

# $\beta$ -Arrestin2 functions as a phosphorylation-regulated suppressor of UV-induced NF- $\kappa$ B activation

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**NF- $\kappa$ B activation is an important mechanism of mammalian UV response to protect cells. UV-induced NF- $\kappa$ B activation depends on the casein kinase II (CK2) phosphorylation of I $\kappa$ B $\alpha$  at a cluster of C-terminal sites, but how it is regulated remains unclear. Here we demonstrate that  $\beta$ -arrestin2 can function as an effective suppressor of UV-induced NF- $\kappa$ B activation through its direct interaction with I $\kappa$ B $\alpha$ . CK2 phosphorylation of  $\beta$ -arrestin2 blocks its interaction with I $\kappa$ B $\alpha$  and abolishes its suppression of NF- $\kappa$ B activation, indicating that the  $\beta$ -arrestin2 phosphorylation is critical. Moreover, stimulation of  $\beta_2$ -adrenergic receptors, a representative of G-protein-coupled receptors in epidermal cells, promotes dephosphorylation of  $\beta$ -arrestin2 and its suppression of NF- $\kappa$ B activation. Consequently, the  $\beta$ -arrestin2 suppression leads to promotion of UV-induced cell death, which is also under regulation of  $\beta$ -arrestin2 phosphorylation. Thus,  $\beta$ -arrestin2 is identified as a phosphorylation-regulated suppressor of UV response and this may play a functional role in the response of epidermal cells to UV.**

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## Introduction

The UV response in mammalian cells is mediated through activation of several transcription factors, including NF- $\kappa$ B (Shaulian *et al*, 2000). In resting cells, NF- $\kappa$ B is predominantly sequestered in the cytoplasm through association with specific inhibitors I $\kappa$ B (reviewed by Silverman and Maniatis, 2001). The binding of I $\kappa$ B to NF- $\kappa$ B masks the nuclear localization sequences (NLS) of the latter and thus traps NF- $\kappa$ B in the cytoplasm. UV irradiation of mammalian cells leads to phosphorylation of I $\kappa$ B by casein kinase II (CK2) and triggers the ubiquitination-dependent degradation of I $\kappa$ B

(Bender *et al*, 1998; Kato *et al*, 2003). NF- $\kappa$ B is then released and translocated to the nucleus and promotes target genes transcription through binding to specific promoters (Israel, 2000; Karin and Ben-Neriah, 2000). NF- $\kappa$ B pathway is under various regulations. For example, Gao *et al* (2004) have shown that  $\beta$ -arrestin2, one of the major regulatory and signaling molecules in G-protein-coupled receptor (GPCR) signal transduction, directly interacts with I $\kappa$ B $\alpha$  and prevents phosphorylation and degradation of I $\kappa$ B $\alpha$ . Similar results are also obtained from Witherow *et al* (2004). It has been shown that deficiency of the murine NF- $\kappa$ B results in embryonic lethality as a result of fetal hepatocyte apoptosis (Beg and Baltimore, 1996), indicating that NF- $\kappa$ B plays a critical role in regulation of apoptosis. NF- $\kappa$ B exerts its antiapoptotic effect through inducing various antiapoptotic genes, such as *Bcl-xL*, *X-IAP*, *IAP1* and *2*, *IEX-1L*, and *Bfl-1*, following treatment with diverse stimuli, including UV irradiation, inflammatory cytokines, and chemotherapeutic drugs (Barkett and Gilmore, 1999; Baldwin, 2001).

GPCRs comprise a large gene family of up to 1000 members that mediate many distinct physiological functions as diverse as phototransduction, olfaction, vascular tone, cardiac output, digestion, and pain (Oakley *et al*, 2000). Recently, increasing evidence indicates the potential role of GPCRs in epidermal functions (Takahashi *et al*, 1996; Schallreuter *et al*, 1997; Denda *et al*, 2003; Gillbro *et al*, 2004; Steckelings *et al*, 2004).  $\beta_2$ AR, one of the well-studied GPCRs, are ubiquitously expressed in the epidermis and function to maintain homeostasis in normal physiological settings as well as pathological states in epidermis (Koizumi *et al*, 1991; Steinkraus *et al*, 1996; Denda *et al*, 2003). Epinephrine and other catecholamines, which stimulate  $\beta_2$ AR, mediate signals not only in autocrine regulation among epidermal cells but also from the sympathetic nervous system (Steckelings *et al*, 2004). However, the molecular mechanism underlying  $\beta_2$ AR regulation of epidermis remains to be investigated.

$\beta$ -Arrestins are ubiquitous multifunctional adaptor proteins that regulate numerous aspects of GPCR functions and have been recently shown to serve as mediators that link the receptors to other signaling pathways (Goodman *et al*, 1996; Luttrell *et al*, 1999; McDonald *et al*, 2000; Perry *et al*, 2002; Chen *et al*, 2003). Recent studies showed that  $\beta$ -arrestins are subjected to several post-translational modifications. Upon stimulation with  $\beta_2$ AR,  $\beta$ -arrestin2 is rapidly and transiently ubiquitinated through its association with Mdm2, and the ubiquitination of  $\beta$ -arrestin2 is required for the GPCR internalization (Shenoy *et al*, 2001). The functions of  $\beta$ -arrestins can also be regulated by phosphorylation. Cytosolic  $\beta$ -arrestin1 is constitutively phosphorylated at Ser412 by ERKs, and stimulation of  $\beta_2$ AR leads to dephosphorylation of  $\beta$ -arrestin1 on the plasma membrane, which is essential for targeting receptor to clathrin-coated pits and for binding to c-Src to induce ERK activation (Lin *et al*, 1997; Luttrell *et al*, 1999).

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$\beta$ -Arrestin2 is also reported to be constitutively phosphorylated by CK2, predominantly at residues Ser361 and Thr383, and  $\beta_2$ AR stimulation promotes dephosphorylation of  $\beta$ -arrestin2 (Kim *et al*, 2002; Lin *et al*, 2002). However, the functional role of  $\beta$ -arrestin2 phosphorylation remains unclear.

