The Cannabinoid Receptors Are Required for Ultraviolet-Induced Inflammation and Skin Cancer Development

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Abstract

Solar UV irradiation is an important carcinogen that leads to the development of skin cancer, which is the most common human cancer. However, the receptors that mediate UV-induced skin carcinogenesis have not yet been unequivocally identified. Here we showed that UV irradiation directly activates cannabinoid receptors 1 and 2 (CB1/2). Notably, our data indicated that the absence of the CB1/2 receptors in mice results in a dramatic resistance to UVB-induced inflammation and a marked decrease in UVB-induced skin carcinogenesis. A marked attenuation of UVB-induced activation of mitogen-activated protein kinases and nuclear factor-κB was associated with CB1/2 deficiency. These data provide direct evidence indicating that the CB1/2 receptors play a key role in UV-induced inflammation and skin cancer development. [Cancer Res 2008;68(10):3992–8]

Introduction

Skin cancer is the most common type of human cancer and is associated with excessive exposure to UV solar irradiation. Exposure to UV irradiation can activate various oncogenes and inactivate many tumor suppressor genes. The net result is abnormal proliferation of keratinocytes that might harbor DNA damage leading to the onset of skin cancer (1). Exposure to UVB irradiation has been shown to induce activation, aggregation, and internalization of cell-surface receptors for epidermal growth factor (EGF), tumor necrosis factor α (TNF-α), and interleukin-1 (IL-1; refs. 2, 3).

Manipulation of the cannabinoid receptors has been useful in the pain management and treatment of osteoporosis, inflammation, and cancer (4), but the mechanisms of these effects are still not fully understood. Two cannabinoid receptors from mammalian tissues have been cloned and characterized (5, 6). Cannabinoid receptor 1 (CB1) is highly expressed in the brain, whereas the CB2 receptor is found mainly in the immune system (5, 6). However, no direct experimental evidence has confirmed that these membrane-bound receptors are required for UV-induced skin carcinogenesis.

CB1 and CB2 are members of a superfamily of seven-transmembrane-spanning (7-TM) receptors, which have a protein structure defined by an array of seven-membrane-spanning helices with intervening intracellular loops and a COOH-terminal domain that can associate with G proteins. A CB1 (7) and CB2 homology model (8) was constructed using the published X-ray crystal structure of bovine rhodopsin (9), a photosensitive G-protein-coupled receptor (GPCR) found in the retina. Arrestin-1 (or visual arrestin) and arrestin-4 (or cone arrestin) modulate the action of rhodopsin (10, 11) and arrestin-2 and arrestin-3 modulate non-retinal GPCRs (12, 13). Recently, desensitization of the CB1 receptor was reported to be mediated by the interaction of the COOH-terminal residues (419–439) of CB1 with arrestin-2 (14), indicating that CB1 and CB2 might act as photoreceptors in nonretinal tissues. However, a role for CB1 and CB2 in skin cancer has not been reported. The identification of the “UV receptor” associated with skin cancer is still to be elucidated and the CB1/2 receptors are potential candidates.

The function of CB1 and CB2 in cancer is controversial. The expression levels of both CB1 and CB2 are significantly higher in CA-human papillomavirus-10 cells, which are virally transformed cells derived from human prostate adenocarcinoma, and also in other human prostate cancer cell lines, including LNCaP, DU145, PC3, and CWR22Rv1, compared with human prostate epithelial and PZ-HPV-7 cells that are virally transformed cells derived from normal human prostate tissues (15). Moreover, the CB1 antagonist Rimonabant or SR141716 inhibited human breast cancer cell proliferation and was more effective in highly invasive metastatic MDA-MB-231 cells than in the less invasive T47D or MCF-7 cells (16). In leukemic precursor cells, the CB2 receptor is an oncoprotein that blocks neutrophilic differentiation when overexpressed in myeloid precursor cells (17). However, treatment of LNCaP cells with WIN# 55212-2, a mixed CB1/CB2 agonist, resulted in inhibition of cell growth and induction of apoptosis (15, 18). Furthermore, cannabinoids have been shown to suppress the growth of several models of tumor xenografts in rats and mice (19). The CB1 and CB2 receptors are expressed in normal skin and skin tumors, and local activation of cannabinoid receptors induced the apoptotic death of tumorigenic epidermal cells, suppressed the proliferation of melanoma cells, and inhibited the growth and angiogenesis of skin tumor xenografts in nude mice (20, 21). However, cannabinoids may inhibit human keratinocyte proliferation through a non–CB1/CB2 mechanism (22). Therefore, although CB1 and CB2 are physiologically linked with cancer, the functional role of these receptors in cancer is not clear.

