# Enhancement of DNA repair using topical T4 endonuclease V does not inhibit melanoma formation in *Cdk4*$^{R24C/R24C}$/*Tyr-Nras*$^{Q61K}$ mice following neonatal UVR

Elke Hacker[1, 2], H. Konrad Muller[3,4], Nicholas Hayward[1], Paul Fahey[1] and Graeme Walker[1].

[1]Genetics & Population Health Division, Queensland Institute of Medical Research, Brisbane, Queensland, Australia

[2]AusSun Research Laboratories, Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, Queensland, Australia

[3]University of Tasmania, School of Medicine, Hobart, Tasmania, Australia

[4]Royal Brisbane and Women's Hospital, Pathology Queensland, Brisbane, Queensland, Australia

Running Title: Enhanced DNA repair did not prevent melanoma

Corresponding author: Dr Elke Hacker,
Queensland Institute of Medical Research
300 Herston Rd
Herston
QLD 4029, Australia

Tel: 61-7-3362 0308
Fax: 61-7-3845 3508
E-mail: Elke.Hacker@qimr.edu.au

Total Word Count = 5034

**Summary**

To further investigate the use of DNA repair-enhancing agents for skin cancer prevention, we treated *Cdk4*$^{R24C/R24C}$/*Nras*$^{Q61K}$ mice topically with the T4 endonuclease V DNA repair enzyme (known as Dimericine) immediately prior to neonatal ultraviolet radiation (UVR) exposure, which has a powerful effect in exacerbating melanoma development in the mouse model. Dimericine has been shown to reduce the incidence of basal-cell and squamous cell carcinoma. Unexpectedly, we saw no difference in penetrance or age of onset of melanoma after neonatal UVR between Dimericine-treated and control animals, although the drug reduced DNA damage and cellular proliferation in the skin. Interestingly, epidermal melanocytes removed cyclobutane pyrimidine dimers (CPDs) more efficiently than surrounding keratinocytes. Our study indicates that neonatal UVR-initiated melanomas may be driven by mechanisms other than solely that of a large CPD load and/or their inefficient repair. This is further suggestive of different mechanisms by which UVR may enhance the transformation of keratinocytes and melanocytes.

Keywords- melanoma, DNA repair, ultraviolet radiation, Dimericine, melanocyte activation

**Significance**

The results of Dimericine treatment in basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) have led to much discussion about the potential use of DNA repair-enhancing agents in skin cancer prevention. The development of "super-sunscreens" that in addition to shielding the skin from ultraviolet radiation (UVR) also contain active compounds that function to enhance DNA repair of damaged "sunburnt" cells, is underway. We have used the $Cdk4^{R24C/R24C}/Nras^{Q61K}$ mouse model to test how enhanced repair of DNA following UVR will impact on the development of melanoma and found that it had no effect. Our findings argue that DNA repair enhancement may not be helpful, at least for some types of melanoma, and are suggestive of potentially different mechanisms of UVR carcinogenesis between melanocytes and keratinocytes.

**Introduction**

Exposure to solar ultraviolet radiation (UVR) is the main environmental risk factor for melanoma development. The precise mechanism by which sunlight induces melanoma is still unclear, however sun exposure can cause the generation of various types of cellular and DNA damage, immune suppression, and inflammation (Abdel-Malek et al., 2008). The importance of effective DNA repair is highlighted in patients with the rare genetic disease xeroderma pigmentosum (XP), in which a defect in DNA repair is associated with the development of melanoma and other skin cancers much earlier in life and with increased risk compared to the general population (Kraemer et al., 1994). Tanaka et al (1975)- first showed that treating XP cells in culture with the bacterial DNA excision repair enzyme T4 endonuclease V reduced DNA damage induced by UVR. Further clinical studies have demonstrated that the repair enzyme encapsulated in a liposomal delivery vehicle can be transported to intracellular compartments of human and mouse skin (Wolf et al., 2000, Yarosh et al., 1992). The hydrophobicity afforded by the liposomal complex allows for the penetration of the enzyme through the stratum corneum, delivering T4 endonuclease V to epidermal keratinocytes (Yarosh et al., 1994). The T4 endonuclease V protein lacks a nuclear localization sequence (NLS), entering the nucleus through passive diffusion after initially accumulating in the cytoplasm of cells (Yarosh et al., 1994).