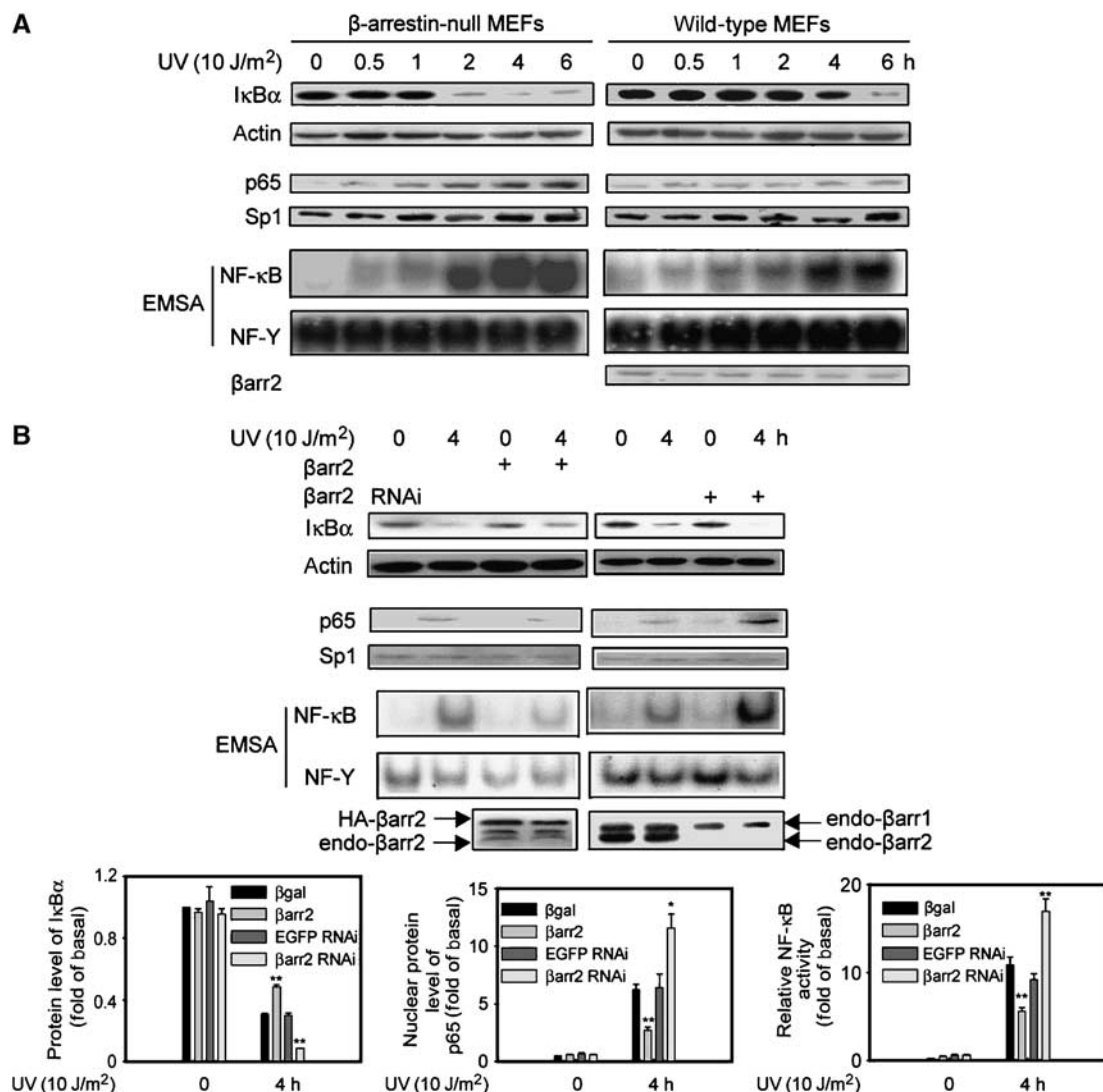
## Results

### $\beta$ -Arrestin2 suppresses UV-induced NF- $\kappa$ B activation

Previous studies have shown that  $\beta$ -arrestin2 could inhibit NF- $\kappa$ B activation induced by activators such as TNF $\alpha$  (Gao *et al*, 2004, Witherow *et al*, 2004), LPS, and IL-1 $\beta$  (our unpublished data). As UV irradiation also induced NF- $\kappa$ B activation (Li and Karin, 1998; Shaulian *et al*, 2000), we first investigated whether  $\beta$ -arrestin2 had a similar function in UV pathway.  $\beta$ -Arrestin-null murine embryonic fibroblasts (MEFs) and wild-type MEFs were exposed to UV irradiation

for 1 min ( $10\text{ J/m}^2$ ), and then incubated for up to 6 h. Cells were harvested at the indicated time points and the level of I $\kappa$ B $\alpha$  was detected by immunoblot. As shown in Figure 1A, UV irradiation led to a time-dependent I $\kappa$ B $\alpha$  degradation in  $\beta$ -arrestin-null MEFs, which was much less in wild-type MEFs. Next, UV-induced NF- $\kappa$ B p65 nuclear translocation, which follows I $\kappa$ B $\alpha$  degradation, was examined. UV irradiation caused a much stronger nuclear translocation of NF- $\kappa$ B p65 and NF- $\kappa$ B DNA-binding activity in  $\beta$ -arrestin-null MEFs than that in their wild-type counterparts (Figure 1A). The purity of nuclear and cytoplasmic fractions was demonstrated with antibodies specific for Sp1, a known nuclear protein (Figure 1A), and tubulin, known to be cytoplasmic (data not shown). The specificity of NF- $\kappa$ B DNA-binding activity was ascertained as reported previously (Gao *et al*, 2004).

To further analyze  $\beta$ -arrestin2's function in UV-induced NF- $\kappa$ B activation, human epidermal carcinoma A431 cells



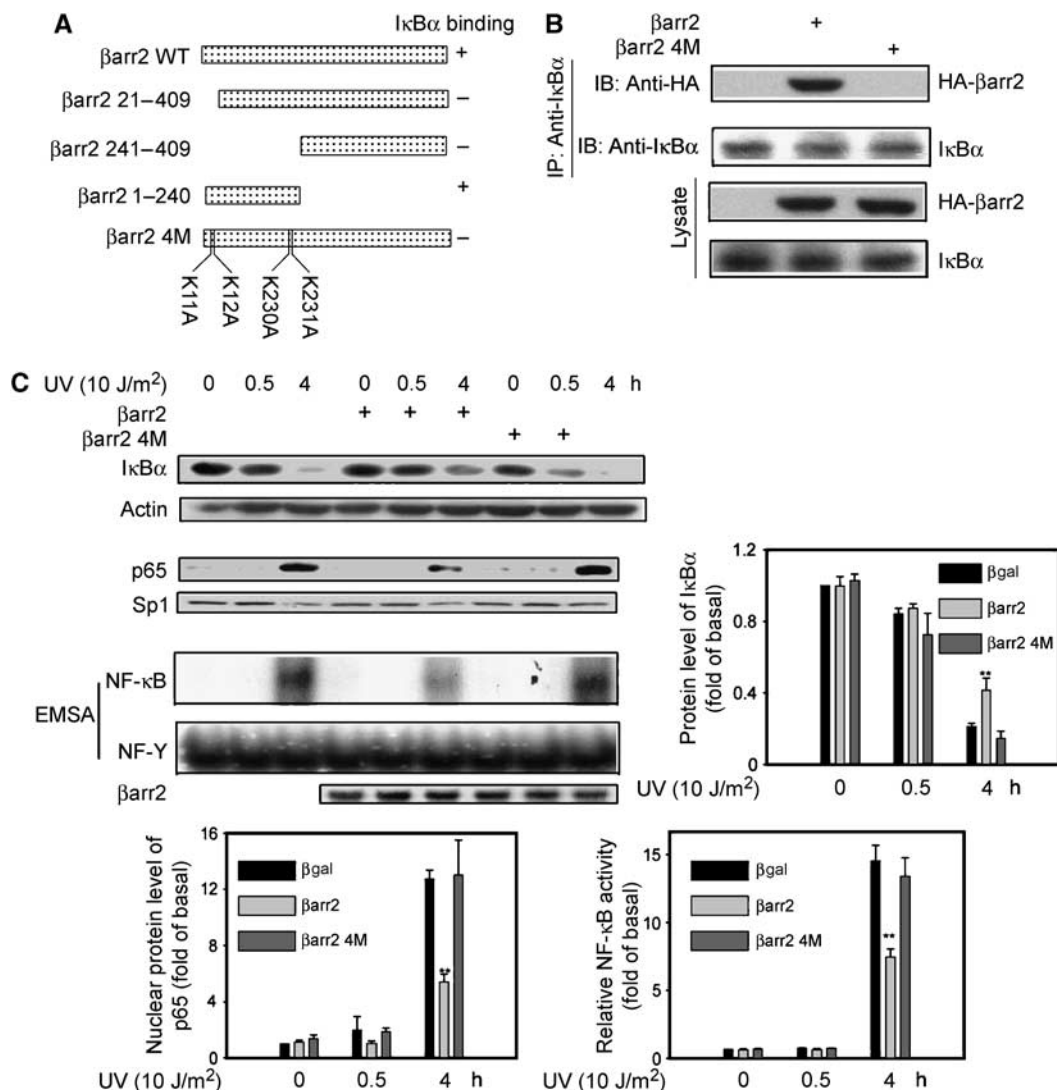
**Figure 1**  $\beta$ -arrestin2 suppresses UV-induced NF- $\kappa$ B activation. (A) Time course of UV-induced I $\kappa$ B $\alpha$  degradation measured by immunoblot, nuclear translocation of NF- $\kappa$ B p65 by immunoblot, and NF- $\kappa$ B DNA-binding activities by EMSA using nuclear extracts in  $\beta$ -arrestin-null MEFs and wild-type MEFs. (B) UV-induced degradation of I $\kappa$ B $\alpha$  was measured by immunoblot in A431 cells expressed with  $\beta$ -arrestin2 or  $\beta$ -arrestin2 siRNA. UV-induced nuclear translocation of NF- $\kappa$ B p65 was measured by immunoblot and UV-induced NF- $\kappa$ B DNA-binding activities by EMSA using nuclear extracts of A431 cells expressed with  $\beta$ -arrestin2 or  $\beta$ -arrestin2 siRNA. The actual increase (upon overexpression) is four-fold of basal and decrease (with siRNA) is about 95% of basal  $\beta$ -arrestin2 expression. Data are representative of at least three independent experiments and shown as mean  $\pm$  s.e. \* $P < 0.05$ , and \*\* $P < 0.01$  versus control.  $\beta$ arr2:  $\beta$ -arrestin2.