Here, we used wild-type CB1/2 (CB1/2+/+) and CB1/2–deficient (CB1/2−/−) mice and respective mouse embryonic fibroblasts (MEF) to elucidate the function of endogenous CB1 and CB2 in UVB-induced inflammation and skin cancer development.

Materials and Methods

Plasmids and chemicals. The mouse CB1 plasmid (pcDNA3-CB1) was kindly provided by Dr. Beat Lutz and the mouse CB2 plasmid (pcDNA3-CB2) was kindly provided by Dr. Ruud Delwel. Chemical reagents for molecular biology and buffer preparation, including (R)-(+)-WIN55212-2, pertussis toxin, Tris, NaCl, and SDS, were purchased from Sigma-Aldrich Corp. The luciferase assay substrate was from Promega. Restriction enzymes were purchased from Roche Diagnostics Corp. and Taq DNA polymerase was from Qiagen, Inc. The DNA ligation kit (version 2.0) was used.
purchased from TAKARA Bio, Inc. Cell culture media and other supplements were purchased from Life Technologies, Inc. [3H]CP55940 and membrane fractions, which were isolated from HEK293 EBNA cells overexpressing human CB1 or CB2 receptors, were obtained from Perkin-Emer Corp. The HisG antibody was from Invitrogen Corp. and antibodies against phosphorylated (p-) c-Jun NH2-terminal kinases (JNK), total JNKs, p-p38, total p38, p-extracellular signal–regulated kinases (ERK), and total ERKs were from Cell Signaling Technology, Inc. The antibody to detect phosphorylated serine residues was from Abcam, Inc., and the antibody against TNF-α was purchased from Santa Cruz Biotechnology, Inc.

**Mice.** The CB1/2-deficient and wild-type mice were provided by Dr. Andreas Zimmer.

**In vivo mouse studies.** Age- and gender-matched CB1/2+/+ and CB1/2−/− mice were divided into groups and initiated by topical application of 200 nmol of 7,12-dimethyl benz(a)anthracene (DMBA), and then 2 wk later, 1 group each of CB1/2+/+ and CB1/2−/− mice were treated with increasing doses (increase of 1.5 kJ/m2/wk) of UVB thrice a week as described above (23), and the R* state is appropriate for functional coupling to G proteins. Because an agonist has a high relative affinity for R* conformations compared with the inactive form, receptor binding of the agonist results in a shift of the equilibrium toward the active state and G protein coupling can occur virtually concomitantly (23). Activation of the CB1/2 receptors by UV treatment produced a similar shift in affinity for the agonist, (R)(−)-WIN55212-2, compared with the untreated control, which was detected by an agonist-specific increase in binding affinity. Competition binding experiments using membrane fractions from cells overexpressing CB1 and exposed to UVB (9 kJ/m2) or UVA (60 or 120 kJ/m2) showed a 1.9-, 2.1-, or 2.9-fold lower

| Cell culture and transfections. CB1/2+/+ and CB1/2−/− MEFs were treated with increasing doses (increase of 1.5 kJ/m2/wk) of UVB thrice a week as described above (23). When cells reached 50% to 60% confluence, transfection of the expression vectors was done with the Lipofectamine reagent (Invitrogen) following the manufacturer's suggested protocol.

**Construction of His-fusion proteins.** The His-fusion proteins for the CB1 and CB2 receptors were constructed with PCR-amplified open reading frame DNA fragments and the pcDNA3/HisMax vector (Invitrogen). The reading frame of each plasmid construct was confirmed by restriction mapping and DNA sequencing.