Once inside the nucleus, T4 endonuclease V, a T4 pyrimidine dimer glycosylase, acts on cyclobutane pyrimidine dimers (CPDs). The enzyme scans through DNA, specifically binding to any CPDs. Upon recognition of a CPD, it bends the duplex DNA and rotates the nucleotide base complementary to the 5' residue of the CPD, resulting in a cleavage event and base excision of the dimer (Morikawa and Shirakawa, 2000). It is likely that an exonuclease degrades the area surrounding the single-strand break, and a polymerase fills in the gap, however the precise mechanism downstream of T4 endonuclease V is unclear.

Topical application of the T4 endonuclease V liposome lotion (known as Dimericine) accelerated the removal of UVR-induced DNA damage in XP patients and reduced the rate of actinic keratoses and basal-cell carcinoma (BCC) (Yarosh and Klein, 1996, Yarosh et al., 2001). Dimericine has also been shown to reduce the incidence of squamous cell carcinoma (SCC) in UVR exposed SKH-1 hairless mice (Yarosh et al., 1992). We established a mouse model of melanoma using transgenic animals carrying a melanocyte-specific Nras activating mutation and the cell cycle-deregulating *Cdk4 R24C* mutation (Hacker et al., 2006). Exposing these mice to a single neonatal dose of UVR greatly increased melanoma penetrance in this model. The aim of this study was to determine whether treatment with Dimericine would prevent, or reduce, UVR-induced melanoma development in this model.

## Results

### Dimericine treatment reduced the level of DNA damage following UVR exposure

Topical Dimericine treatment 30 minutes prior to UVR exposure reduced the level of DNA damage present in mouse epidermal keratinocytes after a single neonatal UVB dose of 5.9 kJ/m$^2$. Evidence of significant erythema (peeling) was observed 72 h after UVR in placebo-treated, but not in Dimericine-treated animals (Figure S1). We observed only a relatively small amount of 6-4 photoproduct damage in the epidermis at 3 h post-UVR, with an average of only 7.5% and 3.4% of cells staining in placebo-treated (n=3) and Dimericine-treated (n=3) animals respectively (Mann Whitney p=0.014) (Figure 1A and 2A). We observed no evidence of 6-4 photoproducts in non-UVR treated animals (Figure 1A). The majority of 6-4 photoproducts were removed by 24 h in both placebo and Dimericine treated animals (data not shown). We rarely observed any melanocytes harbouring 6-4 photoproducts and found no significant difference between Dimericine +UVR treated animals and placebo treated animals.

Dimericine treatment significantly reduced the percentage of epidermal cells harbouring CPDs post-UVR, the greatest difference between Dimericine and placebo treated animals was observed at the 3 h post-UVR timepoint (Mann Whitney p= 0.004)(Figure 1B). No CPDs were observed in the epidermis of non-UVR treated animals (data not shown). We analysed 128 epidermal melanocytes from three placebo +UVR treated animals and found that at 3 h after UVR 92% carried CPDs while in the Dimericine +UVR cohort (n=3) we analysed 87 epidermal melanocytes and found that only 59% carried CPDs (Pearson Chi-Squared p<0.001)(Figure 2B). Dimericine treatment also reduced the number of extra-follicular dermal melanocytes carrying CPDs at 3 h post-UVR, with 36% of 330 melanocyte harbouring CPD damaged compared with 75% of 260 extra-follicular melanocytes in placebo treated animals (Pearson Chi-Squared p<0.001). These results suggest that Dimericine penetrates the skin and reduces DNA damage in melanocytes in the epidermis and dermis. Interestingly, even without Dimericine CPDs were undetectable in both epidermal and extra-follicular melanocytes by 24 h post-UVR. In contrast, epidermal keratinocytes (both basal and suprabasal) still exhibited CPD damage at 24 h post-UVR (Figure 1B). Dimericine sped up the initial removal of CPDs from these cells (at the 3 h timepoint), but by 24 h post UVR there was no significant difference in the level of CPDs between placebo and Dimericine treated animals.

