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## Role of AMPK in UVB-induced DNA damage repair and growth control

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### Abstract

Skin cancer is the most common cancer in the U.S., while DNA-damaging UVB radiation from the sun remains the major environmental risk factor. Reducing skin cancer incidence is becoming an urgent issue. The energy-sensing enzyme 5'-AMP-activated protein kinase (AMPK) plays a key role in the regulation of cellular lipid and protein metabolism in response to stimuli such as exercise and changes in fuel availability. However, the role AMPK in the response of skin cells to UVB damage and in skin cancer prevention remains unknown. Here we show that AMPK activation is reduced in human and mouse squamous cell carcinoma as compared with normal skin, and by UVB irradiation, suggesting that AMPK is a tumor suppressor. At the molecular level, AMPK deletion reduced the expression of the DNA repair protein xeroderma pigmentosum C (XPC) and UVB-induced DNA repair. AMPK activation by its activators AICAR (5-aminoimidazole-4-carboxamide ribonucleoside) and metformin (N',N'-dimethylbiguanide), the most widely used anti-diabetic drug, increased the expression of XPC expression and UVB-induced DNA repair in mouse skin, normal human epidermal keratinocytes, and AMPK wild-type cells but not in AMPK deficient cells, indicating an AMPK-dependent mechanism. Topical treatment with AICAR and metformin not only delayed onset of UVB-induced skin tumorigenesis but also reduced tumor multiplicity. Furthermore, AMPK deletion increased ERK activation and cell proliferation, while AICAR and metformin inhibited ERK activation and cell proliferation in keratinocytes, mouse skin, AMPK wild-type and AMPK deficient cells, suggesting an AMPK-independent mechanism. Finally, in UVB-damaged tumor-bearing mice, both topical and systemic metformin prevented the formation of new tumors and suppressed growth of established tumors. Our findings not only suggest that AMPK is a tumor suppressor in the skin by promoting DNA

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### Conflict of interest

The authors declare no conflict of interest.

repair and controlling cell proliferation, but also demonstrate previously unknown mechanisms by which the AMPK activators prevent UVB-induced skin tumorigenesis.

## Keywords

AMPK; Tumorigenesis; UVB; DNA repair; XPC; proliferation; ERK

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## Introduction

Non-melanoma skin cancer (NMSC) is the most common type of cancer in the US, with more than one million new cases of skin cancer being diagnosed each year, accounting for 40% of all newly diagnosed cancer cases. The number of NMSC continues to rise each year. The major risk factor for NMSC is environmental UV radiation, in which UVB in sunlight is the dominant skin carcinogen (1–3). UVB damages DNA, causes somatic mutations, and thus disrupts genomic integrity.

The predominant DNA photoproducts caused by UVB radiation are pyrimidine(6-4)pyrimidone dimers (6-4PP) and cyclobutane pyrimidine dimers (CPD) (4, 5). CPD are also a major source of DNA breaks (6) that cause genomic instability (7). Replication of damaged DNA can cause mutations that may ultimately lead to skin carcinogenesis (8–14). In response to DNA damage, the cells activate a specific DNA repair mechanism, global genome nucleotide excision repair (GG-NER), which involves well-coordinated actions of DNA damage-binding proteins 1 and 2 (DDB1 and DDB2) and the xeroderma pigmentosum (XP) proteins (XPA-G) (11, 12, 15–18). A deficiency in repairing UV-induced DNA damage substantially accelerates skin cancer development, as seen in xeroderma pigmentosum (XP) patients with genetic defects in the repair of UV-induced DNA damage (11, 12).

The energy-sensing enzyme 5'-AMP-activated protein kinase (AMPK) plays a key role in the regulation of cellular lipid and protein metabolism in response to stimuli such as exercise and changes in fuel availability, and is conserved among animals, plants, and fungi (19, 20). AMPK is a heterotrimer that contains  $\alpha$ -,  $\beta$ -, and  $\gamma$ -subunits, each of which has at least two isoforms. Emerging evidence indicates that AMPK is a promising metabolic tumor suppressor and a target for cancer prevention and therapy (21). The AMPK pathway intersects with the oncogenic Ras/PI3K/mTOR and ERK pathways at multiple points in growth control pathways (19). AMPK signaling also interacts with the p53 and ATM pathways, two essential tumor suppressors and genomic gatekeepers, to coordinate metabolic checkpoints and DNA damage response (22–24). As more functions and targets of AMPK are decoded, the challenge will be in determining the role of AMPK activity in malignancies and the precise interactions of AMPK with a specific organ and its carcinogenic causes. These future findings will provide a fundamental basis for AMPK activators as new agents and for strategies to better prevent and treat cancer.

There are two AMPK activators that have been explored for cancer treatment. One of them is the most widely used anti-diabetic drug, metformin (N',N'-dimethylbiguanide), which belongs to the biguanide class of oral hypoglycemic agents. It is now prescribed to almost

120 million people worldwide and has become the first line anti-hyperglycemic agent in the treatment of type 2 diabetes (25). Metformin works mainly by activating the AMPK pathway and via an AMPK-independent mechanism (26, 27). Retrospective studies suggest that diabetics treated with metformin have a substantially reduced cancer burden compared with other diabetics. It is unclear whether this reflects a chemopreventive effect, and whether these data have relevance to people without diabetes. Over the past few years, however, impressive evidence from several studies indicates that metformin exhibits cancer prevention effects *in vitro* and in animal models (28, 29). In addition, another AMPK activator, AICAR (5-aminoimidazole-4-carboxamide ribonucleoside), has been shown to suppress glioblastoma growth *in vivo* (30) and melanoma cell growth *in vitro* (31). AICAR also inhibits keratinocyte growth *in vitro* (32). However, the role AMPK in the response of skin cells to UVB and in skin cancer prevention remains unknown.

Here we have investigated the role of AMPK in UVB-induced DNA damage repair and cell proliferation, two critical processes determining skin cancer susceptibility, and the impact of AICAR and metformin on UVB-induced skin tumorigenesis and the role of AMPK activation. We found that the activation of the energy-sensing enzyme 5'-AMP-activated protein kinase (AMPK) was reduced in human and mouse squamous cell carcinomas as compared with normal skin and by UVB damage. AMPK plays important roles in UVB-induced DNA damage repair and cell growth. Both AICAR and metformin reduced UVB-induced skin tumorigenesis. Furthermore, both topical and systemic metformin inhibited growth of established tumors and prevented new tumor formation in mice with previous UVB damage. At the molecular level, both AMPK-dependent- and independent-mechanisms are involved.