**Extraction of membrane and cytotoxic proteins.** HEK293 cells were harvested, suspended in cell lysis buffer (20 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl), and disrupted by freezing and thawing. The supernatant fraction including cytosolic and nuclear soluble proteins was recovered by centrifugation at 15,000 × g for 10 min at 4°C and the pellet was resuspended with cell suspension buffer (50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% Triton X-100). The cell suspension was stirred on ice for 1 h, centrifuged at 15,000 × g for 10 min at 4°C, and membrane proteins were recovered in the supernatant fraction.

**Immunoprecipitation and Western blotting.** Immunoblotting was done following the instructions from Cell Signaling Technology. In brief, HEK293 cells or CB1/2−/− or CB1/2+ MEFs were starved for 24 h in DMEM supplemented with 0.1% FBS. Cells were then treated with UVB (4 kJ/m2) and harvested in cell lysis or suspension buffer. Samples were equalized for protein and analyzed by Western blotting. To analyze CB1 or CB2 phosphorylation, a HisG antibody was used for immunoprecipitation with membrane proteins (500 μg). The antibody binding was carried out at 4°C overnight and proteins were visualized by Western blotting with an antibody against phosphorylation of serine residues.

**Nuclear factor-κB activity assay.** The nuclear factor-κB (NF-κB) luciferase reporter plasmid (pGL2-NF-κB-Luc) was transfected into CB1/2+/+ or CB1/2−/− MEFs together with the phRL-SV40 plasmid. The MEFs were cultured for an additional 36 h, starved for 24 h, and then exposed to UVB (4 kJ/m2). The cells were disrupted with lysis buffer 12 or 18 h later, and luciferase activity was measured.

**Results.**

**CB1 and CB2 are activated by UVA or UVB.** The CB1/2 receptors are seven-transmembrane-helix receptors that belong to the superfamily of GPCRs (5, 6). A GPCR isomerizes between two different states, an inactive (R) and an active (R*) conformation (23), and the R* state is appropriate for functional coupling to G proteins. Because an agonist has a high relative affinity for R* conformations compared with the inactive form, receptor binding of the agonist results in a shift of the equilibrium toward the active state and G protein coupling can occur virtually concomitantly (23). Activation of the CB1/2 receptors by UV treatment produced a similar shift in affinity for the agonist, (R)(−)-WIN55212-2, compared with the untreated control, which was detected by an agonist-specific increase in binding affinity. Competition binding experiments using membrane fractions from cells overexpressing CB1 and exposed to UVB (9 kJ/m2) or UVA (60 or 120 kJ/m2) showed a 1.9-, 2.1-, or 2.9-fold lower

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**CB1/2 double-knockout mice show resistance to UV-induced skin inflammation.** Because of the potential role of cannabinoids in cell growth inhibition and inflammation (24), we investigated

| Table 1. Effect of UV radiation in CB1 or CB2 on ligand binding affinities
<table>
<thead>
<tr>
<th>Receptor</th>
<th>UV radiation</th>
<th>Dose (kJ/m²)</th>
<th>Control/UV treated Kᵢ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB1</td>
<td>UBV</td>
<td>9</td>
<td>1.9:1</td>
</tr>
<tr>
<td>CB1</td>
<td>UVA</td>
<td>60</td>
<td>2.1:1</td>
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<tr>
<td>CB1</td>
<td>UVA</td>
<td>120</td>
<td>2.9:1</td>
</tr>
<tr>
<td>CB2</td>
<td>UBV</td>
<td>9</td>
<td>1.4:1</td>
</tr>
<tr>
<td>CB2</td>
<td>UVA</td>
<td>60</td>
<td>2.6:1</td>
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NOTE: Competition binding studies, using [3H]CP55940 as a tracer, were conducted with membrane preparations isolated from cells overexpressing CB1 or CB2 (Perkin-Elmer).
whether CB1/2-/- mouse skin would exhibit an altered susceptibility to UVB-induced inflammation. At the established peak response time of 24 hours after irradiation (25), CB1/2+/+ and CB1/2-/- mouse skin was harvested and analyzed. Histologic analysis indicated that UVB-treated CB1/2+/+ mouse skin showed a pronounced inflammation with the emergence of subcorneal pustules and epidermal erosion (Fig. 1A and B), whereas the epidermis of CB1/2-/- mice was far more resistant to UVB-induced inflammation (Fig. 1C and D).