were transiently transfected with  $\beta$ -arrestin2 or  $\beta$ -arrestin2 siRNA and irradiated with UV. The I $\kappa$ B $\alpha$  degradation, p65 nuclear translocation, and NF- $\kappa$ B DNA-binding activity were all decreased by expression of  $\beta$ -arrestin2, while they were all augmented by application of  $\beta$ -arrestin2 siRNA (Figure 1B). As a control, AP1 transcription activity was unaffected under similar conditions (data not shown).

Next we analyzed whether  $\beta$ -arrestin2 stabilized I $\kappa$ B $\alpha$  after UV irradiation via its direct binding to I $\kappa$ B $\alpha$  as in the case of TNF $\alpha$  stimulation (Gao *et al*, 2004). Previous work had shown that deletion of amino acids 1–19 from the N-terminus or 186–409 from the C-terminus of  $\beta$ -arrestin2 resulted in complete loss of its ability to bind to I $\kappa$ B $\alpha$ , while deletion of amino acids 240–409 did not. These results indicated that  $\beta$ -arrestin2 possessed two binding sites to I $\kappa$ B $\alpha$  (one among the amino acids 1–19 and another 186–240) (Figure 2A), and positively charged amino acids in  $\beta$ -arrestin2 were important

for its interaction with other proteins like GPCR and Mdm2 (data not shown). The mutations of the positively charged amino acids in those two fragments were screened, and the mutations of Lys11, Lys12, Lys230, and Lys231 to Ala of  $\beta$ -arrestin2 ( $\beta$ -arrestin2 4M) resulted in nearly complete loss of its ability to bind I $\kappa$ B $\alpha$  (Figure 2B).

We then compared the effect of  $\beta$ -arrestin2 and the 4M mutant on NF- $\kappa$ B signaling pathway by re-expression in  $\beta$ -arrestin-null MEFs.  $\beta$ -Arrestin2 re-expression decreased degradation of I $\kappa$ B $\alpha$ , NF- $\kappa$ B p65 nuclear translocation, and NF- $\kappa$ B DNA-binding activity upon UV irradiation (Figure 2C), further confirming  $\beta$ -arrestin2's function in UV pathway. In contrast to wild-type  $\beta$ -arrestin2,  $\beta$ -arrestin2 4M did not rescue the suppressive function in  $\beta$ -arrestin-null MEFs, as it did not change any of these responses to UV irradiation. These results strongly suggest that  $\beta$ -arrestin2 suppresses UV-induced NF- $\kappa$ B activation via its interaction with I $\kappa$ B $\alpha$ .



**Figure 2** Interaction of  $\beta$ -arrestin2 with I $\kappa$ B $\alpha$  is required for its suppression on NF- $\kappa$ B activation. (A) Schematic representation of  $\beta$ -arrestin2 and its mutants. (B) No interaction between  $\beta$ -arrestin2 4M and I $\kappa$ B $\alpha$ . Cell lysates from  $\beta$ -arrestin-null MEFs expressed with  $\beta$ -arrestin2 or  $\beta$ -arrestin2 4M were subjected to immunoprecipitation and then immunoblot with antibody as indicated. (C) No effects of  $\beta$ -arrestin2 4M on I $\kappa$ B $\alpha$  degradation, NF- $\kappa$ B p65 translocation, and NF- $\kappa$ B DNA-binding activity.  $\beta$ -Arrestin-null MEFs expressed with  $\beta$ -arrestin2 or  $\beta$ -arrestin2 4M were exposed to UV irradiation and I $\kappa$ B $\alpha$  degradation by immunoblot, nuclear translocation of NF- $\kappa$ B p65 by immunoblot, and NF- $\kappa$ B DNA-binding activity by EMSA were examined. Data are representative of at least three independent experiments, and are shown as mean  $\pm$  s.e. \* $P$ <0.05 and \*\* $P$ <0.01 versus control. IP, immunoprecipitated; IB, immunoblot.

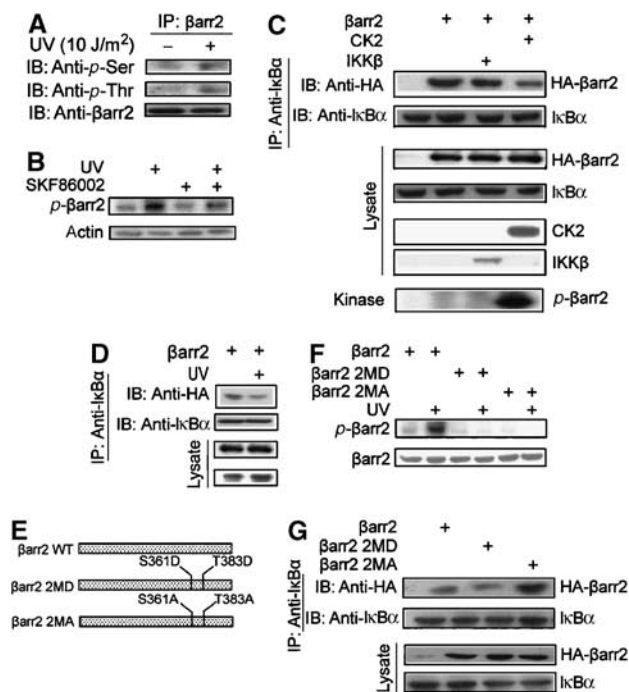
**$\beta$ -Arrestin2 phosphorylation regulates its suppression of NF- $\kappa$ B activation**

$\beta$ -Arrestin2 is constitutively phosphorylated by CK2 at the C-terminus, but its functions remain unclear up to now (Lin *et al*, 2002). Moreover, CK2 kinase activity towards I $\kappa$ B $\alpha$  is increased after UV irradiation (Kato *et al*, 2003). Therefore, we first examined whether  $\beta$ -arrestin2 was further phosphorylated under UV irradiation.  $\beta$ -Arrestin2 re-introduced into  $\beta$ -arrestin-null MEFs with or without UV irradiation was immunoprecipitated and then analyzed by antibody against p-Ser and p-Thr to detect its phosphorylation status. Figure 3A shows that both Ser and Thr phosphorylation of

