Palmitoylation-dependent activation of MC1R prevents melanomagenesis

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The melanocortin-1 receptor (MC1R), a G-protein-coupled receptor, has a crucial role in human and mouse pigmentation1-8. Activation of MC1R in melanocytes by α-melanocyte-stimulating hormone (α-MSH)9 stimulates CAMP signalling and melanin production and enhances DNA repair after ultraviolet irradiation10-16. Individuals carrying MC1R variants, especially those associated with red hair colour, fair skin and poor tanning ability (denoted as RHC variants), are associated with higher risk of melanoma17-20. However, how MC1R activity is modulated by ultraviolet irradiation, why individuals with red hair are more prone to developing melanoma, and whether the activity of RHC variants might be restored for therapeutic benefit are unknown. Here we demonstrate a potential MC1R-targeted intervention strategy in mice to rescue loss-of-function MC1R in MC1R RHC variants for therapeutic benefit by activating MC1R protein palmitoylation. MC1R palmitoylation, primarily mediated by the protein-acyl transferase ZDHHC13, is essential for activating MC1R signalling, which triggers increased pigmentation, ultraviolet-B-induced G1-like cell cycle arrest and control of senescence and melanomagenesis in vitro and in vivo. Using C57BL/6-Mc1r<sup>−/−</sup> mice, in which endogenous MC1R is prematurely terminated, expressing McIr RHC variants, we show that pharmacological activation of palmitoylation rescues the defects of McIr RHC variants and prevents melanomagenesis. The results highlight a central role for MC1R palmitoylation in pigmentation and protection against melanoma.

A preliminary small molecule screen to identify modulators of RHC-variant MC1R activity suggested that palmitic acid increased cAMP levels in human primary melanocytes (HPMs) with an endogenous MC1R(R151C) variant (HPM-RHC), or engineered RHC-variant B16 melanoma cells (B16-RHC cells) in which MC1R(R151C) was reintroduced after deletion of the endogenous gene (MC1R-reconstituted cells). To validate this result, HPM-RHC and B16-RHC cells were serum starved, pretreated with α-MSH and exposed to standard erythema dose of ultraviolet B (UVB) light (100 J m<sup>−2</sup>), before being treated with medium containing BSA-conjugated fatty acids for 3 h. Palmitic acid, but not other lipids, increased cAMP levels in both HPM-RHC and B16-RHC cells (Fig. 1a, Extended Data Fig. 1a).

As palmitic acid can induce palmitoylation of cysteine residues (Extended Data Fig. 1b), we treated cells with the general palmitoylation inhibitor 2-bromopalmitate. 2-bromopalmitate prevented palmitic acid-induced cAMP levels in both HPM-RHC and B16-RHC cells (Fig. 1b, Extended Data Fig. 1c), as well as in wild-type B16 cells and HPMS, with the effect of palmitic acid being dependent on α-MSH and stimulated by ultraviolet irradiation (UVR; Extended Data Fig. 1d, e).

Using acyl-biotin exchange (ABE) assay in HPM-RHC cells (Extended Data Fig. 1f), free cysteine thiol groups were irreversibly blocked by N-ethylmaleimide, palmitoylated cysteines exposed by hydroxylamine and biotinylated. Labelled proteins pulled down using streptavidin-coupled Dynabeads were analysed by mass spectrometry (Extended Data Fig. 1g) to reveal that MC1R is palmitoylated (Extended Data Fig. 1h), consistent with G-protein-coupled receptors such as MC1R<sup>7</sup> being palmitoylated during activation. Palmitoylation site prediction analysis<sup>21</sup> highlighted MC1R cysteines 78 and 315 as potential palmitoylation sites (Extended Data Fig. 1i–k).

Palmitoylation of both endogenous MC1R and exogenously expressed Flag–MC1R was confirmed using streptavidin to detect MC1R protein labelled using ABE, following immunoprecipitation from B16 cells and HPMS stimulated with α-MSH (Fig. 1c, d, Extended Data Fig. 2a, b). The Flag–MC1R(C78S) mutant, but not Flag–MC1R(C315S), was also palmitoylated (Fig. 1e, Extended Data Fig. 2c), indicating that C315 is the major MC1R palmitoylation site.

Using MC1R-reconstituted cells, we showed that, in the presence of α-MSH, both R151C and R160W MC1R variants exhibited reduced palmitoylation compared to wild type (Fig. 1f, Extended Data Fig. 2d). Moreover, a C315S mutation in the context of the R151C variant completely blocked MC1R(R151C) palmitoylation, indicating that Cys151 is not a neo-palmitoylation site (Extended Data Fig. 2e, f). Notably, UVR stimulated palmitoylation of endogenous MC1R and exogenously expressed Flag–MC1R (Fig. 1g, h,Extended Data Fig. 2g, h), but not the MC1R(C315S) mutant (Fig. 1i, Extended Data Fig. 2i), whereas UVR-induced palmitoylation of the R151C and R160W RHC variants was reduced compared to the wild-type protein (Fig. 1j, k, Extended Data Fig. 2j–m).

To identify which protein S-acyl transferases were responsible for MC1R modification, 21 haemagglutinin (HA)-tagged ZDHHC protein S-acyl transferases<sup>22</sup> were co-expressed with Flag–MC1R. Detectable MC1R palmitoylation was only observed following expression of ZDHHC2, 3, 7, 13 and 17 (Extended Data Fig. 3a), with ZDHHC13 being the most efficient (Fig. 2a). MC1R palmitoylation was substantially reduced in cells expressing a C456S mutant form of ZDHHC13 in which the enzymatic DQHC motif was mutated (Fig. 2b, Extended Data Fig. 3b). Silencing ZDHHC13 diminished UVR-stimulated palmitoylation of wild-type MC1R (Fig. 2c, Extended Data Fig. 3c), as well as the R151C RHC variant (Fig. 2d, Extended Data Fig. 3d), whereas ZDHHC13 overexpression activated MC1R(R151C) palmitoylation (Fig. 2e, Extended Data Fig. 3d–f). The MC1R(C315S) mutant was used as a negative control.

