-7 lysate with overexpressed Mdm2] where necessary. Reaction mixtures were incubated for 1 hour at 30°C. Ubiquitinated receptor (vesicles) was isolated by repeated washes with LB and centrifugation at 250,000*g*, 45 min. After addition of SDS–sample buffer, protein samples were incubated at 37°C for 2 hours before electrophoresis.
27. A cDNA construct of β-arrestin2 was cloned into pET-29a and was used to isolate recombinant S·TAG-β-arrestin2His6 purified on S-protein agarose beads (Novagen), which was then used as the substrate in the β-arrestin2 in vitro ubiquitination reaction containing 20 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 2 mM DTT, 2 mM ATP, 5 μg of ubiquitin, 20 μM MG132, and crude RRL, either supplemented or not with 100 μg of COS cell extract (clarified by centrifugation at 21,000*g* for 15 min) with or without overexpressed Mdm2.
28. K. Nakamura, M. F. Lazari, S. Li, C. Korgaonkar, M. Ascoli, *Mol. Endocrinol.* **13**, 1295 (1999).
29. P. Tsao, T. Cao, M. von Zastrow, *Trends Pharmacol. Sci.* **22**, 91 (2001)
30. Mdm2 truncation mutants were prepared by standard polymerase chain reaction protocols and subcloned into pcDNA3. All sequences were confirmed with an automated ABI DNA sequencer (Howard Hughes Nucleic Acid Facility, Duke University).
31. J. Chen, V. Marechal, A. J. Levine, *Mol. Cell. Biol.* **13**, 4107 (1993).
32. A. L. Parola, S. Lin, B. K. Kobilka, *Anal. Biochem.* **254**, 88 (1997).
33. Flag epitope–tagged β_2ARs expressed in HEK293 cells in six-well dishes were treated with or without isoproterenol for 30 min in serum-free medium at 37°C. Cell surface receptors were labeled with Flag mAb M1, and fluorescein isothiocyanate–conjugated goat antibody to mouse immunoglobulin G was used as secondary antibody. Receptor internalization was quantified as loss of cell surface receptors, as measured by flow cytometry.
34. O. B. Goodman Jr. *et al.*, *Nature* **383**, 447 (1996).
35. S. A. Laporte *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 3712 (1999).
36. S. Mori *et al.*, *Eur. J. Biochem.* **247**, 1190 (1997).
37. M. Bouvier *et al.*, *Mol. Pharmacol.* **33**, 133 (1988).
38. We thank D. Addison, M. Holben, and J. Turnbough for secretarial assistance; B. K. Kobilka for 0-Kβ_2AR plasmid; A. J. Levine for the Mdm2 plasmid and mAb 2A10; G. Lozano for providing the Mdm2-p53 null cell line; M. Delahunty for purified rβ_2AR; W. E. Miller for pAS2 constructs and advice in yeast experiments; F. T. Lin for pcDNA3-βarrHis6 constructs; M. Cong for providing pcDNA3-β_2AR^{2-}; S. J. Perry and K. L. Pierce for critical reading; H. E. Kendall and G. P. Irons for technical assistance; W. D. Capel for purified GRK2; and G. J. Sabo for assistance. Supported by NIH grant HL16037. R.J.L is an investigator of the Howard Hughes Medical Institute.

27 June 2001; accepted 17 September 2001
Published online 4 October 2001;
10.1126/science.1063866
Include this information when citing this paper.

Logic Gates and Computation from Assembled Nanowire Building Blocks

Yu Huang,[1*] Xiangfeng Duan,[1*] Yi Cui,[1] Lincoln J. Lauhon,[1] Kyoung-Ha Kim,[2] Charles M. Lieber[1,2†]

Miniaturization in electronics through improvements in established "top-down" fabrication techniques is approaching the point where fundamental issues are expected to limit the dramatic increases in computing seen over the past several decades. Here we report a "bottom-up" approach in which functional device elements and element arrays have been assembled from solution through the use of electronically well-defined semiconductor nanowire building blocks. We show that crossed nanowire p-n junctions and junction arrays can be assembled in over 95% yield with controllable electrical characteristics, and in addition, that these junctions can be used to create integrated nanoscale field-effect transistor arrays with nanowires as both the conducting channel and gate electrode. Nanowire junction arrays have been configured as key OR, AND, and NOR logic-gate structures with substantial gain and have been used to implement basic computation.

Fundamental physical constraints and economics are expected to limit continued miniaturization in electronics by conventional top-down manufacturing during the next one to two decades (*1*, *2*) and have thus motivated efforts world wide to search for new strategies to meet expected computing demands of the future. Bottom-up approaches to nanoelectronics (*2*), where the functional electronic structures are assembled from well-defined nanoscale building blocks, such as carbon nanotubes (*3–8*), molecules (*9–11*), and/or semiconductor nanowires (*12–14*), have the potential to go far beyond the limits of top-down manufacturing. For example, single-walled carbon nanotubes (NTs) have been used as building blocks to fabricate room-temperature field-effect transistors (FETs) (*3–5*), diodes (*6*, *7*) and recently, an inverter (*8*), which represents a key component for logic. However, the inability to control whether NTs are semiconducting or metallic (*2*, *5*) makes specific device fabrication largely a random event and poses a serious issue for integration beyond the single–device element level. A potential solution to the prob-

[1]Department of Chemistry and Chemical Biology, [2]Division of Engineering and Applied Sciences, Harvard University, Cambridge, MA 02138, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: cml@cmliris.harvard.edu