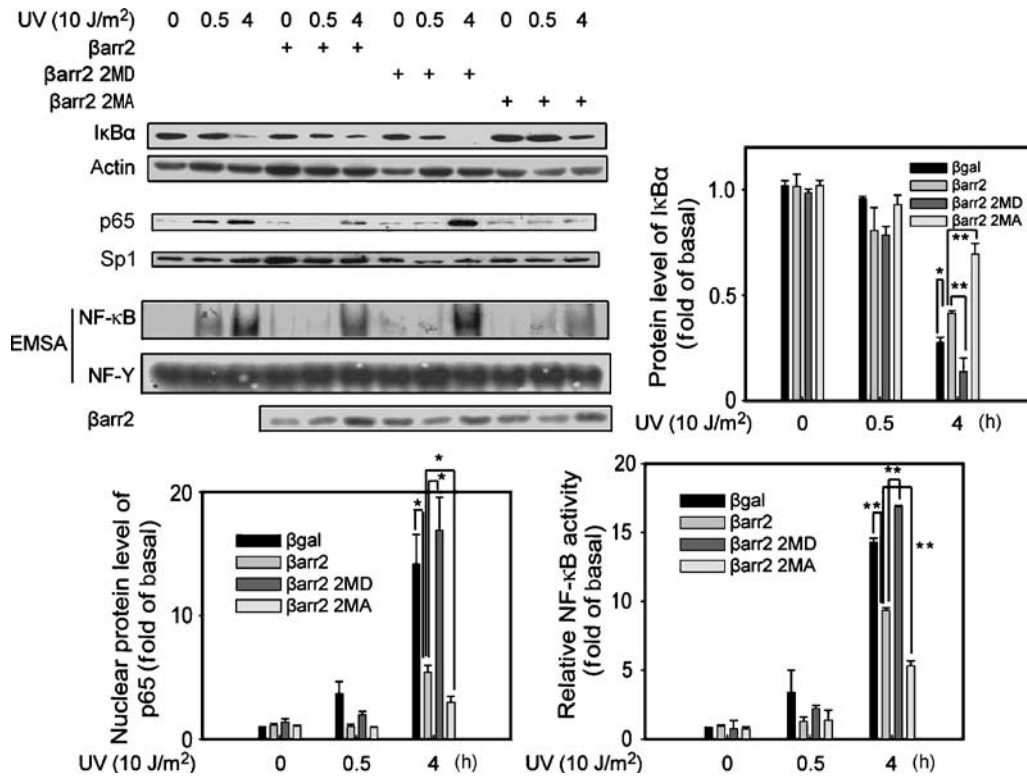
$\beta$ -arrestin2 were increased upon UV irradiation. Increased  $\beta$ -arrestin2 phosphorylation upon UV irradiation was also found in A431 by using metabolic labeling of [<sup>32</sup>P]orthophosphate followed by immunoprecipitation (Figure 3B). Furthermore, treatment of the cells with a p38 inhibitor (SKF86002, 20  $\mu$ M for 90 min) significantly reduced the level of phosphorylation, suggesting that p38 was involved in UV-induced  $\beta$ -arrestin2 phosphorylation. We then examined the effect of CK2 phosphorylation of  $\beta$ -arrestin2 on its interaction with I $\kappa$ B $\alpha$ .  $\beta$ -Arrestin-null MEFs were transfected with plasmids encoding  $\beta$ -arrestin2, CK2, or IKK $\beta$ , and the cells were incubated with 10  $\mu$ M MG132 for 4 h to inhibit I $\kappa$ B $\alpha$  degradation. Under these conditions,  $\beta$ -arrestin2 was phosphorylated by CK2 but not IKK $\beta$ . Cell lysates were prepared and used for coimmunoprecipitation to detect  $\beta$ -arrestin2–I $\kappa$ B $\alpha$  interaction. Figure 3C shows that  $\beta$ -arrestin2–I $\kappa$ B $\alpha$  interaction was significantly reduced by expression of CK2 but not IKK $\beta$ , suggesting that CK2 phosphorylation of  $\beta$ -arrestin2 may regulate its binding to I $\kappa$ B $\alpha$ . Moreover, UV irradiation of  $\beta$ -arrestin-null MEFs with re-expression of  $\beta$ -arrestin2 also reduced the  $\beta$ -arrestin2–I $\kappa$ B $\alpha$  interaction, further confirming our conclusion (Figure 3D). MG132 was also used to inhibit I $\kappa$ B $\alpha$  degradation in these experiments.

$\beta$ -Arrestin2 is phosphorylated mainly at Ser361 and Thr383 by CK2 (Lin *et al*, 2002). Next we generated two  $\beta$ -arrestin2 mutants in which Ser361 and Thr383 were substituted with Asp ( $\beta$ -arrestin2 2MD) or Ala ( $\beta$ -arrestin2 2MA) (Figure 3E). Phosphorylation of these mutants upon UV irradiation was then analyzed after their re-expression in  $\beta$ -arrestin-null MEFs. Phosphorylation of both  $\beta$ -arrestin2 2MD and 2MA mutants was significantly reduced compared to the wild-type  $\beta$ -arrestin2, and their level of phosphorylation was no longer increased by UV irradiation (Figure 3F), confirming that Ser361 and Thr383 are the predominant phosphorylation sites in  $\beta$ -arrestin2. Furthermore, coimmunoprecipitation experiments showed that  $\beta$ -arrestin2 2MD mutant, which mimics the phosphorylated  $\beta$ -arrestin2, exhibited significantly reduced interaction with I $\kappa$ B $\alpha$ , while  $\beta$ -arrestin2 2MA, which mimics the dephosphorylated  $\beta$ -arrestin2, displayed much stronger interaction with I $\kappa$ B $\alpha$  than  $\beta$ -arrestin2 (Figure 3G). These results suggested that phosphorylation of  $\beta$ -arrestin2 regulates its interaction with I $\kappa$ B $\alpha$ . Consistent with this, coexpression of CK2 or UV irradiation could no longer decrease the interaction of  $\beta$ -arrestin2 2MD or 2MA mutant with I $\kappa$ B $\alpha$  (Supplementary data) since they could not be phosphorylated by CK2 (Lin *et al*, 2002).

To assess whether  $\beta$ -arrestin2 phosphorylation and consequent changes in its interaction with I $\kappa$ B $\alpha$  regulate its suppressive function in I $\kappa$ B $\alpha$  degradation, the phosphorylation mutants of  $\beta$ -arrestin2 were expressed in  $\beta$ -arrestin-null MEFs. As shown in Figure 4,  $\beta$ -arrestin2 2MD mutant was not as effective as the wild-type  $\beta$ -arrestin2 in its suppressive function for UV-induced I $\kappa$ B $\alpha$  degradation, NF- $\kappa$ B p65 nuclear translocation, and NF- $\kappa$ B DNA-binding activity. In contrast,  $\beta$ -arrestin2 2MA mutant showed significantly increased suppression of these responses of NF- $\kappa$ B pathway to UV irradiation compared to wild-type  $\beta$ -arrestin2. Taken together, these data indicate that  $\beta$ -arrestin2 phosphorylation negatively regulates its suppression on NF- $\kappa$ B activation through interfering its interaction with I $\kappa$ B $\alpha$ .



**Figure 3** CK2 phosphorylation of  $\beta$ -arrestin2 blocks its interaction with I $\kappa$ B $\alpha$ . (A) Increased phosphorylation of  $\beta$ -arrestin2 after UV irradiation.  $\beta$ -Arrestin-null MEFs re-introduced with  $\beta$ -arrestin2 were irradiated with UV for 1 h and cell lysates were subjected to immunoprecipitation and immunoblot with antibody as indicated. (B) *In vivo* phosphorylation of  $\beta$ -arrestin2. A431 cells treated with or without SKF86002 were metabolically labeled with [<sup>32</sup>P]orthophosphate for 90 min and irradiated with UV for 1 h. Cell lysates were subjected to immunoprecipitation and autoradiography. (C) Blockage of  $\beta$ -arrestin2 and I $\kappa$ B $\alpha$  interaction by CK2. Cell lysates from  $\beta$ -arrestin-null MEFs coexpressed with  $\beta$ -arrestin2, and CK2 or IKK $\beta$  were subjected to immunoprecipitation and then immunoblot with antibody as indicated. MG132 (10  $\mu$ M) for 4 h was used to inhibit I $\kappa$ B $\alpha$  degradation. (D) Blockage of  $\beta$ -arrestin2 and I $\kappa$ B $\alpha$  interaction by UV irradiation.  $\beta$ -Arrestin-null MEFs re-introduced with  $\beta$ -arrestin2 were irradiated with UV for 1 h and cell lysates were subjected to immunoprecipitation and immunoblot with antibody as indicated. MG132 (10  $\mu$ M) for 4 h was used to inhibit I $\kappa$ B $\alpha$  degradation. (E) Schematic representation of  $\beta$ -arrestin2 phosphorylation mutants. (F) Phosphorylation of  $\beta$ -arrestin2 and phosphorylation site mutants with or without UV irradiation in  $\beta$ -arrestin-null MEFs was measured using *in vivo* phosphorylation. (G) I $\kappa$ B $\alpha$  interaction with phosphorylation site mutants of  $\beta$ -arrestin2. Phosphorylation sites of  $\beta$ -arrestin2 were mutated into Asp ( $\beta$ -arrestin2 2MD) or Ala ( $\beta$ -arrestin2 2MA) as indicated. Cell lysates from  $\beta$ -arrestin-null MEFs expressed with  $\beta$ -arrestin2,  $\beta$ -arrestin2 2MD, or  $\beta$ -arrestin2 2MA were subjected to immunoprecipitation and then immunoblot with antibody as indicated. Data are representative of at least three independent experiments and are shown as mean  $\pm$  s.e. \* $P$  < 0.05 and \*\* $P$  < 0.01 versus control.