Interaction between ZDHHC13 and MC1R was detected by co-immunoprecipitation from α-MSH-treated HPMS and was enhanced by UVR (Fig. 2e), reflecting increased MC1R palmitoylation.
following UVB (Extended Data Fig. 3g, h). Interaction between the MC1R RHC R151C and R160W proteins and ZDHHC13 was also increased after UVB, but less so than observed with wild-type MC1R (Fig. 2f).

As ATR is a central effector of the UVB response, we investigated whether ATR-mediated phosphorylation of ZDHHC13 promoted MC1R palmitoylation following exposure to ultraviolet light. HPMs expressing HA–ZDHHC13 were irradiated with UVB, and after anti-HA immunoprecipitation, phosphorylation of ZDHHC13 was detected by specific phospho-SQ/TQ antibody. The results revealed that UVB treatment increased ZDHHC13 phosphorylation (Fig. 2g) and MC1R–ZDHHC13 interaction (Fig. 2h) that was substantially reduced in cells stably expressing ATR-targeting shRNA (Fig. 2i). Mutation of an evolutionarily conserved SQ site at Ser8 (Extended Data Fig. 3i) prevented UVR-stimulated ZDHHC13 phosphorylation (Extended Data Fig. 3j) and wild-type ATR, but not a kinase-dead mutant, robustly phosphorylated recombinant wild-type ZDHHC13, but not the S8A mutant (Extended Data Fig. 3k). Compared to wild-type ZDHHC13, the S8A mutant only weakly interacted with MC1R (Extended Data Fig. 3l) and failed to enhance MC1R palmitoylation upon UVB irradiation (Extended Data Fig. 3m). Collectively, these results suggest that ZDHHC13 phosphorylation by ATR following UVB irradiation promotes its interaction with MC1R to stimulate palmitoylation of MC1R.

To test whether MC1R palmitoylation regulates MC1R signalling, cAMP levels were measured after UVB irradiation and α-MSH stimulation of MC1R-reconstituted cells. Whereas wild-type MC1R significantly increased cAMP levels following UVV, the palmitoylation-deficient mutant C315S failed to do so, and RHC variants partially lost the ability to stimulate cAMP production (Extended Data Fig. 4a). The C315S mutant also failed to upregulate microphthalmia-associated transcription factor (Mitf) and tyrosinase (Tytr) mRNA expression (Extended Data Fig. 4b, c) downstream of MC1R signalling. The MC1R RHC variants only weakly activated Mitf and Tytr expression compared to wild type, strongly suggesting that palmitoylation is essential for MC1R signalling.

To examine the role of MC1R palmitoylation in α-MSH–MC1R-regulated DNA repair, endogenous MC1R-reconstituted HPMs, cyclobutane pyrimidine dimer (CPDs) and 6–4 photoproducts were measured following UVB exposure. Although wild-type MC1R promoted clearance of CPDs and 6–4 photoproducts, the palmitoylation-defective C315S mutant and the RHC variants failed to repair the lesions efficiently (Extended Data Fig. 4d).

Consistent with previous work, silencing MC1R augmented low-dose UVB-induced premature senescence that was bypassed by reintroduction of wild-type MC1R (Extended Data Fig. 4e). The R151C and R160W RHC variants also exhibited a defective senescence bypass phenotype, whereas the C315S mutant was ineffective (Extended Data Fig. 4f). Using engineered human immortal melanocytes (hTERT/ p53DD/CDK4(R24C) melanocytes) in colony formation and soft agar growth assays we found that BRAF(V600E)-mediated cellular transformation was suppressed by wild-type MC1R, but not the C315S mutant, whereas the R151C and R160W variants exhibited an intermediate phenotype (Fig. 3a, Extended Data Fig. 4g–k).

To examine its role in CAMP production, ZDHHC13 was silenced or overexpressed in MC1R-reconstituted B16 cells and HPMs, and cAMP were levels measured following UVB irradiation and stimulation.

Figure 1 | Palmitoylation of MC1R in melanocytes. a, b, RHC-HPMs exposed to α-MSH and UVB-irradiated were treated with BSA-conjugated fatty acids. b, Cells were exposed to palmitic acid ± 2-bromopalmitate (2-BP). cAMP were calculated by three independent experiments, shown as mean ± s.d. c, f, HPMs (c), HPMs expressing wild-type, mutant or variant Flag–MC1R (d–f) were incubated with α-MSH and processed for ABE analysis. g–k, HPMs (g), HPMs expressing wild-type, mutant or variant Flag–MC1R (h–k) were treated with α-MSH, irradiated with UVB, and collected for ABE analysis. Western blots shown are representative of three independent experiments. **P < 0.01, ***P < 0.001, unpaired Student’s t-test. For gel source data, see Supplementary Fig. 1.
Figure 2 | ZDHHC13 is a major MC1R palmitoyl acyltransferase. a, b, B16 cells co-expressing Flag–MC1R and HA–ZDHHCs (a) and HPMs expressing Flag–MC1R and ZDHHC13 wild-type or C456S mutant (b) were incubated with α-MSH and processed for ABE analysis. c–f, HPMs expressing ZDHHC13-targeting shRNAs (c), HPMs expressing Flag–MC1R together with ZDHHC13-targeting shRNAs and/or wild-type HA–ZDHHC13 (d), HPMs (e) and HPMs expressing Flag–MC1R with α-MSH. ZDHHC13 overexpression increased cAMP levels in cells expressing wild-type MC1R and also rescued the defect in cAMP production in cells expressing the R151C variant (Extended Data Fig. 5a). ZDHHC13 overexpression increased MITF expression in wild-type cells and rescued the defect in α-MSH and MC1R RHC-variant-induced MITF upregulation (Extended Data Fig. 5b).

The palmitoylation-defective C315S mutant was inactive irrespective of ZDHHC13 expression. ZDHHC13 overexpression enhanced UVR-induced DNA damage repair and rescued the defect in clearance of 6–4 photoproducts in melanocytes with the MC1R(R151C) variant, whereas ZDHHC13 depletion reduced repair (Extended Data Fig. 5c).