## Results

### AMPK pathway is inhibited in human and mouse skin tumors and in UVB-irradiated mouse skin

To determine the role of the AMPK pathway in skin tumorigenesis, we analyzed the activation of AMPK in human and mouse skin tumors and the regulation of AMPK by UVB irradiation. As compared with normal human skin, human cutaneous squamous cell carcinomas (SCC) showed reduced AMPK phosphorylation (Fig. 1A), implying that AMPK is inhibited in human SCC. As compared with sham-irradiated mouse skin, phosphorylation of ACC, a known AMPK target, was reduced not only in UVB-induced mouse tumors but also in non-tumor mouse skin chronically irradiated by UVB (Fig. 1B), suggesting that AMPK inactivation is an early event in UVB-induced skin tumorigenesis. In mouse skin, UVB increased the phosphorylation of AMPK at 6 h, while it suppressed the phosphorylation of AMPK and ACC at 24 h post-UVB, suggesting that, although UVB activates AMPK at an earlier time point, it inhibits AMPK activation at a later time point (Fig. 1C). ). It is possible that AMPK activation is followed by its down-regulation. These findings suggest that AMPK acts as a tumor suppressor in UVB-induced skin cancer.

## AMPK is required for enhancing UVB-induced DNA damage repair by AICAR and metformin

The ability of a keratinocyte to carry out proper DNA repair is vital to its genomic integrity following UVB damage. Replication of damaged DNA can cause mutations that may ultimately lead to skin carcinogenesis (8–14). To determine whether UVB-induced DNA damage repair is affected by AMPK inhibition, AICAR, or metformin, we analyzed the difference in DNA repair between AMPK wild-type (WT) mouse embryonic fibroblast (MEF) cells and AMPK knockout MEF cells, and between vehicle-treated (Veh) mouse skin and AICAR- or metformin (Met)-treated skin and normal human epidermal keratinocytes (NHEK). To determine the specific role of AMPK in UVB-induced DNA damage repair, we elected to use conditions that neither caused apoptosis, which can remove damaged cells, nor allowed cell proliferation, which will lead to overestimation of DNA repair due to dilutions of DNA damage. We elected to use a low dose of UVB at 5 mJ/cm<sup>2</sup> for MEF cells, 100 mJ/cm<sup>2</sup> for mouse skin, and 20 mJ/cm<sup>2</sup> for NHEK cells, which do not cause apoptosis (data not shown). In addition, we used low serum medium (2%) for MEF cells or reduced growth factors (20% of normal levels) for NHEK cells, together with functional checkpoint pathway, to assure growth arrest after UVB radiation within 24 h for NHEK cells and mouse skin, and 48 h for MEF cells (data not shown). In MEF cells, AMPK deletion significantly reduced CPD repair (Fig. 2A-B;  $P < 0.05$ , Student's *t*-test and two-way ANOVA), while it had no effect on 6-4PP repair (Fig. 2A). These data indicate that AMPK is required for efficient CPD repair and suggest that AMPK acts as a tumor suppressor, as failure to repair CPD but not 6-4PP is the principal cause of skin cancer (9). In mouse skin, topical treatment with AICAR or metformin accelerated CPD repair at 6 h post-UVB (Fig. 2C-D;  $P < 0.05$ , Student's *t*-test) significantly, while it did not affect either the repair of CPDs at 24 h or that of 6-4PPs at 6 or 24 h post-UVB (Fig. 2E-F), indicating that the AMPK activators AICAR and metformin promote CPD repair.

To further determine the role of AMPK activation in the action of AICAR and metformin, we assessed whether AMPK deletion abolishes the promoting effect of AICAR and metformin on CPD-repair. In AMPK WT MEF cells, AICAR (AI) and metformin accelerated CPD repair at 6 h post-UVB, while they had no effect in AMPK KO MEF cells (Fig. 2G), indicating that AMPK is required for promoting DNA repair by AICAR and metformin. To determine the molecular mechanism by which AMPK regulates DNA repair, we analyzed the difference in the protein levels of DDB1, DDB2, and XPC, crucial specific factors required for repairing the majority of UVB-induced DNA damage (11, 12, 15–18). AMPK deletion in MEF cells reduced the protein levels of XPC, while it had no effect on DDB1 and DDB2 levels (Fig. 2H). However, AMPK deletion neither affected XPC transcription (Fig. s1A) nor increased the nuclear localization of E2F4/p130 (Fig. s1B), the repressor complex for XPC transcription, suggesting that AMPK regulates XPC expression through a post-transcriptional mechanism. In normal epidermal human keratinocytes (NHEK), AICAR and metformin (Met) increased the expression of XPC (Fig. 2I) and CPD repair (Fig. s1C). Similarly topical AICAR and metformin increased XPC protein levels in mouse skin (Fig. s1D). Taken together, these data indicate that AICAR and metformin promote UVB-induced DNA repair in an AMPK-dependent manner.

### Activation of the AMPK pathway reduces UVB-induced skin tumorigenesis in mice

To determine whether activation of AMPK affects UVB-induced skin tumorigenesis, we treated SKH-1 hairless mice, which are widely used in photocarcinogenesis as a clinically relevant animal model, with the AMPK activators AICAR and metformin and then exposed the mice to UVB radiation three times a week for 23 weeks. Treatment of mice with topical AICAR or metformin at 24 h after the final treatment increased ACC phosphorylation (Fig. 3A), indicating that both AICAR and metformin activate the AMPK pathway in mouse skin. Topical treatment with either AICAR or metformin in parallel with UVB treatment not only significantly delayed the onset of UVB-induced skin tumorigenesis (Fig. 3B;  $P < 0.05$ , Log-rank test between vehicle and AICAR or metformin groups) but also reduced tumor multiplicity (Fig. 3C;  $P < 0.05$ , Student's *t*-test), including both large malignant tumors (diameter  $> 1$ cm, all SCC) and small pre-malignant lesions (diameter  $< 1$ cm, all papilloma) (Fig. 3D). These data indicate that the AMPK activators AICAR and metformin reduce UVB-induced skin tumorigenesis.