**Figure 4**  $\beta$ -Arrestin2 phosphorylation regulates its suppression on NF- $\kappa$ B activation. Regulation of UV-induced I $\kappa$ B $\alpha$  degradation, NF- $\kappa$ B p65 nuclear translocation, and NF- $\kappa$ B DNA-binding activity by  $\beta$ -arrestin2 phosphorylation.  $\beta$ -Arrestin-null MEFs expressed with  $\beta$ -arrestin2 WT,  $\beta$ -arrestin2 2MD, or  $\beta$ -arrestin2 2MA were exposed to UV irradiation and I $\kappa$ B $\alpha$  degradation, NF- $\kappa$ B p65 nuclear translocation, and NF- $\kappa$ B DNA-binding activity were examined. Data are representative of at least three independent experiments and are quantified as mean  $\pm$  s.e. \* $P$  < 0.05 and \*\* $P$  < 0.01 versus control.

### $\beta$ -Arrestin2 suppression is stimulated by $\beta_2$ AR

As a major regulatory molecule in GPCR signal transduction,  $\beta$ -arrestin2's functions are stimulated by GPCRs (Luttrell *et al*, 1999; Wang *et al*, 2003), and  $\beta_2$ AR promotes dephosphorylation of  $\beta$ -arrestin2 at Ser361 and Thr383 (Lin *et al*, 2002). To evaluate a potential role of  $\beta_2$ AR in the regulation of  $\beta$ -arrestin2's function in UV-induced NF- $\kappa$ B activation, we first confirmed that  $\beta_2$ AR promoted  $\beta$ -arrestin2 dephosphorylation in our system. As shown in Figure 5A, Iso stimulation reduced  $\beta$ -arrestin2 phosphorylation in A431 cells, while phosphatase inhibitor okadaic acid (50 nM for 30 min) abolished this effect. As a result,  $\beta$ -arrestin2-I $\kappa$ B $\alpha$  interaction was enhanced by  $\beta_2$ AR stimulation (Figure 5B). In contrast, the interaction of  $\beta$ -arrestin2 phosphorylation mutants with I $\kappa$ B $\alpha$  was not affected by  $\beta_2$ AR stimulation, indicating that GPCR stimulation of  $\beta$ -arrestin2-I $\kappa$ B $\alpha$  interaction is through promoting dephosphorylation of  $\beta$ -arrestin2.

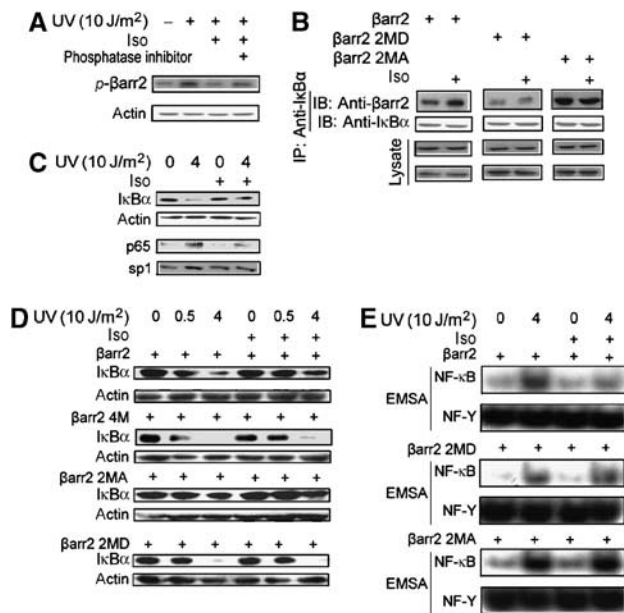
Next we examined whether  $\beta$ -arrestin2 suppression of NF- $\kappa$ B activation induced by UV was stimulated by  $\beta_2$ AR. As shown in Figure 5C, treatment of  $\beta_2$ AR agonist (Iso) significantly suppressed the degradation of I $\kappa$ B $\alpha$  and NF- $\kappa$ B p65 nuclear translocation in A431 cells with endogenous  $\beta_2$ AR, and pretreatment with  $\beta_2$ AR antagonist (Pro) totally abolished the Iso effect (data not shown). We then examined the effect of  $\beta_2$ AR stimulation in  $\beta$ -arrestin-null MEFs that had been transfected with  $\beta_2$ AR and wild-type  $\beta$ -arrestin2 or its mutants. We found that  $\beta$ -arrestin2 suppression of UV-induced I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B DNA-binding activity were further enhanced by  $\beta_2$ AR stimulation in  $\beta$ -arrestin-null

MEFs with re-expression of  $\beta$ -arrestin2, but not those with re-expression of the 2MD or 2MA mutants (Figure 5D and E). Moreover,  $\beta_2$ AR stimulation did not alter UV-induced NF- $\kappa$ B activation in  $\beta$ -arrestin-null MEFs with re-expression of  $\beta$ -arrestin2 4M, which did not bind to I $\kappa$ B $\alpha$  or suppress NF- $\kappa$ B activation. These results clearly demonstrate that  $\beta_2$ AR functionally crosstalks to UV pathway and that this crosstalk is mediated by its stimulation of  $\beta$ -arrestin2-I $\kappa$ B $\alpha$  interaction.

### $\beta$ -Arrestin2 functions to promote UV-induced cell death

UV-induced I $\kappa$ B $\alpha$  degradation leads to the activation of NF- $\kappa$ B (Kato *et al*, 2003), which is an important antiapoptotic transcription factor via inducing expression of various antiapoptotic target genes, such as bfl-1 and bcl-xl (Barkett and Gilmore, 1999; Baldwin, 2001). We investigated the potential physiological consequences of  $\beta$ -arrestin2's suppression of the NF- $\kappa$ B activation by examining UV-induced cell death in our systems. As shown in Figure 6A and B, UV-induced expression of bfl-1 and bcl-xl was decreased at mRNA level as determined by real-time PCR after expression of  $\beta$ -arrestin2 in either A431 cells or  $\beta$ -arrestin-null MEFs. Conversely, knockdown of  $\beta$ -arrestin2 in A431 cells by RNAi significantly increased UV-induced expression of bfl-1 and bcl-xl (Figure 6A).

Consistent with the effects of  $\beta$ -arrestin2 on the expression of these antiapoptotic genes, re-expression of  $\beta$ -arrestin2 in  $\beta$ -arrestin-null MEFs increased the sensitivity of these cells to UV-induced cell death as measured by both MTT and fluorescence-activated cell sorting (FACS) assays (Figure 6C).