Similarly, ZDHHC13 overexpression protected against UVB-induced

Figure 3 | Activating MC1R palmitoylation rescues the defect of MC1R RHC variants. a, b, MC1R-depleted hTERT/p53DD/CDK4(R24C)/BRAF(V600E) melanocytes expressing Flag–MC1R (a) or cells further infected with ZDHHC13-targeting shRNAs and/or wild-type HA–ZDHHC13 virus (b) were pre-incubated with α-MSH, UVB irradiated and assayed for clonogenic survival. Results were calculated as mean ± s.d. from three independent experiments. c–e, Growth curves (c), dissected tumours (d) and tumour weight (e) for the xenograft experiments with indicated cells inoculated subcutaneously into each flank of nude mice (n = 8). Visible tumours were measured at the indicated days. Error bars represent ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001, unpaired Student’s t-test.
premature senescence in melanocytes expressing wild-type and MC1R(R151C), but not those expressing the C315S mutant, whereas shRNA depletion of ZDHHC13 enhanced senescence in cells expressing wild-type or MC1R(R151C) (Extended Data Fig. 5d).

Soft agar and colony formation assays using the human hTERT/p53DD/CDK4(R24C) melanocytes revealed that ZDHHC13 depletion increased transformation by BRAF(V600E) in the presence of wild-type MC1R, whereas enhanced transformation by the R151C mutant was suppressed by ZDHHC13 overexpression (Fig. 3b, Extended Data Fig. 5e). Elevated transformation in the presence of the C315S mutant was unaffected by ZDHHC13 overexpression/depletion. Subcutaneous xenograft tumour assays also indicated that ZDHHC13 overexpression inhibits MC1R(R151C)-augmented BRAF(V600E)-induced malignant transformation (Fig. 3c–e).

We next used MC1R loss-of-function (C57BL/6-Mc1r<sup>−/−</sup>) mice, backcrossed to C57BL/6 for more than 24 generations, to generate transgenic mice re-expressing melanocyte-specific wild-type, RHC-variant and C315S-mutant MC1R (Extended Data Fig. 6a). Unlike previous transgenic MC1R RHC mice developed by Healy et al., using bacterial artificial chromosomes, our Mc1r<sup>R151C</sup> variant offspring show a mosaic coat colour that could arise as a consequence of the promoter used, the integration site or the mouse strain background. Our other RHC variants exhibited a paler coat colour than the wild type (Fig. 4a, Extended Data Fig. 6b). All mice are at generation F26 or later with stable fur phenotypes and exhibit similar expression levels of the human transgenic Mc1r mRNAs (Extended Data Fig. 6c). The MC1R(R151C) variant mice also exhibit higher skin pheomelanin/eumelanin ratios than wild-type mice (Fig. 4b). Notably, the palmitoylation-deficient MC1R(C315S)-expressing mice had a similar coat colour and skin pheomelanin content to the Mc1r<sup>−/−</sup> mice (Fig. 4b, Extended Data Fig. 6b), with no significant difference in the number/distribution/location of melanocytes detected in mice with Mc1r<sup>R151C</sup>, Mc1r<sup>R151C</sup> and Mc1r<sup>C315S</sup> (Extended Data Fig. 6d, e).

Tyr-Cre-BRAF<sup>V600E</sup>-<sup>Cre-BRAF<sup>V600E</sup></sup>-<sup>Tyr-Cre-BRAF<sup>V600E</sup></sup>-<sup>Mc1r<sup>C315S</sup></sup> mice were next crossed to the Braf<sup>−/−</sup> knock-in mouse model in which melanocytes express BRAF(V600E) protein following Cre-mediated recombination. Tyr-Cre-BRAF<sup>V600E</sup>-<sup>Tyr-Cre-BRAF<sup>V600E</sup></sup>-<sup>Braf<sup>−/−</sup></sup>, Mc1r<sup>R151C</sup> mice, and BRAF(V600E) expression induced melanomagenesis. a

![Image](image1.png)

**Figure 4** MC1R palmitoylation controls melanomagenesis. a, C57BL/6 MC1R variant transgenic mice. b, Eumelanin and pheomelanin content of whole skin from C57BL/6 MC1R variant transgenic mice. Data shown represent the mean ± s.d. of three independent experiments. c, Melanoma-free survival. Tyr-Cre, n = 15; Tyr-Cre-BRAF(V600E)-Mc1r<sup>−/−</sup>, n = 23; Tyr-Cre-BRAF(V600E)-Mc1r<sup>R151C</sup>, n = 23; Tyr-Cre-BRAF(V600E)-Mc1r<sup>R151C</sup>Mc1r<sup>R151C</sup>, n = 26; Tyr-Cre-BRAF(V600E)-Mc1r<sup>C315S</sup>Mc1r<sup>C315S</sup>, n = 20. By log-rank test, P = 0.0001 (e/e, +/+)/, P = 0.0179 (e/e, R151C), P = 0.8943 (e/e, C315S), P = 0.0232 (+/– –, R151C), P = 0.0001 (+/+, C315S), P = 0.0233 (R151C, C315S), P = 0.3711 (Mc1r<sup>R151C</sup> and Mc1r<sup>C315S</sup>–Palm-B). *P < 0.05, **P < 0.01, ***P < 0.001, unpaired Student’s t-test.

Tyr-Cre-BRAF<sup>V600E</sup>CA mice were next crossed to the Tyr-Cre-BRAF<sup>V600E</sup>-<sup>Tyr-Cre-BRAF<sup>V600E</sup></sup>-<sup>Mc1r<sup>C315S</sup></sup> mice, and BRAF(V600E) expression induced by tamoxifen administration. After UVB irradiation each week for four weeks (Extended Data Fig. 5f), melanomas developed early and frequently in MC1R loss-of-function (e/e) or palmitoylation-defective MC1R transgenic (C315S) mice, much later with wild-type MC1R mice, and at an intermediate time with the MC1R(R151C) variant (Fig. 4c).

All of the diagnosed melanomas displayed similar morphological and histologic features (Extended Data Fig. 6g).