### AICAR and metformin reduce cell proliferation through an AMPK-independent mechanism

Deregulated cell proliferation is not only critical for tumor growth but also essential for tumor formation. To determine whether AICAR or metformin plays a role in cell proliferation *in vivo*, we assessed the difference in epidermal thickness and the number of Ki67-positive epidermal cells between vehicle-, AICAR-, or metformin-treated mouse epidermis. Chronic UVB irradiation increased epidermal thickness about 10-fold, while AICAR and metformin significantly reduced UVB-induced epidermal hyperplasia (Fig. 4A-B,  $P < 0.05$ , Student's *t*-test). In addition, AICAR and metformin reduced the number of Ki67-positive epidermal cells in sham- or UVB-irradiated mice (Fig. 4C-D;  $P < 0.05$ , Student's *t*-test). These data indicate that AICAR and metformin inhibit cell proliferation *in vivo*. To determine whether AMPK plays a role in the anti-proliferative action of AICAR and metformin, we analyzed the effect of AMPK deletion and the effect of AICAR and metformin on cell proliferation in AMPK WT and KO MEF cells. AMPK deletion increased cell proliferation significantly at 2 and 3 days in culture (Fig. 4E,  $P < 0.05$  using Student's *t*-test and two way ANOVA), indicating that AMPK is critical for controlling cell growth. AICAR and metformin inhibit cell proliferation in both WT and KO cells at the third day after plating (Fig. 4F), indicating that AMPK is dispensable for the anti-proliferating effect of AICAR and metformin.

### ERK pathway is inhibited by AICAR and metformin

To determine the molecular pathway that mediates the effect of AICAR and metformin on cell growth, we analyzed the involvement of ERK, a major growth-promoting pathways. Repeated topical treatment with either AICAR or metformin in mice reduced the phosphorylation of ERK (Fig. 5A). One time treatment also had an inhibitory effect (Fig. s1D). These data suggest the ERK pathway as a target for AICAR and metformin in the skin. Similarly, in NHEK cells AICAR and metformin decreased ERK activation (Fig. 5B). To determine the role of AMPK in ERK activation, we analyzed the difference in ERK activation in AMPK WT and KO cells. AMPK deletion increased the phosphorylation of ERK and EGFR (Fig. 5C), an upstream pathway of ERK activation, and the protein levels of

cyclin D1, a downstream target of ERK activation. In AMPK KO cells, inhibiting the EGFR pathway decreased ERK phosphorylation and cyclin D1 levels, and inhibiting the ERK pathway reduced cyclin D1 levels (Fig. 5D). These data indicate that AMPK is a suppressor for the EGFR/ERK/cyclin D1 pathway, a key mitogenic signal in response to growth factor stimulation. However, AICAR and metformin reduced ERK phosphorylation not only in AMPK WT cells but also in AMPK KO cells (Fig. 5E), indicating that AICAR and metformin inhibit ERK through an AMPK-independent pathway. In contrast, neither AICAR nor metformin affected EGFR phosphorylation, suggesting that AICAR and metformin target EGFR downstream but ERK upstream pathways. These data suggest that the anti-proliferative action of AICAR and metformin is mediated through AMPK-independent inhibition of ERK signaling.

### **Metformin suppresses growth of established tumors and prevents new tumor formation in UVB-irradiated mice**

To further advance our findings on the chemopreventive effect of AICAR and metformin toward potential clinical applications, we analyzed the impact of metformin on tumor formation and growth in tumor-bearing mice that had been chronically exposed to UVB radiation. We elected to focus on metformin, as it has been widely used for years for anti-diabetic treatment, and thus is more likely to be applied for cancer prevention and intervention in, for example, non-diabetic high risk individuals who have a history of skin cancer. To mimic a skin tumorigenesis scenario, we irradiated SKH-1 mice with UVB for 17 weeks until they developed 3–4 tumors on average that were 2–4 mm in diameter. Then these mice were treated with vehicle, topical metformin (Met-T), or systemic metformin through oral gavage (Met-G) and continued to be irradiated with UVB (Fig. 6A-B). Both topical and systemic metformin increased phosphorylation of ACC in non-tumor mouse skin, indicating AMPK activation (Fig. 6C). Either treatment prevented new tumor formation (Fig. 6D;  $P < 0.05$ , Student's *t*-test). In addition, both topical and systemic metformin treatment significantly inhibited growth of established tumors (Fig. 6E,  $P < 0.05$ , Student's *t*-test), while systemic treatment was more effective than topical treatment. This may be due to the limited penetration of topical treatment for established tumors.

To determine the role of cell proliferation, we assessed the effect of topical and systemic metformin on epidermal hyperplasia and the number of Ki67-positive cells in UVB-damaged non-tumor skin and established tumors. Both topical and systemic metformin reduced epidermal thickness and the number of Ki67-positive cells in non-tumor skin, while systemic but not topical metformin reduced the number of Ki67-positive cells in established tumors (Fig. 6F). Taken together, these data demonstrated that, in tumor-bearing mice, the most widely used anti-diabetic drug metformin given topically or systemically prevents new tumor formation and suppresses growth of established tumors in association with inhibiting cell proliferation, suggesting a potential chemopreventive benefit for individuals with skin cancer histories and at high risk for skin cancer.

## Discussion

Recent studies have demonstrated that the energy-sensing enzyme AMPK inhibits growth and/or survival of cancer cells, and thus AMPK activators including AICAR and the anti-diabetic drug metformin might be used to enhance cancer therapy (19, 20). However, the role AMPK in the response of skin cells to UVB damage and in skin cancer prevention remains unknown. Here we have shown that the AMPK pathway is down-regulated in human and mouse skin tumors and in UVB-irradiated mouse skin. The AMPK pathway is critical for UVB-induced DNA damage repair and growth control. In mice, topical treatment with the AMPK activators AICAR and metformin prevents UVB-induced skin tumorigenesis. Topical or systemic metformin prevents new tumor formation and suppresses growth of established tumors in UVB-irradiated mice with skin tumors. AICAR and metformin promote UVB-induced DNA damage repair through an AMPK-dependent mechanism, while they inhibit cell proliferation through an AMPK-dispensable pathway. Our findings strongly indicate that AICAR and metformin promote the genomic integrity of normal skin cells following UVB damage and thus reduce skin tumorigenesis.