**The CB1/2 receptors are phosphorylated in response to UV treatment.** Current theories for GPCR regulation predict that the active state conformation is the target for phosphorylation, internalization, and desensitization (26). Phosphorylation hinders further association of the receptor with G proteins, thereby terminating the signaling pathways (27). In the continued presence of agonist, receptors are targeted to lysosomes for degradation (28) and agonist treatment has been shown to result in rapid phosphorylation of the CB1 and CB2 receptors in transfected cell systems (29, 30). The quick internalization of the CB1 and CB2 receptors after agonist exposure has been reported (30, 31). Activation of the CB1/2 receptors by UV irradiation might produce a similar rapid phosphorylation and internalization of these receptors. To test this hypothesis, we transfected His-epitope-tagged CB1 or CB2 into HEK293 cells and treated the cells with UVB followed by immunoprecipitation and Western blotting. Results indicated that treatment with UVB induced a rapid phosphorylation of the CB1 and CB2 receptors, which was observed at 5 minutes and remained at this level until 30 minutes after UVB treatment (Fig. 2A and B). Treatment with UVB also induced rapid internalization of CB1 and CB2 as indicated by the time-dependent decrease in protein level of CB1 and CB2 in membrane fractions after UVB treatment (Fig. 2C and D). These results indicated that the CB1 and CB2 receptors are phosphorylated and internalized in response to UVB treatment.

**CB1/2 modulate JNK and ERK signaling cascades in response to UV treatment.** The GPCRs can stimulate the mitogen-activated protein (MAP) kinase cascades, which have prominent roles in the control of cell growth, differentiation, and proliferation (32). To show a potential role for the cannabinoid receptors in UV-induced signal transduction, we examined the phosphorylation of ERKs, JNKs, and p38 in CB1/2+/+ and CB1/2-/- MEFs following UVB irradiation (4 kJ/m²). Results of Western blot analysis indicated that phosphorylation of ERKs, JNKs, and p38 was induced in a time-dependent fashion after exposure of CB1/2+/+ MEFs to UVB (Fig. 3A). In marked contrast, UVB-induced phosphorylation of ERKs and JNKs in CB1/2-/- cells was almost abolished (Fig. 3A). Although p38 phosphorylation was somewhat attenuated, the decrease was not as substantial as the decrease in ERK and JNK phosphorylation (Fig. 3A). These data provide direct evidence supporting the involvement of the CB1/2 receptors in mediating the activation of the MAP kinase pathways in the cellular response to UVB.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** CB1/2 deficiency reduces UV-induced skin inflammation. CB1/2+/+ (A and B) or CB1/2-/- (C and D) mice were untreated or treated with UVB (4 kJ/m²). Dorsal skin from irradiated (B and D) or nonirradiated (A and C) mice was harvested 24 h after UVB exposure. For assessment of inflammation, tissues were fixed in 10% neutral-buffered formalin and stained with H&E. All UV irradiation experiments were done in triplicate for each condition and representative images are shown. Bar, 25 μm; magnification, ×200.
CB1/2 are involved in TNF-α-mediated NF-κB activation. Manipulation of the cannabinoid receptors has been useful in the pain management and treatment of osteoporosis, inflammation, and cancer (4). Human keratinocytes constitutively secrete only a small amount of TNF-α and other cytokines, including IL-1, IL-6, IL-8, colony-stimulating factor, transforming growth factors, and platelet-derived growth factor. However, UV exposure significantly induces the production of cytokines (37, 38). TNF-α is an important member of the cytokine cascade and might be involved in the mediation of the inflammatory response to UV exposure, either directly or through the induction of other cytokines (39). We therefore examined the effect of UVB on the expression of TNF-α in CB1/2+/+ and CB1/2−/− mice. Interestingly, compared with adult wild-type CB1/2−/− mice, adult CB1/2+/+ mouse skin showed a more pronounced inflammatory cytokine TNF-α level (Fig. 4A and B) following UVB treatment, corresponding with the observed elevated inflammatory response (Fig. 1A–D). Thus, the CB1/2 receptors are involved in orchestrating events leading to the inflammation observed in UVB-treated mouse skin. Furthermore, CB1 or CB2 activation has been reported to result in enhanced NF-κB transactivation (40). To test whether CB1/2 deficiency is associated with suppression of NF-κB activity, we transfected a NF-κB-linked luciferase promoter plasmid into CB1/2+/+ or CB1/2−/− MEFs and stimulated the cells with UVB (4 kJ/m²). The results indicated that NF-κB activation was induced in CB1/2+/+ MEFs, but not in CB1/2−/− MEFs (Fig. 4C), suggesting that the CB1/2 receptors are involved in UVB-induced NF-κB activation.