To explore whether MC1R palmitoylation is dynamic, we removed -MSH and Palm-B before UVB irradiation and assayed for clonogenic survival. Results were calculated as mean ± s.d. from three independent experiments. e–g, Growth curves (e), dissected tumours (f) and tumour weight (g) for subcutaneous xenograft experiments in nude mice (n = 10) using indicated cells. Error bars represent ± s.e.m. b, Melanoma-free survival of Tyr-Cre-BRAF(V600E)-Mc1r<sup>R151C</sup>, n = 20; Tyr-Cre-BRAF(V600E)-Mc1r<sup>R151C</sup> + Palm-B, n = 20; Tyr-Cre-BRAF(V600E)-Mc1r<sup>R151C</sup>Mc1r<sup>R151C</sup>, n = 17; Tyr-Cre-BRAF(V600E)-Mc1r<sup>R151C</sup>Mc1r<sup>R151C</sup> + Palm-B, n = 18. By log-rank test, P = 0.0241 (R151C and R151C + Palm-B), P = 0.3711 (Mc1r<sup>R151C</sup> and Mc1r<sup>C315S</sup> + Palm-B). *P < 0.05, **P < 0.01, ***P < 0.001, unpaired Student’s t-test.
from the R151C variant to levels comparable to those observed from α-MSH-stimulated wild-type MC1R in the absence of Palm-B (Extended Data Fig. 7c). Signalling downstream of the R151C variant, as well as wild-type MC1R, was functional as both MIF and Tyr mRNA expression were upregulated by Palm-B (Extended Data Fig. 7d, e). Palm-B also accelerated clearance of UV-induced 6-4 photoprotocols in melanocytes with the MC1R(R151C) variant (Extended Data Fig. 7f). By performing ABE on extracts from mouse skin irradiated with UVB, we confirmed that Palm-B administration to mice increased MC1R palmitoylation in Mc1R\textsuperscript{R151C} and Mc1r\textsuperscript{+/+} in vivo (Extended Data Fig. 7g). Following UVB treatment, both CDP and 6-4 photoprototype DNA lesions were significantly higher in R151C mice than in wild-type mice, with Palm-B reducing the levels of damage detected in both (Extended Data Fig. 7h, i). Notably, Palm-B also repressed low-dose UVB-induced premature senescence in HPMs or B16 cells reconstituted with the MC1R(R151C) variant (Extended Data Fig. 7j). In human immortal hTERT/p53DD/CDK4(R24C) melanocytes, Palm-B largely reversed the increased colony formation observed using the R151C variant (Fig. 4d, Extended Data Fig. 7k) and in xenograft studies, Palm-B inhibited MC1R(R151C)-augmented BREF(V600E) melanogenesis (Fig. 4e–g). Importantly, Palm-B had no effect in any assay using the C315S mutant.

Finally, to confirm the preventive effect of Palm-B in melanoma genesis, the transgenic mice re-expressing melanocyte-specific MC1R(R151C) (Tyr-Cre-BRAF\textsuperscript{CA, Mc1R\textsuperscript{R151C}}) were UVB-irradiated each week for four weeks, with 10 mg per kg body weight Palm-B injected intraperitonealat ence before irradiation. Although no strong side effects were observed, in Palm-B-treated mice melanogenesis was substantially delayed and the incidences reduced (Fig. 4h). Similar results were obtained using Palm-B and the MC1R(R160W) variant (Extended Data Fig. 8).

Collectively, our results highlight a central role for MC1R palmitoylation in protecting against melanoma, and suggest that rescuing MC1R palmitoylation by upregulation of ZDHHC13 or inhibition of depalmitoylation might be a potential clinical preventive strategy for melanoma in individuals carrying red hair colour variants.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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METHODS

Data reporting. Sample sizes were determined based on previous model-specific experience and no statistical methods were used to predetermine sample size. Mice were randomly assigned to treatment groups. The investigators were blinded to allocation during experiments and outcome assessment whenever possible.

Cell lines, animals and UV exposure. Cell lines and ultraviolet (UV) exposure were as described previously24–26. B16 and HEK293 cell lines were purchased from ATCC, authenticated by ATCC, and mycoplasma negative. Briefly, cells were washed three times by PBS to remove UV irradiation. For the UV exposure experiments, cells were exposed to ultraviolet radiation in the Stratalinker UV chamber (Stratagen) with UVB bulbs (UVL LLC). UV emittance was measured with the use of a UV photometer (UVL LLC). Adherent cells were irradiated through a small volume of PBS at a dose of 100 mJ m\(^{-2}\). A UVB dose of 100 mJ m\(^{-2}\) is equivalent to one standard erythema dose of UVB (SED), commonly used as a measurement of sunlight. As a reference, the ambient exposure over an entire sunny summer day in Europe (Switzerland) is approximately 30–40 SED31. Human primary melanocytes were isolated from normal discarded foreskins as described before32,33 and were cultured (Switzerland) is approximately 30–40 SED31. Human primary melanocytes were isolated from normal discarded foreskins as described before32,33 and were cultured (Switzerland) is approximately 30–40 SED31. Human primary melanocytes were isolated from normal discarded foreskins as described before32,33 and were cultured in vitro. Cell lines, animals and UV exposure.

Mice were randomly assigned to treatment groups. The investigators were blinded to assignment within staining solution (0.3% Coomassie blue, 45% methanol, 10% glacial acetic acid). The expression levels of MC1R region (−1,200 bp forward 5′-GGCATGGTATCAAACTCCAAGA-3′; reverse 5′-ACACTTAAAGCGCGTGCACCGC-3′) were used in assays. pcDNA3-3′-ATR wild-type (Addgene, #31611) and pcDNA3-3′-ATR Kinase dead (Addgene, #31612) were gifts from A. Sancar34.