We demonstrated that AMPK is required for efficient repair of UVB-induced DNA damage, linking energy metabolism with genomic stability. This was supported by the following evidence: (1) AICAR and metformin increased UVB-induced DNA damage repair in mouse skin; (2) deletion of AMPK inhibited DNA repair; and (3) AMPK deletion diminished the promoting action of AICAR and metformin on DNA repair. At the molecular level, AMPK inhibition specifically down-regulated XPC, a key protein required for global genome nucleotide excision repair (GG-NER), while AICAR and metformin increased XPC levels, suggesting that AMPK positively regulates GG-NER through XPC. Our previous studies have supported a model in which XPC mediates the function of the deacetylase SIRT1 and the tumor suppressor PTEN in GG-NER (33, 34). Both SIRT1 and PTEN regulate XPC at the transcriptional level. However, the regulation of XPC by AMPK seems to be independent of its transcription or its transcription repressor factors. It is possible that AMPK regulates the mRNA stability through the RNA binding protein HuR (35). Further investigation is needed to elucidate the molecular mechanism by which AMPK regulates XPC. Nevertheless, our results strongly indicate that AMPK activation is required for fully operational GG-NER capacity to remove the tumorigenic DNA lesions, i.e., CPD, as failure to repair CPD is the principle cause of skin cancer (9), and effective GG-NER protects mice against UVB-induced skin tumorigenesis.

In addition, our results indicate that AICAR and metformin inhibit cell proliferation in mouse skin and reduce UVB-induced epidermal hyperplasia. AMPK suppresses cell proliferation through inhibiting the mitogenic EGFR/ERK pathway. In contrast, both AICAR and metformin inhibit the ERK pathway but they had no effect on EGFR activation. The anti-proliferative action and ERK inhibition of AICAR and metformin are independent of AMPK action, as AMPK deletion had no effect. Interestingly, previous studies have identified an opposing effect of AICAR/AMPK on ERK activation. In both NIH-3T3 and cardiac fibroblasts, AICAR inhibits growth factor-induced ERK activation (36, 37), in an AMPK-dependent but EGFR-independent manner (36). In contrast, in erythroleukemia K562 cells, AMPK is required for ERK activation (38). It is possible that the regulation of

ERK activation by AICAR is cell type-specific. In the skin as well as in MEF cells in this study, AICAR and metformin inhibit the ERK pathway through an AMPK- and EGFR-independent mechanism. Furthermore, although AMPK and metformin have been shown to suppress the mTOR pathway (19, 20, 39–41), in glioblastomas, AICAR is more effective in blocking cell proliferation than the mTOR inhibitor rapamycin, despite less efficient inhibition of mTOR signaling (30), thus challenging the dominant role of mTOR inhibition. We are currently working on identifying the molecular mechanism of AICAR and metformin for ERK inhibition and proliferation control and elucidating the importance of mTOR in the chemopreventive action of AICAR and metformin.

In summary, we have demonstrated that AMPK may play a critical role in inhibiting skin carcinogenesis by promoting UVB-induced DNA damage repair and growth control. The AMPK activators AICAR and metformin reduce UVB-induced skin tumorigenesis. AICAR and metformin promote UVB-induced DNA damage repair through AMPK activation, while they decrease cell proliferation through AMPK-independent ERK inhibition. Our findings suggest that AICAR and the most widely used anti-diabetic drug metformin are potential chemopreventive agents for skin cancer, especially for individuals with skin cancer history.

## Materials and Methods

### Human normal and tumor samples

All human specimens were studied after approval by the University of Chicago Institutional Review Board. Frozen tissues were obtained under the consent (Department of Medicine, University of Chicago) as in our previous studies (34).

### Cell culture

AMPK wild-type (WT) and knockout (KO) mouse embryonic fibroblast (MEF) cells (42) were maintained in a monolayer culture in 95% air/5% CO<sub>2</sub> at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units per mL penicillin, and 100 mg per mL streptomycin (Invitrogen, Carlsbad, California). MEF cells were cultured for less than 20 passages. Normal human epidermal keratinocytes (NHEK) were obtained from Clonetics (Lonza) and cultured in KGM Gold BulletKit medium (Clonetics, Lonza) according to the manufacturer's instructions. NHEK cells were cultured for less than 4 passages. For DNA repair analysis, MEF or NHEK cells were cultured in low serum medium (2%) for MEF cells or growth factors (20% of normal levels) for NHEK cells overnight prior to UVB irradiation and after irradiation to prevent cell growth.

### UVB radiation

UVB radiation was performed as described previously (43). Our UVB radiation was monitored every other week to measure the exposure output and dose. Our UVB system does not emit UVC radiation.

## Animal Treatments

All animal procedures have been approved by the University of Chicago Institutional Animal Care and Use Committee. Hairless SKH-1 mice were obtained from Charles River. Mice were exposed to UVB (100 mJ/cm<sup>2</sup>, dose selected to avoid visible sunburn) dorsally or sham-irradiated, three times a week for up to 25 weeks, to monitor tumor formation and growth. One hour prior to each UVB irradiation, mice were treated with vehicle (acetone), AICAR (1 μmol) or metformin (2 μmol). In UVB-irradiated tumor-bearing mice, metformin was given either topically (2 μmol) or by gavage (300 mg/kg). Mouse skin samples were fixed in formalin for histological analysis or immunohistochemical analysis for Ki67-positive cells (Immunohistochemistry Core facility), or snap-frozen for immunoblotting analysis. Mice were housed five animals per cage, and there was no evidence of dorsal wounds caused by fighting or sunburn.

## Western blotting

Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL, USA). Equal amounts of protein were subjected to electrophoresis. Western blotting was performed as described previously using film detection (13, 43). Antibodies used included phospho-ERK (p-ERK), ERK, phospho-EGFR (p-EGFR), AMPK, ACC, DDB1, DDB2, XPC, E2F4, p130, Lamin B, β-actin, GAPDH (Santa Cruz), cyclin D1 (BD Bioscience) p-AMPK (T172) and p-ACC (S79) (Cell Signaling Technology).

## In vitro cell proliferation assay, immunohistochemistry, promoter reporter assay and cytosol-nuclear fractionation

Cell proliferation of MEF cells were analyzed using the MTS assay (Promega) according to the manufacturer's instructions as in our recent studies (44). Immunohistochemical analysis of Ki67-positive cells in the mouse epidermis was conducted in the Immunohistochemistry Core facility. The promoter reporter assay and cytosol-nuclear fractionation were performed as described in our recent studies (33).