### Proliferation of melanocytes inhibited by Dimericine treatment

At 72 h post-UVR there was an increase in proliferating epidermal keratinocytes as measured by Ki67 positivity. Animals treated with placebo lotion (n=3) had on average 16% of epidermal cells (both basal and suprabasal keratinocytes) proliferating, while Dimericine-treated animals (n=4) had on average 9% and non UVR-treated animals (n=3) only 4% of epidermal cells proliferating (Figure 3A and 2C). These differences are statistically significant (ANOVA, p<0.001), and post hoc analyses (Tukey's test) revealed that all three means were statistically significantly different from each other. There was a higher percentage of epidermal melanocytes proliferating at 72 h post-UVR in placebo-treated animals (55%, 133/241) compared with Dimericine-treated (36%, 45/126) and non-UVR treated animals (24%, 8/25). Dimericine treatment significantly reduced the proliferation of epidermal melanocytes (Pearson chi squared

p<0.01). We also analysed Ki67 positivity of extra-follicular dermal melanocytes at 72 hours post-UVR and found no difference between animals treated with placebo +UVR (26%, 63/247) and Dimericine +UVR (23%, 61/263) (Pearson chi squared p= 0.6). There were a higher percentage of hair follicle outer root sheath (ORS) melanocytes proliferating at 72 h post-UVR in placebo-treated animals (68%, 141/206) compared with Dimericine-treated (43%, 54/125) and non-UVR treated animals (31% , 35/112) (Pearson chi squared p<0.001).

**Migration of melanocytes to the epidermis following UVR exposure**

The numbers of epidermal melanocytes increased following UVR exposure and peaked at 72 h post-UVR (Figure 3B). Dimericine treatment prior to UVR resulted in a slight decrease in epidermal melanocyte number at 72 h post-UVR (Figure 2D and 2E). The average number of epidermal melanocytes per field reduced from 14.4 in placebo treated animals (n=3) to 9.3 in animals treated with Dimericine (n=4) (ANOVA contrast *t*-test p<0.01) (Figure 3B). In comparison the non-UVR cohort did not exhibit an increase in the number of epidermal melanocytes over the period of the experiment (Figure 3B).

Dermal melanocytosis is part of the phenotype of animals carrying the melanocyte-specific Nras mutation (Ackermann et al., 2005). There was a much greater number of dermal extrafollicular melanocytes in *Cdk4*$^{R24C/R24C}$/*Nras*$^{Q61K}$ mice compared to homozygous *Cdk4*$^{R24C/R24C}$ animals regardless of treatment regimen (Placebo + UVR, Dimericine + UVR or non-UVR) (Figure 3C). These differences were statistically significant between *Cdk4*$^{R24C/R24C}$/*Nras*$^{Q61K}$ and homozygous *Cdk4*$^{R24C/R24C}$ animals (ANOVA p<0.01)(Figure 3C).

**Dimericine did not prevent melanoma development in *Cdk4*$^{R24C/R24C}$/*Nras*$^{Q61K}$ mice**

Thirty-three percent of mice homozygous for the *Cdk4*-R24C mutation, and also carrying the melanocyte-specific activated *Nras* mutation (*Cdk4*$^{R24C/R24C}$/*Nras*$^{Q61K}$), developed melanoma spontaneously by 250 days (Table 1). A single neonatal UVR treatment increased the tumour penetrance and significantly decreased the age of onset (Log rank test Bonferroni correction, p>0.001) (Table 1). We compared the penetrance of melanoma in *Cdk4*$^{R24C/R24C}$/*Nras*$^{Q61K}$ mice, both with and without Dimericine treatment after a single neonatal UVR dose. We observed no statistically significant difference in the onset of melanoma development between the placebo + UVR and the Dimericine + UVR treated cohorts (Log rank test Bonferroni correction, Table 1).