**Figure 5**  $\beta$ -arrestin2 suppression is stimulated by  $\beta_2$ AR. (A)  $\beta_2$ AR promoted  $\beta$ -arrestin2 dephosphorylation. A431 cells were exposed to UV irradiation after stimulation with or without Iso (10  $\mu$ M for 1 min) and phosphorylation of  $\beta$ -arrestin2 was measured using *in vivo* phosphorylation. Okadaic acid (50 nM) for 30 min was used before Iso stimulation or not. (B) Enhancement of  $\beta$ -arrestin2 and I $\kappa$ B $\alpha$  interaction by  $\beta_2$ AR stimulation.  $\beta$ -Arrestin-null MEFs coexpressed with  $\beta_2$ AR, and  $\beta$ -arrestin2,  $\beta$ -arrestin2 2MD, or  $\beta$ -arrestin2 2MA were stimulated with or without Iso (10  $\mu$ M) for 1 min. Cell lysates were then subjected to immunoprecipitation and immunoblot with antibodies as indicated. (C) Enhancement of  $\beta$ -arrestin2 effects on I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B p65 nuclear translocation by  $\beta_2$ AR stimulation. A431 cells were exposed to UV irradiation after stimulation with or without Iso (10  $\mu$ M for 1 min), and then I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B p65 nuclear translocation were examined by immunoblot. (D, E) Dependence of  $\beta_2$ AR stimulation on  $\beta$ -arrestin2 phosphorylation.  $\beta$ -Arrestin-null MEFs coexpressed with  $\beta_2$ AR and  $\beta$ -arrestin2,  $\beta$ -arrestin2 2MD, or  $\beta$ -arrestin2 2MA were exposed to UV irradiation after stimulation with or without Iso (10  $\mu$ M for 1 min) and then cell lysates were used to examine I $\kappa$ B $\alpha$  degradation by immunoblot (D). Nuclear extracts of the cells were separated to examine the NF- $\kappa$ B DNA-binding activity by EMSA (E). Data are representative of at least three independent experiments and quantified as mean  $\pm$  s.e. \* $P$  < 0.05 and \*\* $P$  < 0.01 versus control. IP, immunoprecipitated; IB, immunoblot.

In contrast, re-expression of  $\beta$ -arrestin2 4M mutant, which does not bind to I $\kappa$ B $\alpha$ , did not alter UV-induced cell death in these cells. Similar results were obtained in p53-null Saos2 cells, which excluded the possible involvement of Mdm2/p53 pathway in which  $\beta$ -arrestin2 also plays a functional role (Wang *et al*, 2003) (Supplementary data). Analysis of the  $\beta$ -arrestin2 phosphorylation site mutants further supported a functional role of  $\beta$ -arrestin2's suppression on NF- $\kappa$ B activation in the regulation of UV-induced cell death. Consistent with their effects on NF- $\kappa$ B activation,  $\beta$ -arrestin2 2MD mutant was less effective than the wild-type  $\beta$ -arrestin2 on its promotion of UV-induced cell death. In contrast,  $\beta$ -arrestin2 2MA mutant showed much higher effect on this cell death as compared to wild-type  $\beta$ -arrestin2 in both  $\beta$ -arrestin-null MEFs (Figure 6D) and p53-null Saos2 cells (Supplementary data). Lastly, the effect of  $\beta$ -arrestin2 and its various mutants on UV-induced cell death was further confirmed by TUNEL assays (Figure 6E).

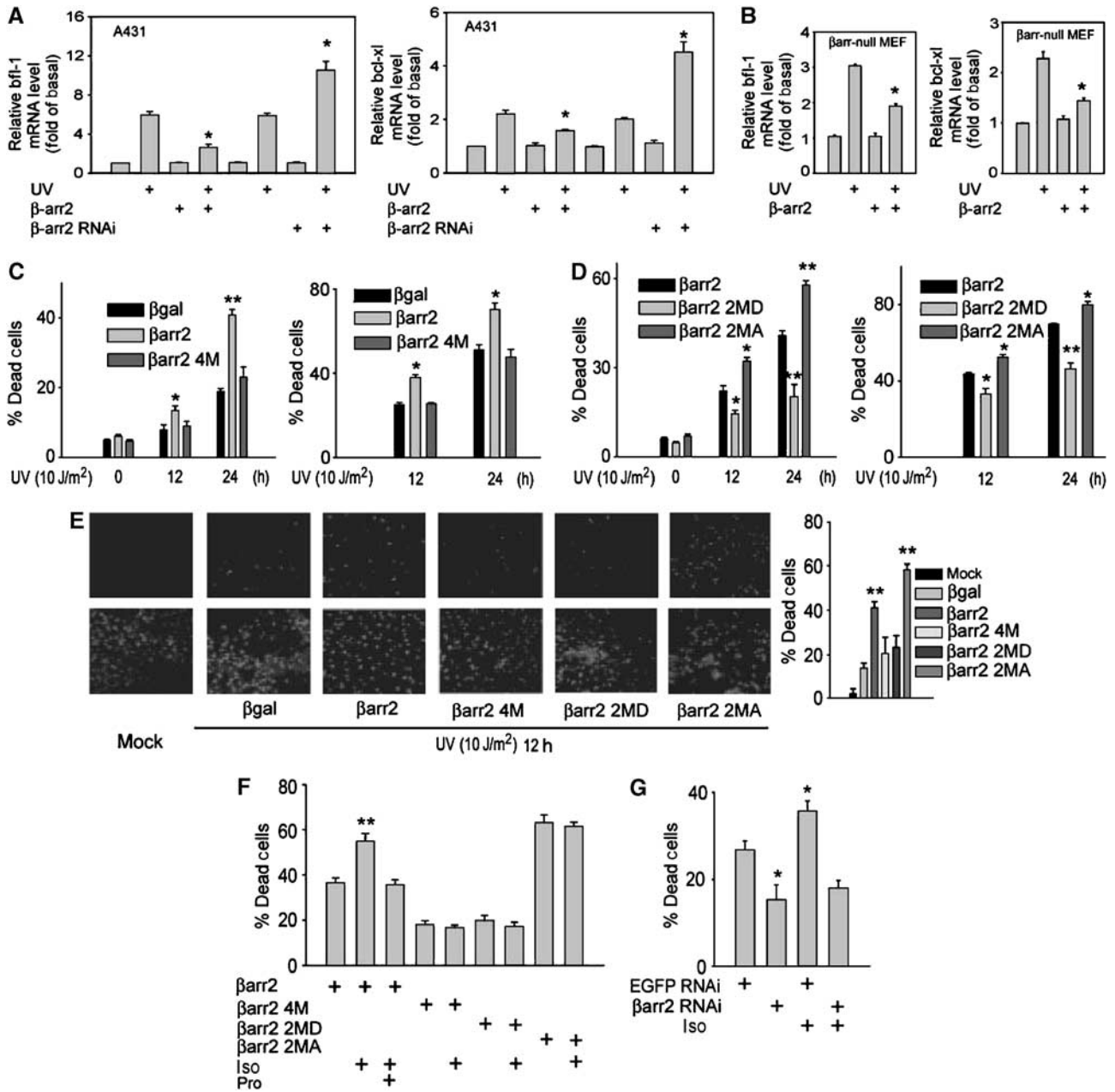
We also examined the effect of  $\beta_2$ AR in UV-induced cell death. As shown in Figure 6F,  $\beta$ -arrestin2 promotion on UV-induced cell death was further enhanced by  $\beta_2$ AR stimulation only in the expression of  $\beta$ -arrestin2 but not its mutants 2MD and 2MA, and pretreatment with  $\beta_2$ AR antagonist (Pro) totally abolished the Iso effect. Moreover,  $\beta_2$ AR stimulation did not alter UV-induced cell death in the expression of  $\beta$ -arrestin2 4M, which did not bind to I $\kappa$ B $\alpha$  or suppress NF- $\kappa$ B activation (Figure 6F). Finally, we found that knockdown of endogenous  $\beta$ -arrestin2 by  $\beta$ -arrestin2 RNAi in human epidermal carcinoma A431 cells greatly suppressed UV-induced cell death and that the stimulatory effect of  $\beta_2$ AR activation by Iso in UV-induced cell death was also lost (Figure 6G).

## Discussion

UV response in mammalian cells mainly involves NF- $\kappa$ B activation, which protects epidermis against apoptosis (reviewed by Baldwin, 1996). NF- $\kappa$ B activation induced by UV irradiation differs from that induced by other stimuli such as TNF $\alpha$  and LPS: it makes use of CK2 instead of IKK to phosphorylate I $\kappa$ B $\alpha$ . Our current work clearly showed that  $\beta$ -arrestin2, the multifunctional signal molecule, functions as a GPCR-stimulated suppressor of UV-induced NF- $\kappa$ B activation and its antiapoptotic function. Previously, we have found that  $\beta$ -arrestin2 can modulate TNF $\alpha$ -induced NF- $\kappa$ B activation and subsequent expression of NF- $\kappa$ B target genes (Gao *et al*, 2004). Thus, activation of NF- $\kappa$ B by a wide variety of biological stimuli, mediated by either IKK or CK2, is negatively regulated by  $\beta$ -arrestin2, demonstrating that  $\beta$ -arrestin2 functions as an intrinsic suppressor of NF- $\kappa$ B pathway.