## Determination of two major forms of UVB-induced DNA damage in genomic DNA by slot blot assay

Slot blot assay of CPD and 6-4PP were performed as described previously (45). Briefly, mouse skin or cells were collected at different time points post-UVB and DNA was isolated using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA). The DNA concentration was calculated from the absorbance at 260 nm using NanoDrop 1000 (NanoDrop products, Wilmington, DE). The CPD and 6-4PP in DNA were quantified by slot blot (Bio-Rad) with monoantibodies (TDM-2 for CPD and 64 M-2 for 6-4PP, COSMO BIO Co., Koto-Ku, Tokyo, Japan) as described previously (45). The chemiluminescence was detected with a Carestream Imaging Station (Carestream). For examining repair kinetics, the percentage (%) of repair was calculated by comparing the optical density at the indicated time to that of the corresponding absorbance at time zero when there was no opportunity for repair and 100% of CPDs (or 6-4PPs) were present post-UVB.

## Statistical analyses

Statistical analyses were performed using Prism 5 (GraphPad software, San Diego, CA). Data were expressed as the mean of at least three independent experiments and analyzed by Student's *t*-test and ANOVA. Error bars indicate standard error of means (S.E.). Log-rank tests were used to evaluate tumor onset. Student's *t*-tests were used to analyze tumor number per mouse. A *P* value of less than 0.05 was considered statistically significant.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

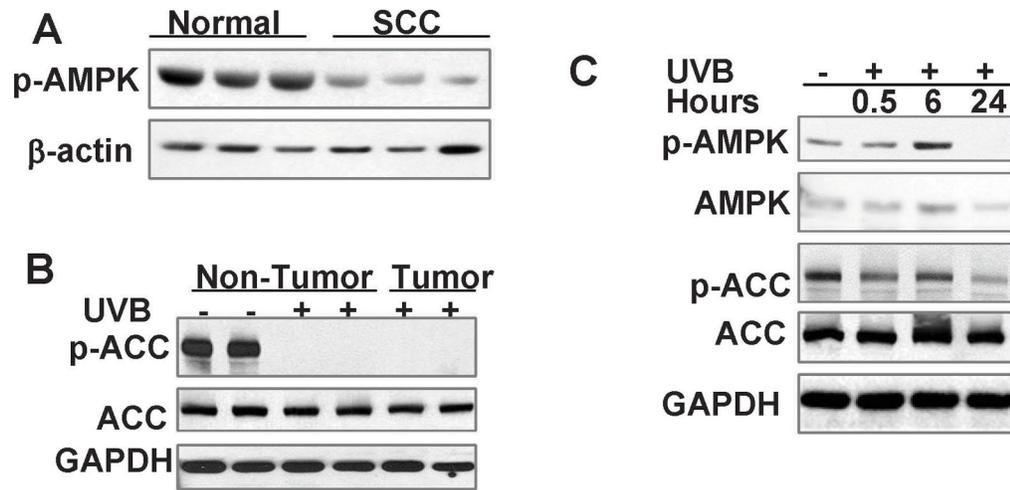
<b>6-4PP</b>	pyrimidine(6-4)pyrimidone dimmers
<b>ACC</b>	Acetyl-CoA carboxylase
<b>AICAR</b>	AI, 5-aminoimidazole-4-carboxamide ribonucleoside
<b>AMPK</b>	5'-AMP-activated protein kinase
<b>CPD</b>	cyclobutane pyrimidine dimers
<b>EGFR</b>	epidermal growth factor receptor
<b>ERK</b>	extracellular signal-regulated kinase
<b>KO</b>	knockout
<b>Met</b>	metformin
<b>mTOR</b>	mammalian target of rapamycin
<b>mTORC1</b>	mTOR complex 1
<b>NHEK</b>	normal human epidermal keratinocytes
<b>NMSC</b>	non-melanoma skin cancer
<b>SCC</b>	squamous cell carcinoma
<b>UVB</b>	Ultraviolet B
<b>Veh</b>	vehicle
<b>WT</b>	wild-type
<b>XP</b>	xeroderma pigmentosum
<b>XPC</b>	xeroderma pigmentosum group C