Immunoblot analysis. High Sensitivity Streptavidin–HRP (21130) and Dynabeads MyOne Streptavidin C1 (65001) were purchased from Thermo Fisher Scientific Inc. Anti-ZDHHC13 antibody (ab28759) was purchased from Abcam. Anti-MC1R antibody (N-19) (sc-6875) was purchased from Santa Cruz Biotechnologies, Inc. Monoclonal anti-β-actin – peroxidase antibody (AC15), monoclonal anti-Flag M2–peroxidase antibody (A4592), monoclonal anti-HA–peroxidase antibody (H6533), anti-Flag agarose beads (A2202), anti-HA agarose beads (A2095), peroxidase-conjugated anti-mouse secondary antibody (A4416) and peroxidase-conjugated anti-rabbit secondary antibody (A-4914) were purchased from Sigma-Aldrich. All western blots shown are representatives of three independent experiments.

Co-immunoprecipitation. Briefly, cells were washed twice with ice-cold phosphate-buffered saline and lysed in lysis buffer containing 50 mM Tris pH 7.4; 1% Triton X-100; 0.5 mM EDTA; 0.5 mM EGTA; 150 mM NaCl; 10% glycerol; 1 mM phenylmethylsulfonyl fluoride, complete protease inhibitor cocktail (Roche) on ice for 30 min. The supernatant was collected after centrifugation at 15,000g for 15 min at 4°C, and 500μg of total cell lysate was treated by DNase (15 U ml\(^{-1}\), Pierce), precleared by 20μl Protein G Agarose Beads (Thermo Fisher Scientific Inc.) and then incubated with primary antibodies overnight at 4°C or Flag/HA beads (Sigma-Aldrich) for 2 h. 20μl Protein G Agarose Beads was added into the samples with rotation at 4°C for 1 h. After three washes with 1 ml of lysis buffer, the bound proteins were released by boiling in 30μl of SDS loading buffer and detected as described above.

Acyt-3′-biotin exchange palmitoylation assay. Briefly, cells were lysed by buffer (1% IGEPLAC-630, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol) directly for 2 h. 20μl Protein G Agarose beads (Thermo Fisher Scientific Inc.) were purified on MC1R protein was divided into two groups, and one treated with lysis buffer + 1 μM hydroxyamine (Sigma-Aldrich) for 1 h at room temperature. Finally, the beads were gently washed with lysis buffer pH 6.2 and incubated with lysis buffer pH 6.2 + 2μl M biotin-BMCC (Thermo Fisher Scientific Inc.) for 1 h at 4°C. Proteins were washed three times and used for immunoblot analysis.

Mass spectrometry. Mass spectrometry protein identification was performed by the Taplin Biological Mass Spectrometry Facility, Harvard Medical School. Protein samples were mixed with sample buffer and heated at 100°C for 5 min, then loaded to SDS–PAGE for separation. When running was finished, gel was immersed in 50 mM N-ethylmaleimide (Sigma–Aldrich) and endogenous MC1R or FLAG-MC1R was then purified by 6-plex CNBr antibodies and beads. Purified MC1R protein was divided into two groups, and one treated with lysis buffer + 1 μM hydroxyamine (Sigma-Aldrich) for 1 h at room temperature. Finally, the beads were gently washed with lysis buffer pH 6.2 and incubated with lysis buffer pH 6.2 + 2μl M biotin-BMCC (Thermo Fisher Scientific Inc.) for 1 h at 4°C. Proteins were washed three times and used for immunoblot analysis.

Immunosassay. DNA damage immunosassay was performed using the anti-CBP (MC-062) and anti-6-4-photoproducts (KTM-50) antibodies. Briefly, the heat-denatured genomic DNA were coated onto the microplate wells with Pierce DNA Coating Solution (17250) (Thermo Fisher Scientific Inc.). After washing and blocking by PBS with the purified anti-rabbit IgG Ab of 2 μg ml\(^{-1}\) the mouse monoclonal anti-rabbit IgG (MC-062) and anti-6-4-photoproducts (KTM-50) antibodies were diluted 1:1,000 in blocking buffer and added to each well. Optical density at 405 nm was determined. Intracellular ACP levels were measured by ACP Direct Immunoblot Kit (ab65355) (Abcam) following the manufacturer’s protocol, optical density at 450 nm was determined. For CAPM measurement with fatty acid treatment, fatty acids were dissolved in 100% ethanol (250 mM) mixed with 25 mM BSA.

Other mutants were generated by site-directed mutagenesis using the QuickChange II Site-Directed Mutagenesis kit (Agilent). To generate stable knockdown of MC1R or ZDHHC13 in B16 and HPMS, mouse or human specific short hairpin RNAs in pLKO.1 against MC1R or ZDHHC13 were co-transfected with psPAX2 (Addgene #12260) and pmD2-G (Addgene, #12295) in HEK293–FT (ATCC) using Lipofectamine 3000 (Thermo Fisher Scientific Inc.). Lentiviruses were collected after 48 h, and then infected cells for 24 h in the presence of polybrene (8μg ml\(^{-1}\)) and the infected cells were selected with puromycin (2μg ml\(^{-1}\)). To generate stable knockdown of MC1R variants or HA-ZDHHC13, HEK293T cells were co-transfected with MC1R variants or HA–ZDHHC13 in pQXClIP, VSVG-G and pUVMC (Addgene, #8449) plasmids using Lipofectamine 3000. Retroviruses were harvested after 48 h and cells were infected with retroviruses in the presence of polybrene (8μg ml\(^{-1}\)). After 24 h, cells were selected with puromycin (2μg ml\(^{-1}\)). shRNA constructs targeting human MC1R (RH54353–EG4157), mouse MC1R (RMM4534–EG1799), human ZDHHC13 (RH54533–EG5403), mouse ZDHHC13 (RMM4534–EG243983) and human ATR (RH5433–EG545) were purchased from OpenBiosystems. The most efficient knockdown cell lines with shMC1R (target sequence: 5′-AAAGGGCTTCTTGAAGACGCT-3′) or shMC1R (target sequence: 5′-AAAGGGCTTCTTGAAGACGCT-3′) were used in assays. pcDNA3-3′-ATR wild-type (Addgene, #31611) and pcDNA3-3′-ATR Kinase dead (Addgene, #31612) were gifts from A. Sancar34.
and added to serum-free DMEM/medium 254 at a final concentration of 500 μM. Cells were serum-starved for 6 h. For the last 30 min, cells were incubated with 1 μM α-MSH, followed by 100 J m⁻² UVB treatment. Lastly cells were treated with indicated BSA-conjugated fatty acid medium for 3 h with or without 2-BP (25 μM). All results are calculated by three independent experiments.