In TNF $\alpha$  activation of NF- $\kappa$ B pathway,  $\beta$ -arrestin2-I $\kappa$ B $\alpha$  interaction remains unchanged after TNF $\alpha$  stimulation. In UV responses, however,  $\beta$ -arrestin2-I $\kappa$ B $\alpha$  interaction is regulated by  $\beta$ -arrestin2's phosphorylation status upon UV irradiation. A working model depicting the role of  $\beta$ -arrestin2 in cellular responses to UV is proposed in Figure 7. Upon UV irradiation, CK2 phosphorylates  $\beta$ -arrestin2, diminishes its interaction with I $\kappa$ B $\alpha$ , and releases I $\kappa$ B $\alpha$  for degradation. This model suggests that  $\beta$ -arrestin2, which binds to phosphorylated signal molecules and thus regulates their functions, is itself regulated by phosphorylation. Our current work provides further support for the functional role of  $\beta$ -arrestin2 phosphorylation, as reported previously in the case of  $\beta$ -arrestin2 binding to clathrin (Kim *et al*, 2002; Lin *et al*, 2002). This study also proposes a new model of one kinase in a signal cascade, which can phosphorylate both the downstream signal molecule (i.e. I $\kappa$ B $\alpha$ ) and its suppressor (i.e.  $\beta$ -arrestin2) in a synchronized way to more dedicatedly and accurately transduce the signal.

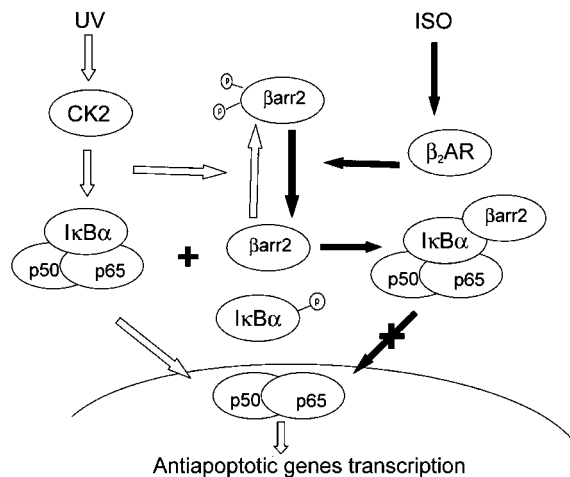
Although  $\beta$ -arrestin2 and  $\beta$ -arrestin1 share similar functional roles in many circumstances like GPCR signal transduction, accumulating evidence also revealed potential functional differences between them (Luttrell and Lefkowitz, 2002). For example,  $\beta$ -arrestin1 binds some GPCRs such as  $\beta_2$ AR, MOR, and endothelin type receptor with lower affinity than  $\beta$ -arrestin2, and is less efficient in membrane translocation upon agonist stimulation, while  $\beta$ -arrestin1 and  $\beta$ -arrestin2 bind other receptors, including angiotensin II type 1A receptor, neurotensin 1 receptor, and substance P receptor, with similar high affinities and translocate to the membrane



**Figure 6**  $\beta$ -arrestin2 functions to promote UV-induced cell death. (A) A431 cells expressed with  $\beta$ -arrestin2 or  $\beta$ -arrestin2 siRNA were subjected to RT-qPCR for detection of bfl-1 and bcl-xl expression after UV irradiation for 12 h. (B)  $\beta$ -Arrestin-null MEFs expressed with  $\beta$ -arrestin2 were subjected to RT-qPCR for detection of bfl-1 and bcl-xl expression after UV irradiation for 12 h. (C, D)  $\beta$ -Arrestin-null MEFs expressed with  $\beta$ -arrestin2,  $\beta$ -arrestin2 4M (C),  $\beta$ -arrestin2 2MD (D), or  $\beta$ -arrestin2 2MA (D) were exposed to UV irradiation for the indicated times, and cell death was measured using MTT and FACS assay. (E)  $\beta$ -Arrestin-null MEFs expressed with  $\beta$ -arrestin2 or its mutants were exposed to UV irradiation, and cell death was measured using TUNEL assay. Data are representative of at least three independent experiments. (F)  $\beta$ -Arrestin-null MEFs coexpressed with  $\beta_2$ AR and  $\beta$ -arrestin2,  $\beta$ -arrestin2 4M,  $\beta$ -arrestin2 2MD, or  $\beta$ -arrestin2 2MA were exposed to UV irradiation after stimulation with or without Iso (10  $\mu$ M for 1 min). Pro (10  $\mu$ M) was added 5 min before Iso. Cell death was measured using TUNEL assay. (G) A431 cells expressed with  $\beta$ -arrestin2 siRNA were exposed to UV irradiation after stimulation with or without Iso (10  $\mu$ M for 1 min). Cell death was measured using TUNEL assay. Data were quantified as mean  $\pm$  s.e. \* $P$  < 0.05, and \*\* $P$  < 0.01 versus control.

with similar efficiencies (Oakley *et al*, 2000). Further, it has been reported that  $\beta$ -arrestin2 is predominantly distributed in the cytoplasm, while  $\beta$ -arrestin1 is localized equally in the cytoplasm and nucleus (Scott *et al*, 2002). Our current study also provides evidence that, although  $\beta$ -arrestin1 showed a similar ability to modulate UV-induced NF- $\kappa$ B activation as  $\beta$ -arrestin2,  $\beta$ -arrestin1 Q394L mutant, which is predominantly distributed in the cytoplasm, did not modulate

NF- $\kappa$ B activation, suggesting that  $\beta$ -arrestin1 may exert its effect through different mechanisms in the nucleus. Our unpublished data show that  $\beta$ -arrestin1 could modulate histone H4 acetylation and transcription of genes like *p27* and *c-fos*, indicating that epigenetic regulation of gene expression is one function of  $\beta$ -arrestin1 in the nucleus. Thus,  $\beta$ -arrestin1 may exert its inhibitory function through epigenetic histone modification. The precise mechanism



**Figure 7** Model illustration that  $\beta$ -arrestin2 functions as a phosphorylation-regulated suppressor to UV-induced NF- $\kappa$ B activation.

underlying  $\beta$ -arrestin1 modulation of UV-induced NF- $\kappa$ B activation is worthy of further investigation.

Arrestins, besides their well-established extensive interactions with GPCRs, are recently displayed to functionally interact with growth factor receptors (e.g. insulin-like growth factor receptor; Lin *et al*, 1998) and transforming growth factor- $\beta$  receptor (Chen *et al*, 2003), thus indicating their potential role in regulation of cellular proliferation and apoptosis. Previous studies have revealed that the irreversible association of *Drosophila* arrestin to the activated rhodopsin leads to retinal cell apoptosis (Kiselev *et al*, 2000), and the GPCR-stimulated binding of  $\beta$ -arrestin2 to Mdm2 enhances the p53-mediated apoptosis (Wang *et al*, 2003). Our current study further demonstrates that  $\beta$ -arrestin2 effectively modulates UV-induced NF- $\kappa$ B activation and consequently suppresses the antiapoptotic function of NF- $\kappa$ B, revealing its general role in proapoptosis in different signal pathways. However, it seems that the function of arrestins is not limited to proapoptosis, since they are also reported to be involved in the prevention of apoptosis (Povsic *et al*, 2003; Revankar *et al*, 2004).