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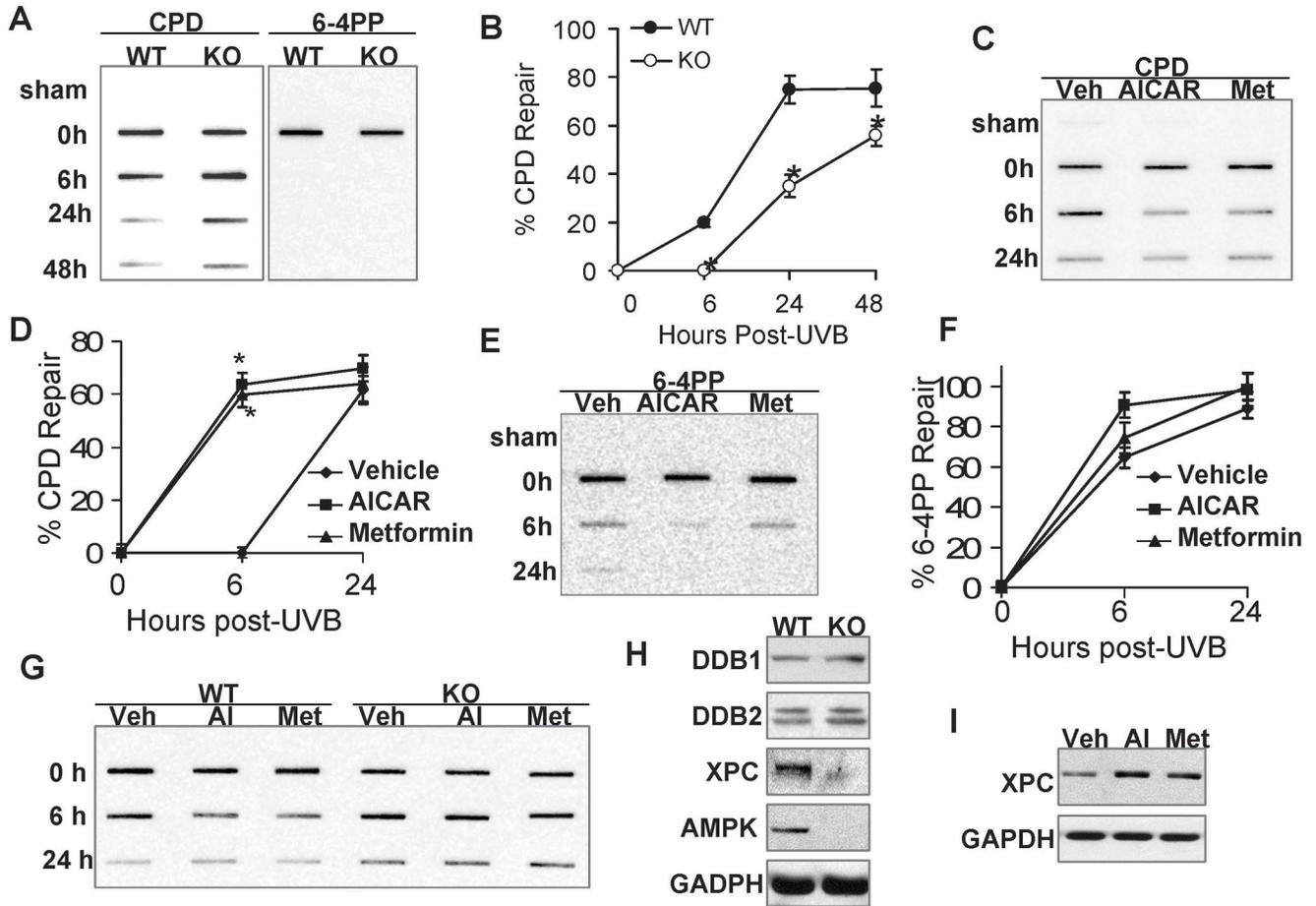
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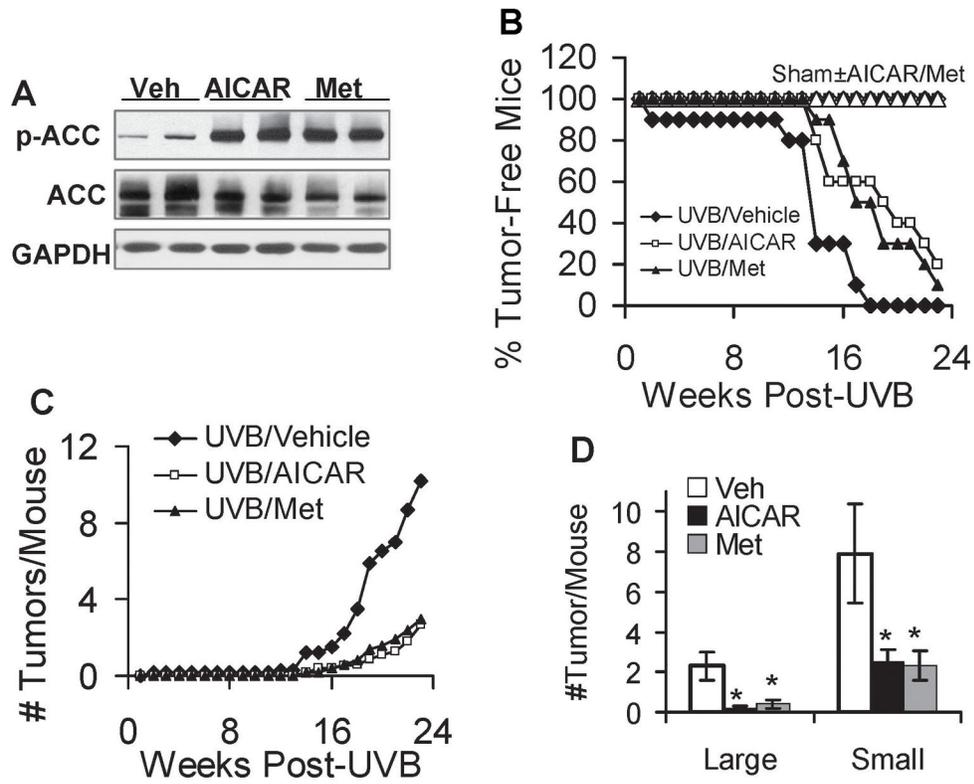
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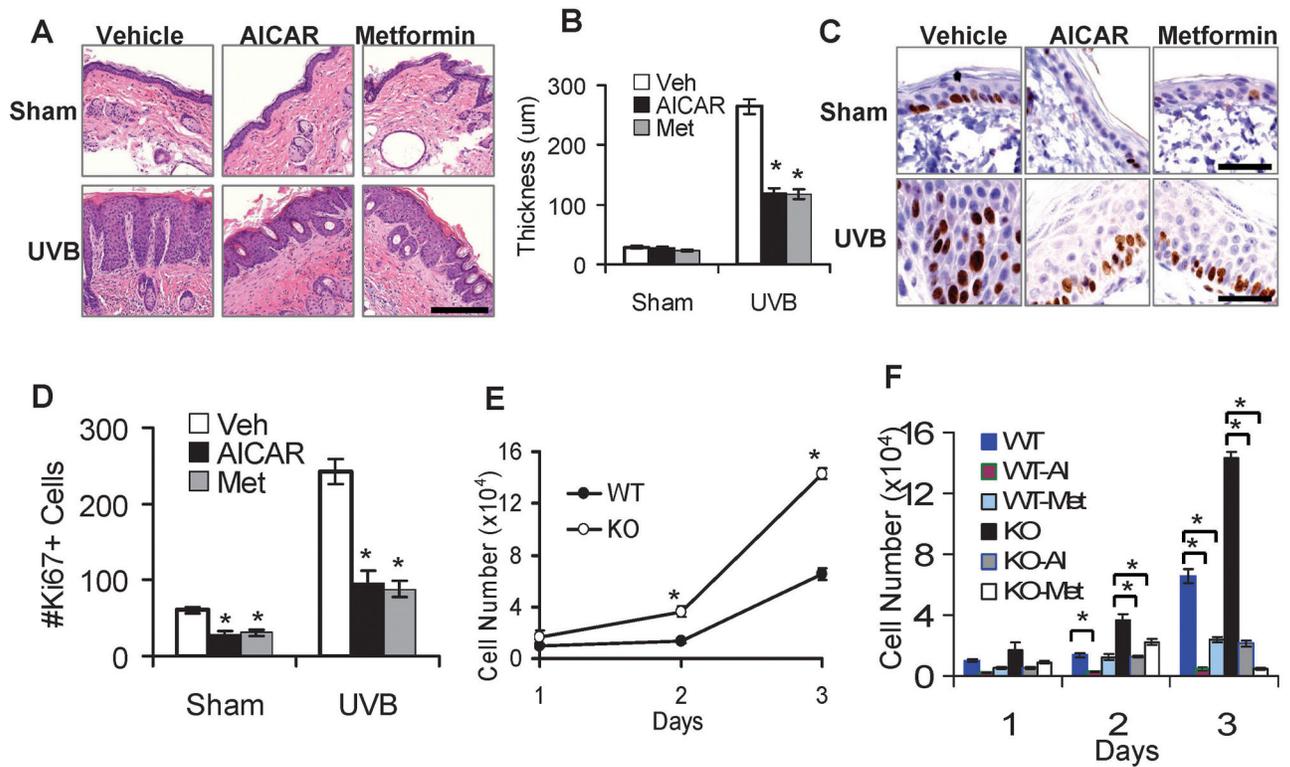
**Fig. 1.** AMPK pathway is inhibited in skin tumors from human and mouse and by UVB. A, immunoblot analysis of p-AMPK (T172) and  $\beta$ -actin in normal human skin and human SCC. B, immunoblot analysis of p-ACC (S79), ACC and GAPDH in sham- or UVB-irradiated non-tumor skin and UVB-induced skin tumors from SKH-1 mice. Mice were irradiated with UVB (100 mJ/cm<sup>2</sup>) three times a week for 23 weeks. Non-tumor skin or tumor was collected at 24 h after the final UVB irradiation or sham irradiation. C, immunoblot analysis of p-AMPK, AMPK, p-ACC, ACC and GAPDH in SKH-1 mouse skin sham-treated or treated with UVB at 0.5, 6 or 24 h post-UVB (100 mJ/cm<sup>2</sup>).