Figure 2. UVB induces CB1 or CB2 phosphorylation and internalization. HEK293 cells transfected with pcDNA4-HisG-CB1 (A) or pcDNA4-HisG-CB2 (B) were irradiated with UVB (4 kJ/m²) followed by incubation at 37°C in a 5% CO₂ incubator for 5, 15, or 30 min. The transfected cells not exposed to UVB were used as a negative control. Cells were harvested at the indicated time and the membrane protein was extracted and subjected to immunoprecipitation with a HisG monoclonal antibody. CB1 or CB2 serine phosphorylation was detected by Western blotting with a phospho-serine antibody. The total protein levels of CB1 (C) or CB2 (D) using the same sample as in A and B, respectively, were determined by Western blotting with the HisG monoclonal antibody to show the localization of the CB1 or CB2 receptor in the membrane fraction after UVB treatment.

Both CB1 and CB2 receptors are coupled to G₁/₀ proteins, through which they stimulate the activity of the MAP kinases (33). The involvement of G₀ proteins in coupling the CB1 and/or CB2 receptor to JNK activation in this system was assessed with pertussis toxin (100 ng/mL), a compound that has widely been used as a reagent to characterize the involvement of heterotrimeric G proteins in signaling (34). Pertussis toxin catalyzes the ADP-ribosylation of specific G-protein α subunits of the G₁ family, which prevents the occurrence of the receptor and G-protein interaction (34). Pertussis toxin pretreatment before UVB treatment partially prevents UVB-induced JNK activation in CB1/2+/+ cells, indicating the involvement of G₁/₀ proteins in UVB-induced JNK activation (Fig. 3B). Cannabinoid receptors are also known to stimulate the phosphatidylinositol 3-kinase (PI3K)/Akt survival pathway (35), although the negative coupling of cannabinoid receptors to Akt has also been reported (36). In CB1/2+/+ and CB1/2−/− cells, LY294002, a PI3K inhibitor, was used to determine whether the PI3K/Akt survival pathway is involved in the UVB-induced JNK activation. Results indicated that UVB-induced JNK phosphorylation is not mediated through PI3K (Fig. 3B). Notably, neither compound affected JNK phosphorylation in CB1/2−/− cells, which was still markedly decreased compared with wild-type cells.

Figure 3. UVB-induced MAP kinase activation is markedly attenuated in CB1/2-deficient (CB1/2−/−) MEFs. A, CB1/2 wild-type (CB1/2+/+) or CB1/2−/− MEFs were irradiated with UVB (4 kJ/m²), and then incubated at 37°C for 5, 15, or 30 min. The cells not exposed to UVB were used as a negative control. Cells were then harvested and phosphorylated ERKs, JNKs, or p38 was detected by immunoblotting with the respective specific phospho-antibodies. Total protein levels of ERKs, JNKs, and p38 were used as loading controls. B, CB1/2+/+ and CB1/2−/− MEFs were treated with pertussis toxin (PTX; 100 ng/mL) or LY294002 (25 μmol/L) before UVB (4 kJ/m²) exposure. Cells were then incubated at 37°C for 15 min and harvested. Phosphorylation of JNK was detected by immunoblotting with a specific phospho-antibody. Total protein levels of JNKs were used as a loading control.
CB1/2 deficiency reduces UV-induced skin papilloma formation. Studies in CB1/2−/−, CB2−/−, or double-knockout mice have revealed responses to cannabinoids that are not mediated by the CB1/2 receptors (41). Candidates for the observed responses included other membrane-bound receptors (42) and the peroxisome proliferator–activated receptor family of nuclear receptor transcription factors (43). These results emphasize the importance of identifying the function of the CB1/2 receptors in carcinogenesis. To elucidate the function of the CB1/2 receptors in skin cancer development, we exposed mice to UVB in the two-stage skin carcinogenesis mouse model. Mice were initiated with DMBA and then treated with increasing doses of UVB beginning 2 weeks after initiation. Notably, CB1/2+/+ mice showed substantially greater overall papilloma development compared with CB1/2+/− mice. Forty percent of wild-type mice developed papillomas compared with <10% for CB1/2+/− mice over the same period of time (Fig. 5A). Furthermore, the papillomas found in CB1/2+/+ mice were more numerous (Fig. 5B) and larger (Fig. 5C) compared with CB1/2−/− mice (Fig. 5D). DMBA alone had no effect on tumor development in either wild-type or CB1/2-deficient mice (data not shown). These results suggested that the CB1/2 receptors might have an enhancing effect on DMBA/UVB-induced skin tumor development.