The epidermis is at the forefront of protection for the organism against multiple environmental insults. One of such insults is UV light, which is considered to be a potent and complete carcinogen causing the most common form of cancers, nonmelanoma skin cancers (Quinn, 1997; Brash and Ponten, 1998; Wikonkal and Brash, 1999). Various studies have shown that the UV insult in epidermis induces three major signal pathways, namely AP1, p53, and NF- $\kappa$ B. While activation of AP1 and p53 is proapoptotic, activation of NF- $\kappa$ B serves an antiapoptotic function (Kato *et al*, 2003). It is generally believed that the skin cancers relevant to UV irradiation are related to the imbalance of proapoptotic and antiapoptotic functions of these signal pathways. The current work showing that effective suppression of UV-induced NF- $\kappa$ B activation by  $\beta$ -arrestin2-mediated GPCR signaling indicates stimulation of GPCRs such as  $\beta_2$ AR may promote UV-induced apoptosis and thus repress the carcinogenesis in epidermis. If this is the case, some of the GPCRs in epidermis might be used as a potential target for prevention and treatment of UV-relevant skin cancers.

## Materials and methods

### Materials and reagents

Anti- $\beta$ -arrestins rabbit polyclonal antibody (A1CT) is a generous gift from Dr Robert J Lefkowitz (Duke University Medical Center, Durham). Antibodies directed against I $\kappa$ B $\alpha$  (rabbit) and p65 (rabbit) were obtained from Santa Cruz Biotechnology, anti-GG from Covance, anti-HA, Flag, actin, and sp1 from Sigma. RhTNF $\alpha$  was from R&D, and phosphatase inhibitor (okadaic acid), p38 inhibitor (SKF86002), MG132, Iso, and Pro from sigma. NF- $\kappa$ B activation inhibitor (sn50 peptide) and CK2 inhibitor (5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole) were from Calbiochem. The peak emission wavelength of the UV-C used was 254 nm.

### Cell transfection and plasmids

Transient transfection of Saos2 cells was performed using the calcium phosphate method as described previously (Wang *et al*, 2003). Wild-type and  $\beta$ -arrestin-null MEF cell lines were kindly provided by Dr Robert J Lefkowitz, and were transfected by LipofectAMINE (Invitrogen). A431 cells was also transfected by LipofectAMINE. For all transfection experiments, CMV- $\beta$ -Gal (for expression) and EGFP RNAi (for siRNA) were used as a control. Full length of human I $\kappa$ B $\alpha$  was cloned into modified pcDNA3 vector in-frame with Flag at the N-terminus. Plasmids cDNA encoding  $\beta$ -arrestin2 and its mutants were generated as described (Wang *et al*, 2003) and were cloned into modified pcDNA3 vector in-frame with HA at the C-terminus. Full length of human IKK $\beta$  was cloned into modified pcDNA3 vector in-frame with Myc at the N-terminus. The GG tagged CK2 $\alpha$  plasmid was kindly provided by Dr Lin Li (Shanghai Institutes for Biological Sciences). The authenticity of the DNA sequences was confirmed by sequencing. Construction of siRNA plasmid for  $\beta$ -arrestin2 was described previously (Sun *et al*, 2002; Wang *et al*, 2003; Gao *et al*, 2004).

### Immunoprecipitation and immunoblot

For transient transfections, endogenous I $\kappa$ B $\alpha$  was immunoprecipitated with anti-I $\kappa$ B $\alpha$  (rabbit) and protein A-Sepharose beads (Amersham Biosciences). After extensive washing and heating in the sample buffer, the complexes were subjected to immunoblot (Wang *et al*, 2003).

### Nuclear extracts preparation

After serum starvation for 12 h, cells were exposed to UV irradiation (10 J/m<sup>2</sup>) for a certain time after stimulation with or without Iso (10  $\mu$ M for 1 min). Nuclear extracts were prepared by the method as reported previously (Gao *et al*, 2004), with minor modifications with 6  $\mu$ l of NP-40 10% (v/v) for MEFs and 5  $\mu$ l of NP-40 10% (v/v) for A431 cells.

### Electrophoretic mobility shift assay (EMSA)

EMSAs were performed using nuclear extracts obtained at various times before or after exposure to UV irradiation (10 J/m<sup>2</sup>) with or without Iso stimulation (10  $\mu$ M for 1 min). Double-stranded gel shift probes (50 ng) corresponding to the human consensus NF- $\kappa$ B sequences (5'-AGT TGA GGG GAC TTT CCC AGG C-3', Promega), the consensus AP1 sequences (5'-CGC TTG ATG ACT CAG CCG GAA-3', Promega), and the consensus NF-Y probes (5'-AGA CCG TAC GTG ATT GGT TAA TCT CTT-3', Santa Cruz Biotechnology) were end-labeled with  $\gamma$ -<sup>32</sup>P-ATP and T4 polynucleotide kinase. The reaction mixtures (20  $\mu$ l) containing about 10  $\mu$ g nuclear extracts and binding buffer (Promega) were incubated at room temperature for 10 min and 0.5 ng <sup>32</sup>P-labeled DNA probes were added, followed by another 20-min incubation. Samples were subjected to electrophoresis in 10% nondenaturing polyacrylamide gels. In competition or antibody supershift experiments, the reaction mixtures were preincubated at room temperature with competing cold probes (50-fold excess) for 10 min or with specific antibody for 1 h before addition of the <sup>32</sup>P-labeled probes.

### In vitro kinase assays

Expressed myc-IKK $\beta$  or GG-CK2 was immunoprecipitated using anti-myc or anti-GG antibody. The beads were extensively washed and resuspended in 30  $\mu$ l of kinase buffer with 10  $\mu$ M ATP and 2  $\mu$ Ci of  $\gamma$ -<sup>32</sup>P-ATP, containing recombinant GST- $\beta$ -arrestin2. The kinase reaction was performed at 30°C for 30 min and stopped by addition



of 6  $\mu$ l of 6  $\times$  SDS sample buffer. The samples were subjected to SDS-PAGE (10%), electroblot, and autoradiography.

#### **In vivo phosphorylation**

For metabolic labeling, cells were starved in phosphate-free Dulbecco's modified Eagle's medium (Life Technologies, Inc.) for 1 h, labeled for 90 min in the same medium containing [<sup>32</sup>P]orthophosphate (0.2 mCi/ml), and harvested for  $\beta$ -arrestin2 immunoprecipitation.

#### **Reverse transcription quantitative real-time PCR**

Total RNAs were extracted with TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse transcription of purified RNA was performed using oligo(dT) priming and superscript II reverse transcriptase (Invitrogen). The quantification of all gene transcripts was performed by qPCR, using Brilliant SYBR Green QPCR Master Mix and a Light Cycler apparatus (Stratagene).

#### **MTT**

After transfection, MEFs or Saos2 cells were cultivated in a 96-well chamber at 1000–10000 cells per well, and exposed to UV irradiation (10 J/m<sup>2</sup>) for various times. MTT reagent was then added and cells were incubated for 4 h. After adding the detergent reagent for 10 h in the dark, absorbance in 570 nm was recorded.

#### **Fluorescence-activated cell sorting**

After exposure to UV irradiation (10 J/m<sup>2</sup>) for various times, at least 10<sup>6</sup> cells were fixed in 70% ethanol for 1 h. Then, propidium iodide staining solution (PI solution) together with Rnase A were added and the sub-G1 peak of cells was analyzed by passage through a FACSCalibur flow cytometer.

#### **DNA fragmentation/fluorescein staining (TUNEL)**

Cells were grown on glass coverslips; 48 h after split, the cells were exposed to UV irradiation (10 J/m<sup>2</sup>) for a certain time and fixed with

4% polyformaldehyde for 15 min. Cells were washed with PBS and incubated in PBS containing 0.2% BSA and 0.2% Tween for 15 min. After fluorescein staining with TdT end-labeling cocktail (TdT buffer, Biotin-dUTP, and TdT) and avidin-FITC, DNA fragmentation was analyzed on a Leica TCS SP2 laser confocal fluorescence microscope.

#### **Statistical analysis**

Data were analyzed by Student's *t*-test for comparison of independent means, with pooled estimates of common variances. For all tests, *P* < 0.05 was considered significant.

#### **Supplementary data**

Supplementary data are available at *The EMBO Journal* Online.

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