We observed no phenotypic or histopathological differences between lesions that developed after Dimericine and placebo treatment (Figure 4). Lesions were mainly dermal melanomas (Figure 4B and C). They were often multicentric, had aberrant nuclear features, and were positive for Tyrp-1 (Figure 4C and D). We did observe a number of melanocytes scattered along the basal layer near the tumour site and superficial dermal nests in adult *Cdk4*$^{R24C/R24C}$/*Nras*$^{Q61K}$ animals regardless of UVR treatment (Figure 4D and E). We also assessed indicators of tumour aggressiveness. We saw no significant difference in the average number of lesions per animal between the non-UVR, +UVR, placebo +UVR and Dimericine +UVR treated cohorts (Table 2, Pearson's Chi Squared p=0.09). Similarly, there was no difference in the

average size of lesions among animals treated with either +UVR, placebo +UVR and Dimericine +UVR (Figure 4F) (ANOVA p=0.37).

**Discussion**

To further investigate the use of nucleotide excision repair (NER)-enhancing agents for skin cancer prevention after UVR exposure we treated mice topically with Dimericine immediately prior to neonatal UVR exposure, which has a powerful effect in exacerbating melanoma development in mouse melanoma models. Unexpectedly, we saw no difference in penetrance or age of onset of melanoma after neonatal UVR between Dimericine-treated and control animals. This is not the case for non-melanoma skin cancer induction in mice – which is significantly reduced after Dimericine treatments (Yarosh et al., 1992). Notably, chronic UVR is necessary for UVR-induced non-melanoma skin cancer induction in mice. This is normally ineffective in inducing melanoma, even in transgenic animals that are highly melanoma-prone after neonatal UVR (Larue and Beermann, 2007). Our results are further suggestive of different mechanisms of UVR tumorigenesis between the respective cell types (melanocytes vs keratinocytes) and possibly a different role for CPDs in melanoma induction after neonatal UVR than in SCC induction after repeated UVR exposures in mice. It is not known how well neonatal UVR in mice corresponds to settings whereby UVR may induce melanoma in humans, but it is suspected to be somewhat analogous to childhood sunburns.

One caveat of this study is that we do not know the dose-responsiveness for melanoma induction after neonatal UVR in the model we have used ($Cdk4^{R24C/R24C}/Nras^{Q61K}$ mice). For instance, melanoma may be induced by relatively low exposures that result in significantly lower levels of CPDs than the dose we delivered. However the work of De Fabo et al (2004), the only study so far to assess the role of different total UVB doses in neonatal UVR-induced melanoma, suggests that the relationship between UVB dose and melanoma induction is close to linear in the albino *Hgf* model. We suspect that non-linearity of response does not explain the lack of reduction in melanoma incidence in our study due to enhancement of DNA repair. Notably, in both this and our previous study, we do not see any evidence that the neonatal melanocytes in $Cdk4^{R24C/R24C}/Nras^{Q61K}$ mice do not efficiently remove CPDs. In fact epidermal melanocytes remove CPDs more efficiently than surrounding keratinocytes. This would fit with the notion that up-regulation by UVR of keratinocyte-derived cytokines (e.g. αMSH, EDN1, KITLG) results not only in stimulation of melanocyte melanin production, differentiation, and proliferation, but also in enhancement of their DNA repair capacity (Abdel-Malek et al., 2008). Interestingly, we also noted that melanocytes were often present in the epidermal basal layer adjacent to tumours. It is unclear whether this effect is driven by changes in the keratinocytes adjacent the tumour, melanoma cells, or both.

It was also interesting to note that treatment with Dimericine, which does not have any direct action on 6-4 photoproducts, did however reduce the amount of 6-4 damage. It could be speculated that Dimericine shifts repair of CPDs into base excision repair from nucleotide excision repair by making a glycosylic bond break and releasing the 5' pyrimidine base. As nucleotide excision repair complexes have to repair both CPDs and 6-4 photoproducts. When the NER is freed from the task of repairing CPDs, there is more available to focus on the 6-4 photoproducts, and the expected result would be somewhat faster repair. Additionally, this study has only examined global genomic repair in the skin following treatment with Dimericine and there may be effects related to transcription coupled repair that may exacerbate tumorigenesis.