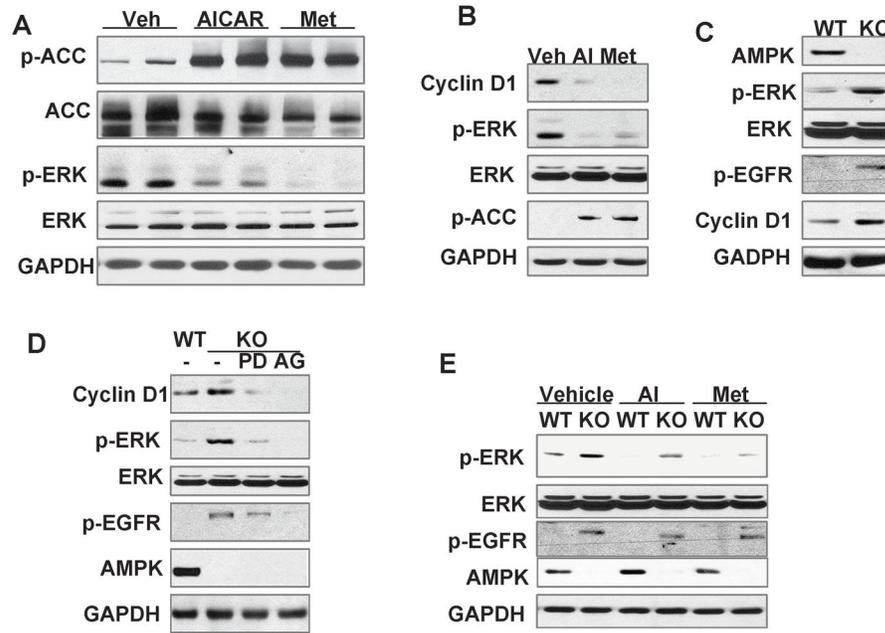
**Fig. 2.** AICAR and metformin enhance UVB-induced DNA repair through activating AMPK. A, slot blot analysis of the levels of CPD and 6-4PP in MEF cells ( $n=3$ ) with wild-type AMPK (WT) or AMPK knockout (KO) at 0, 6, 24, and 48 h post-UVB ( $5 \text{ mJ/cm}^2$ ). B, quantification of percentage (%) of CPD repair from A. \*,  $P < 0.05$ , significant differences between AMPK WT and KO groups. C, slot blot analysis of the levels of CPD in SKH-1 mouse skin ( $n=3$ ) treated with vehicle (Veh), AICAR, or metformin (Met) at different times post-UVB ( $100 \text{ mJ/cm}^2$ ). D, quantification of percentage (%) of CPD repair from C. \*,  $P < 0.05$ , significant differences between vehicle- and AICAR- or metformin-treated groups. E, slot blot analysis of the levels of 6-4PP in SKH-1 mouse skin ( $n=3$ ) treated with vehicle (Veh), AICAR, or metformin (Met) at different times post-UVB ( $100 \text{ mJ/cm}^2$ ). F, quantification of percentage (%) of 6-4PP repair from E. G, slot blot analysis of the levels of CPD and 6-4PP in WT or KO MEF cells ( $n=3$ ) treated with vehicle (Veh), AICAR (AI, 1 mM), or metformin (2 mM) at 0, 6, 24, and 48 h post-UVB ( $5 \text{ mJ/cm}^2$ ). H, immunoblot analysis of DDB1, DDB2, XPC, AMPK and GAPDH in WT and KO AMPK MEF cells. I, immunoblot analysis of XPC and GAPDH in NHEK cells treated with vehicle (Veh), AICAR (AI, 1 mM) or metformin (2 mM). Error bars in panels B, D and F indicate S.E.