Discussion
Solar radiation induces acute and chronic reactions in human and animal skin. Whereas acute UV irradiation causes apoptosis, long-term exposure to UV irradiation induces activation of oncogenes and inactivation of tumor suppressor genes, resulting in abnormal proliferation of keratinocytes that may contain DNA damage leading to the onset of skin cancer (1, 44). In addition, UV irradiation stimulates cell proliferation through signal transduction initiated on the cell membrane. Activation, clustering, and internalization of cell-surface receptors for EGF, TNF-α, and IL-1 after exposure to UVB have been reported (2, 3). The effects of UV may be produced by a perturbation of membrane structure or a conformational change in membrane proteins resulting from energy absorption (3). Whether the CB1 and CB2 receptors are activated by UV radiation is not clear. Our in vitro binding studies indicated that UVB or UVA increased the affinity of the CB1 and CB2 receptors for a selected agonist, and UVB also induced CB1 and CB2 phosphorylation and internalization in the membrane fragments of cells overexpressing these proteins. These data indicated that UV activates the CB1 and CB2 receptors directly. Furthermore, from our in vivo studies, we found that tumorigenesis was significantly increased in wild-type (CB1/2+/+) mice compared with mice lacking the CB1/2 receptors (CB1/2−/−) after chronic or long-term exposure to UVB. These data showed that,
besides the cell-surface receptors for EGF, TNF-α, or IL-1, the CB1/2 receptors are also the target of UV irradiation and have a marked effect on UV-induced skin carcinogenesis.

Our results also indicated that after acute exposure to UVB, CB1/2+/- mice displayed a much higher incidence of inflammation compared with CB1/2-/- mice, coinciding with an increased inflammatory cytokine TNF-α level. These are known effects of acute UV radiation (1). Cannabinoids have been reported to suppress the production of cytokines in animal models and in human cell culture, but they have also been shown to increase the production of cytokines, including TNF, IL-1, IL-6, and IL-10, when they are administered alone or together with bacteria or other antigens (45). Thus, cannabinoids might either suppress or enhance the production of these proinflammatory agents, depending on either the nature of the proinflammatory stimulus or the type of cannabinoids used. Our results showed that activation of the CB1/2 receptors by UV irradiation enhanced the production of TNF-α and inflammation in CB1/2 wild-type mouse skin. One of the crucial mediators of inflammation-induced tumor growth and progression is activation of NF-κB by a classic pathway, which may promote tumor development (46, 47). Our data also indicated that NF-κB activation was decreased in CB1/2-/- mice compared with CB1/2+/- mice. Overall, these observations suggested that the CB1/2 receptors are required in the induction of the proinflammatory cascade-dependent tumor development in response to UVB.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Figure 5. CB1/2 deficiency suppresses skin papilloma formation in the two-stage skin carcinogenesis mouse model. A, percentage of mice developing papillomas over a total of 34 wk. B, average number of papillomas per mouse. C, average papilloma volume per mouse as measured by caliper and calculated according to the following formula: [(short diameter)^2 / (long diameter)] / 2. B and C, points, mean; bars, SD. *, P < 0.05, significantly greater multiplicity (B) or volume (C) of papillomas in wild-type mice compared with CB1/2 knockout (KO) mice. D, representative photographs of UVB-treated CB1/2+/- and CB1/2 KO mice.

References
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