**Quantitative PCR.** The cDNA (40 ng) was used for quantitative PCR amplification with SYBR green PCR master mix (Thermo Fisher Scientific Inc.). The relative levels of expression of genes were normalized according to those of GAPDH. Quantitative PCR (qPCR) data were calculated using the comparative Cₘ method. All quantitative PCR were performed in triplicate, and three independent RNA samples were assayed for each time point.

**Immunohistochemistry.** Mouse melanomas were fixed in 10% formalin solution at 4°C overnight, paraflin-embedded and then cut into 5-μm-thick sections (Dermpath core facility, Boston University). Sections were then deparaffinized, rehydrated, and stained with anti-S-100 (Dako North America, Inc.) and counterstained with haematoxylin. Briefly, for antigen retrieval, sections were heated in a boiling water bath in 10 mM sodium citrate buffer (pH 6.0) for 20 min before immunostaining. Nonspecific staining was blocked by pre-incubation with Tris buffered saline (TBS)/0.1% Tween-20/5% normal goat serum (Jackson ImmunoResearch) for 1 h at room temperature. Tissue sections were incubated with the primary antibodies at 4°C overnight, and were subsequently incubated with secondary antibodies and detected with DAB substrate (Dako EnVision System HRP, Dako) following manufacturer’s instructions. Coverslips were mounted onto glass slides with permanent mounting medium. All images were taken with an Olympus Inverted microscope (Cellular imaging core, Boston University).

**Pigment measurement.** Eumelanin and pheomelanin were quantified by HPLC based on the level of pyrrole-2,3,5-tricarboxylic acid (PTCA) by alkaline hydrogen peroxide oxidation of eumelanin and 4-amino-3-hydroxyphenylalanine (4-AHP) by hydriodic acid reductive hydrolysis of pheomelanin, respectively. Final results were determined by a conversion as described²⁵ (eumelanin = 45 × PTCA, pheomelanin = 9 × 4-AHP). All results are calculated by three independent experiments.

**Clonogenic survival and soft agar assays.** The clonogenic survival and soft agar assays for hTERT/p53DD/CDK4(R24C)/BRAF(V600E) melanocytes were performed as described previously²⁶. Briefly, for the melanocyte growth promoting withdrawal experiments, hTERT/p53DD/CDK4(R24C)/BRAF(V600E) melanocytes were treated with 201 J m⁻² UVB in the presence of 1 μM α-MSH before plating into 6-well plate at 1,000 cells per well, and then subject to clonogenic survival assays 15 days after UVR. Crystal violet was used to stain colonies. DMEM media was used with 10% FBS and penicillin–streptomycin–glutamine. For soft agar assays, cells (10,000 per well) were seeded in 0.5% low-melting-point agarose in DMEM with 10% FBS, layered onto 0.8% agarose in DMEM/10% FBS. The plates were kept in the cell culture incubator for 30 days after which the colonies >50 μm were counted under a light microscope. All results are calculated by three independent experiments.

**In vivo tumorigenesis assay.** In vivo tumorigenesis assay of hTERT/p53DD/CDK4(R24C)/BRAF(V600E) melanocytes was performed as described previously²⁶. Briefly, 3 × 10⁶ melanocytes were mixed with Matrigel (1:1) and injected subcutaneously into the flanks of male nude mice. Tumour size was measured every 3 days with a caliper, and the tumour volume was determined. Three weeks after inoculation, the tumours were dissected to measure their weights.

**Statistical analyses.** All quantitative data were presented as the mean ± s.d. or s.e.m. of at least three independent experiments by Student’s t-test for between group differences and analysis of variance for comparisons among three or more groups (*P < 0.05, **P < 0.01, ***P < 0.001).

**Data availability.** All data that support the findings of this study are available on reasonable request from the corresponding author. The contribution authors declare that all relevant data are included in the paper and its supplementary information files.

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35. Wakamatsu, K. & Ito, S. Advanced chemical methods in melanin determination. Pigment Cell Res. 15, 174–183 (2002).
Extended Data Figure 1 | MC1R is palmitoylated. a, B16-RHC cells exposed to α-MSH and UVB-irradiated were treated with BSA-conjugated fatty acids. b, A schematic showing the general process of protein palmitoylation. c, B16-RHC cells were exposed to palmitic acid ± 2-BP. d, e, MC1R RHC-variant or wild-type B16 melanoma cells (d) and MC1R RHC-variant or wild-type HPMs (e) were treated as indicated in Fig. 1a or ± 2-BP. cAMP were calculated by three independent experiments, shown as mean ± s.d. **P < 0.01, ***P < 0.001, unpaired Student's t-test. f, A schematic showing the general process of the acyl-biotin exchange (ABE) palmitoylation assay. g, Flowchart of palmitoylated protein identification. NEM, N-ethylmaleimide; HAM, hydroxylamine. h, The peptide spectral counts of MC1R from Fig. 1c. i, Palmitoylation site prediction (NBA-palm) analysis of MC1R. The prediction shows two possible sites of MC1R palmitoylation. j, A schematic illustration showing the palmitoylation site at MC1R(C315) predicted by palmitoylation site prediction analysis and PEP-FOLD3, and a schematic showing the conserved C-terminal domain of MC1R. k, Membrane topology of MC1R with indicated RHC, non-RHC and palmitoylation site mutants.
Extended Data Figure 2 | Palmitoylation of MC1R in melanocytes.