**Fig. 3.** AICAR and metformin prevent UVB-induced skin tumorigenesis in SKH-1 hairless mice. A, immunoblot analysis of p-ACC, ACC, and GAPDH in SKH-1 mouse skin at 24 h after the final topical treatment with vehicle (Veh), AICAR (1  $\mu$ mol), or metformin (Met, 2  $\mu$ mol) for 23 weeks. B, percent (%) of tumor-free mice in vehicle (Veh), AICAR, or metformin-treated mice following sham or UVB irradiation (n=10). SKH-1 mice were treated with topical AICAR (1  $\mu$ mol) or metformin (2  $\mu$ mol) 1 h prior to each UVB irradiation (100 mJ/cm<sup>2</sup>) three times a week for 23 weeks. C, Average number (#) of tumors per mouse from mice treated as in B. D, average number (#) of large (> 1cm in diameter) and small (< 1cm in diameter) tumors per mouse. \*,  $P < 0.05$ , significant differences between vehicle- and AICAR- or metformin-treated groups. Error bars in panel D indicate S.E.

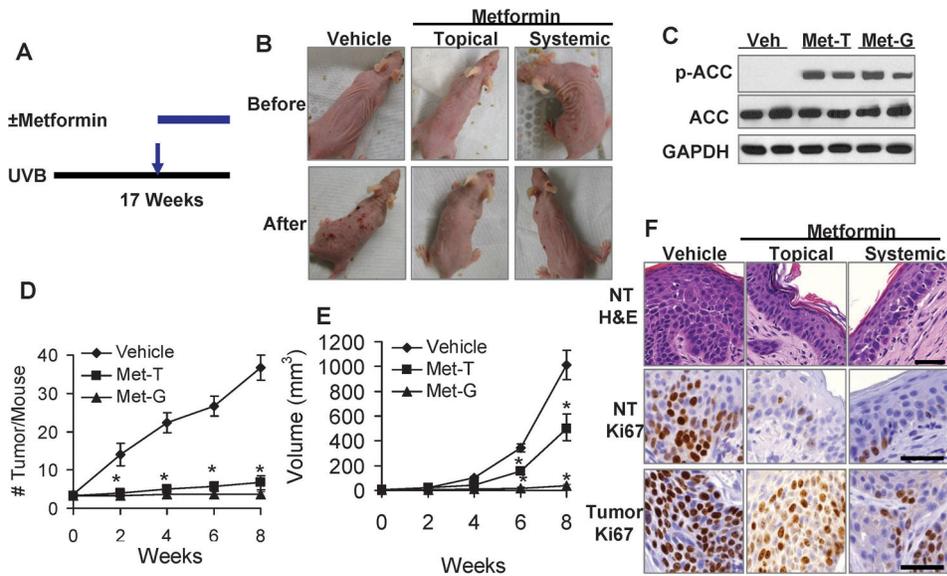
**Fig. 4.**

AICAR and metformin reduce cell proliferation in mouse skin and MEF cells independent of the AMPK pathway. A, histological analysis of non-tumor mouse epidermis ( $n = 10$ ) topically treated with vehicle, AICAR ( $1 \mu\text{mol}$ ) or metformin ( $2 \mu\text{mol}$ ) for 23 weeks post-UVB or –sham. Scale Bar:  $200 \mu\text{m}$ . B, quantification of epidermal thickness ( $\mu\text{m}$ ) in A. C, immunohistochemical analysis of Ki67-positive cells in mouse skin ( $n = 5$ ) topically treated with vehicle, AICAR ( $1 \mu\text{mol}$ ) or metformin ( $2 \mu\text{mol}$ ) for 23 weeks post-UVB or –sham irradiation. Scale Bar:  $50 \mu\text{m}$ . D, quantification of Ki67-positive (Ki67+) cells in C. \*,  $P < 0.05$ , significant differences between vehicle- and AICAR/metformin-treated groups. E, proliferation analysis using the MTS assay (Promega) in WT or KO AMPK MEF cells. \*,  $P < 0.05$ , significant differences between AMPK WT and KO cells. F, proliferation analysis using the MTS assay (Promega) in WT or KO AMPK MEF cells treated with vehicle, AICAR (AI,  $1 \text{ mM}$ ) or metformin (Met,  $2 \text{ mM}$ ). \*,  $P < 0.05$ , significant differences between vehicle- and AI/Met-treated groups in WT and KO cells. Error bars in panels B, D, E, and F indicate S.E.



**Fig. 5.**

AMPK is not required for inhibiting the ERK pathway by AICAR and metformin. A, immunoblot analysis of p-ACC, ACC, p-ERK, ERK, and GAPDH in mouse skin at 24 h after the final topical treatment with vehicle, AICAR (1  $\mu$ mol) or metformin (2  $\mu$ mol) for 23 weeks. B, immunoblot analysis of Cyclin D1, p-ERK, ERK, p-ACC and GAPDH in NHEK cells at 24 h after treatment with vehicle, AICAR (1 mM) or metformin (2 mM). C, immunoblot analysis of AMPK, p-ERK, ERK, p-EGFR, cyclin D1 and GAPDH in AMPK WT and KO MEF cells. D, immunoblot analysis of cyclin D1, p-ERK, ERK, p-EGFR, AMPK, and GAPDH in KO MEF cells treated with vehicle (–), PD (PD98059, 20  $\mu$ M) and AG (AG1478, 1  $\mu$ M), and WT MEF cells. E, immunoblot analysis of p-ERK, ERK, p-EGFR, AMPK and GAPDH in WT and KO MEF cells treated with vehicle, AICAR (1 mM), or metformin (2 mM).



**Fig. 6.** Metformin prevents new tumor formation and suppresses growth of established tumors in mice. A, a schematic diagram of the experimental design for B-F, in which mice were treated with topical metformin (Met-T, 2  $\mu$ mol) or systemic metformin (Met-G, 300 mg/kg body weight) 1 h prior to each UVB treatment at 17 weeks after the initial UVB irradiation, together with continuing UVB irradiation three times a week for 8 weeks. B, representative mouse pictures from experimental design as in A. C, immunoblot analysis of p-ACC, ACC and GAPDH. D, number (#) of new tumors per mouse at different weeks following metformin treatment as in A (n = 3). E, average volume (mm<sup>3</sup>) of established tumors formed at 17 weeks post-UVB at different weeks following treatment as in A. F, histological analysis of non-tumor (NT) epidermis treated with metformin as in A for 8 weeks by hematoxylin and eosin stain (H&E) and immunohistochemical analysis of Ki67-positive (Ki67+) cells in non-tumor (NT) and skin tumors. \*,  $P < 0.05$ , significant differences between vehicle- and metformin-treated groups. Error bars in panels D and E indicate S.E.