Further complicating the interpretation of our results, other studies show that a defective NER response does enhance melanoma development in animals defective in both Ink4a and Arf. *Ink4a/Arf*$^{-/-}$*/Xpa*$^{-/-}$ (van Schanke et al., 2006) and *Ink4a/Arf*$^{-/-}$*/Xpc*$^{-/-}$ (Yang et al., 2007) mice develop melanoma with higher rates after neonatal UVR than for *Ink4a/Arf*$^{-/-}$ alone. The reason for the apparently contrasting results is not obvious. It could involve the germline mutations carried by the mice themselves. Our animals carried a germline cell cycle defect (*Cdk4-R24C*) and a melanocyte-specific *Nras* mutation at the time of UVR exposure - whereas in the other studies the animals did not have a prior Ras pathway mutation. Interestingly, in the Yang et al (2007) study Kras mutations were induced during tumorigenesis in many of the melanomas developing in the *Ink4a/Arf*$^{-/-}$*/Xpc*$^{-/-}$ mice. Thus it is possible that one action of CPD photoproducts in melanomagenesis is to induce mutations in the genes that were already defective in the mice in our study (Cdk4 and Ras). Alternatively, defective NER signalling may exacerbate tumorigenesis in other ways in addition to simply resulting in defective removal of CPDs. These other mechanisms would not manifest in our study. Furthermore, as dimericine and placebo treated animals have the same initial levels of CPDs immediately post-UVR, the initial levels of CPDs and resultant burst of cytokine release within the skin, may be more important for melanomagenesis, than the length of time dimers are present.

Another interpretation of our findings is that melanomagenesis after neonatal UVR is not simply due to the presence of CPDs generated by UVR exposure. Wolnicka-Glubisz et al., (2007) have previously shown that neonatal mice show a defective inflammatory response to UVR suggesting that damaged cells may not be properly recognised and targeted by the immune system and removed when necessary. In addition, other types of DNA (or other) damage such as oxidative lesions have been suggested to play a role in melanomagenesis after neonatal UVR. Notably, treatment of *Hgf* transgenic mice with antioxidants after neonatal UVR delayed the age of onset of melanoma (Cotter et al., 2007), which enhancement of NER did not do in our study. Another possible contributing factor to melanoma genesis after neonatal UVR is the proliferative and migratory response of neonatal melanocytes (Walker et al., 2008). This may contribute to their destabilisation and increase their risk of transformation. Our results reveal the importance of this response in melanocyte transformation as it was reduced by enhancement of NER using Dimericine, but still elevated compared to non-UVR treated animals. In connection with this melanocyte response, *Cdk4*$^{R24C/R24C}$*/Nras*$^{Q61K}$ mice have dermal melanocytosis, which is not present in wild type mice**.** While epidermal melanocytes greatly increase in number after UVR exposure, interfollicular dermal melanocyte numbers in the melanoma-prone mice did not change. All of the proliferative response is centred on the hair follicle, with melanocytes migrating upwards into the basal layer from the outer root sheath (ORS) after UVR exposure (Walker et al., 2008). This opens up the question as to from which population are the melanomas emanating, is it the proliferating epidermal/ORS melanocytes or the non-responding dermal population? In our model and most other mouse models melanomas appear to originate from the dermis. This would suggest that the proliferating melanocyte population may not be one from which the melanomas are initiated.

In summary, treatment with Dimericine prior to UVR exposure reduced CPDs in mouse skin but did not protect against melanoma development. This finding indicates that UVR-induced melanomas are perhaps

driven by mechanisms other than solely that due to persistence of CPD-associated damage.

**Methods**

All experiments were undertaken with the approval of the QIMR animal ethics committee (approval number A98004M).

**Mouse strains.**

*Cdk4*$^{R24C/R24C}$ mice have been previously described (Rane et al., 1999). *Tyr-Nras (Q61K*) transgenic mice express mutant (*Q16K) Nras* in a melanocyte-specific manner via a tyrosinase gene promoter/enhancer cassette previously described (Ackermann et al., 2005). All mice were on a FVB background. Diagnosis of melanoma was confirmed by a pathologist (HKM) and Tyrp-1 staining preformed to confirm melanocytic origin of the tumour cells, as previously described (Hacker et al., 2006).