a–f, B16 cells (a), B16 cells expressing wild-type, mutant or variant Flag–MC1R (b–e), HPMs expressing wild-type, mutant or variant Flag–MC1R as indicated (f) were incubated with α-MSH and processed for ABE analysis. g–m, B16 cells (g), B16 cells expressing wild-type, mutant or variant Flag–MC1R (h–k), HPMs expressing wild-type, mutant or variant Flag–MC1R (l) were treated with α-MSH, irradiated with UVB, and collected for ABE analysis. Western blots shown are representative of three independent experiments. For gel source data, see Supplementary Fig. 1.
Extended Data Figure 3 | ZDHHC13 is a major protein S-acyl transferase of MC1R. a, HEK293 cells co-expressing Flag–MC1R and HA–ZDHHCs were collected for ABE analysis. b, B16 cells infected with Flag–MC1R and the indicated wild-type ZDHHC13- or mutant C456S-encoding retroviral constructs were treated with α-MSH and processed for ABE analysis. c, B16 cells expressing Zdhhc13-targeting shRNAs were treated with α-MSH, irradiated with UVB, and collected for ABE analysis. d, B16 cells expressing Flag–MC1R together with Zdhhc13-targeting shRNAs and/or wild-type HA–ZDHHC13 were treated with α-MSH, irradiated with UVB, and collected for ABE analysis. e, f, B16 cells or HPMs with stable MC1R depletion were infected with the Flag–MC1R(R151C)- or MC1R(R151C/C315S)-double-mutant-encoding retroviral constructs, then cells were infected with wild-type HA–ZDHHC13 expressing virus. After infection, cells were treated with α-MSH, irradiated with UVB, and collected for ABE analysis. g, h, HPMs pre-incubated with α-MSH followed by UVB irradiation were collected for ABE (g) or immunoprecipitation (h) analysis. i, A schematic showing the conserved SQ motif of ZDHHC13. j, HPMs expressing wild-type ZDHHC13 or ZDHHC13(S8A) mutant were irradiated with UVB and collected for immunoprecipitation and immunoblot analysis. k, Wild-type Flag–ATR or the kinase-dead (KD) Flag–ATR mutant transfected HEK293 cells were irradiated before immunoprecipitation with Flag beads. The immunoprecipitated wild-type ZDHHC13 or S8A mutant were then incubated with immunoprecipitated wild-type ATR or the kinase-dead ATR mutant in kinase buffer. After reaction, proteins were collected for IB analysis. l, HPMs expressing wild-type ZDHHC13 or ZDHHC13(S8A) mutant and Flag–MC1R were irradiated with UVB and collected for immunoprecipitation analysis. m, HPMs with stable depletion of ZDHHC13 by shRNA were infected with the indicated ZDHHC13- and Flag–MC1R-encoding retroviral constructs. Cells were then treated with α-MSH, irradiated with UVB and processed for ABE analysis. Western blots shown are representative of three independent experiments. For gel source data, see Supplementary Fig. 1.
Extended Data Figure 4 | Palmitoylation is essential for MC1R function. a–c, B16 cells and HPMs expressing the indicated Flag–MC1R were treated with α-MSH and irradiated with UVB. Cells were collected for cAMP immunoassay (a) or quantitative PCR (qPCR) by specific primers targeting mouse/human MITF or TYR (b, c). Data are represented as mean ± s.d. Three independent experiments were quantified.

d, HPMs expressing indicated Flag–MC1R were treated with α-MSH and irradiated with UVB. Genomic DNA was extracted at the different time points as indicated, and the photoproducts were detected by ELISA. Three independent experiments were measured and shown as mean ± s.d.

e, f, B16 and HPMs with stable depletion of MC1R by shRNA (e), or B16 cells and HPMs expressing indicated Flag–MC1R (f) were pre-treated with α-MSH for 30 min followed by 25 J m⁻² UVB irradiation. Cells were subjected to SA-β-gal staining assay 7 days after UVR. Data are represented as mean ± s.d. from three independent experiments.

g–k, hTERT/p53DD/CDK4(R24C)/BRAF(V600E) melanocytes with stable depletion of MC1R by shRNA (g–i) or expressing the indicated Flag–MC1R (j, k) were pre-incubated with 1 μM α-MSH for 30 min before being irradiated with 20 J m⁻² UVB. Cell lysates were collected for immunoblot analysis (g, j), or cells were seeded for clonogenic survival (h), and soft agar assays (i, k). Results were calculated as mean ± s.d. from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, unpaired Student’s t-test. Western blots shown are representative of three independent experiments. For gel source data, see Supplementary Fig. 1.
Extended Data Figure 5 | Activating MC1R palmitoylation rescues the defect of MC1R RHC variants. a, B16 cells and HPMs expressing Flag–MC1R together with Zdhhc13-targeting shRNAs and/or wild-type HA–ZDHHC13 were treated with α-MSH and irradiated with UVB. Cells were collected for cAMP immunoassay (a) or quantitative PCR (qPCR) by specific primers targeting mouse/human MITF (b). Data are represented as mean ± s.d. Three independent experiments were quantified. c, HPMs expressing Flag–MC1R together with ZDHHC13-targeting shRNAs and/or wild-type HA–ZDHHC13 were treated with α-MSH, irradiated with UVB. Genomic DNA were extracted at the different time points as indicated and photoproducts were detected by ELISA. Three independent experiments were measured and data are represented as mean ± s.d. d, B16 cells and HPMs expressing Flag–MC1R together with Zdhhc13-targeting shRNAs and/or wild-type HA–ZDHHC13 were pre-treated with 1 μM α-MSH for 30 min followed by 25 J m⁻² UVB irradiation. Cells were subjected to SA-β-gal staining assay 7 days after UVR. Data are represented as mean ± s.d. from three independent experiments. e, The cells generated as indicated were subjected for soft agar assay and relative colony numbers were plotted as mean ± s.d. from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, unpaired Student’s t-test.
Extended Data Figure 6 | MC1R variant and mutant transgenic mice. a, Schematic diagrams of MC1R variant constructs. Transgenic mice were designed to express melanocyte-specific MC1R variants or mutants (controlled by the Tyr enhancer/promoter). b, C57BL/6 MC1R variant or mutant transgenic mice. c, Human transgene content in transgenic and control mice. Results were calculated as mean ± s.d. from three independent experiments. d, Whole skins from C57BL/6 MC1R variant transgenic mice (8–12 weeks) were collected and stained with Dct antibody. Melanocytes were then isolated and quantified by FACS sorting. Results were calculated as mean ± s.d. from three independent experiments. e, Frozen sections of skins from C57BL/6 MC1R variant transgenic mice (8–12 weeks) were stained with Dct antibody. The positive staining represents melanocytes. f, Illustrations for UVB-induced melanoma development in Tyr-Cre-BRAFV600E-MC1R variant mice. g, H&E staining of histological sections and immunohistochemistry staining of S100 of representative cutaneous melanomas. Genotypes are indicated.
Extended Data Figure 7 | Palm-B activates MC1R palmitoylation and rescues the defect of MC1R RHC variants. a, HPMs expressing Flag–MC1R were incubated with α-MSH for 3.5 h. The medium was replaced with fresh medium containing vehicle or 1 μM Palm-B, and cells were treated at the indicated times. Cells were then collected for ABE analysis. b–f, B16 cells or HPMs expressing the indicated Flag–MC1R were treated with α-MSH and Palm-B and irradiated with UVB. Cells were processed by ABE analysis (b), cAMP immunoassay (c), qPCR (d, e) and photoproducts were measurement (f). Three independent experiments were quantified. Data are represented as mean ± s.d. g, C57BL/6 mice or C57BL/6-Mc1r<sup>Δ<sup>151C</sup></sup>-transgenic mouse were given a 10 mg kg<sup>−1</sup> Palm-B or vehicle injection intraperitoneally 3 h before UVB irradiation (500 J m<sup>−2</sup>). 3 h after UVB, whole skins were collected and the lysates were subjected for ABE analysis. h, i, C57BL/6 mice or C57BL/6-Mc1r<sup>Δ<sup>151C</sup></sup>-transgenic mouse were injected intraperitoneally with 10 mg kg<sup>−1</sup> Palm-B 3 h before UVB irradiation (500 J m<sup>−2</sup>). Melanocytes were isolated by flow cytometry, then DNA was extracted and subjected to ELISA 3 h after UVB irradiation. Results were calculated as mean ± s.d. from three independent experiments. j, B16 cells and HPMs expressing the indicated Flag–MC1R were pre-treated with α-MSH and Palm-B for 30 min followed by 25 J m<sup>−2</sup> UVB irradiation. Cells were subjected to SA-β-gal staining assay 7 days after UVR. Data are represented as mean ± s.d. from three independent experiments. k, The cells generated as indicated were subjected for soft agar assay and relative colony numbers were plotted as mean ± s.d. from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, unpaired Student's t-test. Western blots shown are representative of three independent experiments. For gel source data, see Supplementary Fig. 1.
Extended Data Figure 8 | Palm-B rescues the defect of MC1R R160W variant. a–d, B16 cells or HPMs expressing the indicated Flag–MC1R were treated with α-MSH and Palm-B and irradiated with UVB. Cells were processed by ABE analysis (a), cAMP immunoassay (b), qPCR (c) and photoproducts were measured (d) (three independent experiments). Data are represented as mean ± s.d. e, B16 cells and HPMs expressing the indicated Flag–MC1R were pre-treated with 1 μM α-MSH and 1 μM Palm-B for 30 min followed by 25 J m⁻² UVB irradiation. Cells were subjected to SA-β-gal staining assay 7 days after UVR. Data are represented as mean ± s.d. from three independent experiments. *P < 0.01, **P < 0.001, unpaired Student’s t-test. Western blots shown are representative of three independent experiments. For gel source data, see Supplementary Fig. 1.

f, g, MC1R-depleted TERT/p53DD/CDK4(R24C)/BRAF(V600E) melanocytes expressing indicated Flag–MC1R were pre-incubated with 1 μM α-MSH and 1 μM Palm-B for 30 min before being irradiated with 20 J m⁻² UVB, and then subjected to clonogenic survival (f) and soft agar assay (g). Results were shown as mean ± s.d. from three independent experiments. **P < 0.01, ***P < 0.001, unpaired Student’s t-test. Western blots shown are representative of three independent experiments. For gel source data, see Supplementary Fig. 1.
Experimental design

1. Sample size
   Describe how sample size was determined.
   
   All sample size and details are included in the manuscript. This is a tumor development experiment, tumor was diagnosed or not, no statistical analysis is required.

2. Data exclusions
   Describe any data exclusions.
   
   No data were excluded.

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   
   All attempts at replications were successful.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   
   All samples were randomly allocated into experimental groups.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   
   The investigators were blinded to group allocation during data collection and analysis.

   Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   n/a Confirmed

   - The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)

   - A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

   - A statement indicating how many times each experiment was replicated

   - The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)

   - A description of any assumptions or corrections, such as an adjustment for multiple comparisons

   - The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted

   - A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)

   - Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

Microsoft excel, GraphPad Prism

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

No unique material were used.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

All antibodies' information is provided in the Method section.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

All cell lines were from ATCC.

b. Describe the method of cell line authentication used.

All cell lines were authenticated by ATCC.

c. Report whether the cell lines were tested for mycoplasma contamination.

All cell lines were negative for mycoplasma contamination.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines were used.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

All mouse study information is provided in the manuscript following the ARRIVE guidelines.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

Our study does not involve any human research participants.
Flow Cytometry Reporting Summary

Data presentation

For all flow cytometry data, confirm that:

1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
3. All plots are contour plots with outliers or pseudocolor plots.
4. A numerical value for number of cells or percentage (with statistics) is provided.

Methodological details

5. Describe the sample preparation. Skin sheets without subcutis were treated with collagenase P and dispase. Single-cell suspensions were then fixed and permeabilized for staining.

6. Identify the instrument used for data collection. BD LSRII

7. Describe the software used to collect and analyze the flow cytometry data. BD FACS Diva 6.2.1, FlowJo

8. Describe the abundance of the relevant cell populations within post-sort fractions. All details were included in the manuscript, the abundance were determined by the software.

9. Describe the gating strategy used. The IgG isotype control was used as a negative control to gate the positive staining cells

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ☑