**UVR and Dimericine treatments.**

At day 2 postnatal, animals were irradiated with a single UVR dose of 5.9 kJ/m$^2$ as described previously (Walker et al., 2008). Animal received either the Dimericine liposome lotion or placebo lotion 30 min prior to irradiation as previously described (Yarosh et al., 2001). Briefly, the Dimericine liposome lotion consisted of 1 mg/L T4 endonuclease V, encapsulated in liposomes, in a 1 % hydrogel lotion and the placebo lotion consisted of equivalent empty liposomes, without enzyme, in a 1% hydrogel lotion. Animals used in the proliferation and DNA damage assays, were monitored and photos taken of each animal prior to euthanasia. The same region of dorsal skin was taken from each animal (Figure S2).

**Proliferation and DNA damage immunofluorescent Assays.**

Paraffin-embedded sections of mice skins were dewaxed, and antigen retrieval performed using Dako retrieval solution, pH 6.0 (Dako, Glostrup, Denmark). Proliferating cells were detected using rat anti-Ki67 (Dako, M7249) diluted 1/100 and melanocytes were identified using tyrosinase-related protein 1 (*Tyrp1*, αPEP1) rabbit polyclonal antibody diluted 1:400 (a gift from Dr V.J. Hearing, NIH, Bethesda). Biotinylated goat anti-rat secondary (Jackson ImmunoResearch laboratories, West Grove, USA) was used for Ki-67 detection with streptavidin linked Alexa-Fluor 488 (Invitrogen, Carlsbad, USA) and Tyrp1 was detected using donkey anti-rabbit Alexa-Fluor 555 (Invitrogen).

Damaged cells were detected using mouse anti-thymine dimer (KTM53) (Kamiya biomedical company, Seattle, USA) diluted 1/200 and melanocytes were identified using Tyrp1 antibody diluted 1:400. A mouse-on-mouse block (Biocare medical, Concord, USA) was conducted for 30 min prior to O/N incubation with primary antibodies. Biotinylated donkey anti-mouse secondary (Jackson) were used for thymine dimmer (CPD) detection with streptavidin linked Alexa-Fluor 488 (Invitrogen) and Tyrp1 detected using donkey anti-rabbit Alexa-Fluor 555 (Invitrogen). Cells with 6-4 photoproducts were detected using mouse anti-6-4 photoproducts (64M-2) (Medical and biological laboratories, Nagoya, Japan) diluted 1/100 and melanocytes were identified using Tyrp1 antibody diluted 1:400. A mouse-on-mouse block (Biocare

medical) was conducted for 30 min prior to O/N incubation with primary antibodies. The same secondary detection system as the thymine dimer assay was used.

All slides were cover-slipped using vectorshield-DAPI mounting media (Vector laboratories, Burlingame, USA). Images were captured using the DeltaVision Microscope system (Applied Precision, Issaquah, USA). The deltaVision tracking software and stage technology allowed non overlapping images to be collected along each skin section. Ten fields were counted using an x20 objective along a skin section from each animal for all assays but the ki67 staining which used 5 fields per animal. The sampled fields were 300µm in length spaced one field apart and we did not select areas for specific cell types or staining patterns. Sections were counted using Image J (available: http://rsbweb.nih.gov/ij/index.html). All staining and quantification procedures were performed blind to the samples ID.

**Statistical Analysis**

The Prism software package (GraphPad Software, La Jolla, USA) was used to generate graphs and the SPSS v15 software package (SPSS, Illinois, USA) was used to perform statistical analyses. Statistical significance was $p<0.05$ and denoted by * in subsequent graphs. Mann Whitney tests were performed to compare the average percentage of epidermal cells carrying DNA damaged amongst placebo and Dimericine treated groups. Pearson's Chi squared tests were performed to compare the percentage of melanocyte populations harbouring CPD damage and proliferating amongst placebo and Dimericine treated cohorts. One-way ANOVAs were used to test for evidence of difference in: mean proliferative response 72 h post-UVR; mean tumour size across cohorts; across the non-UVR, + UVR, Placebo + UVR and Dimericine + UVR cohorts. Statistically significant results were investigated further using Tukey's post hoc test. The incidence of melanoma development was modelled using Kaplan-Meier analysis, and the log-rank test was used to test for pairwise differences between the groups. The statistical significance levels for all tests were adjusted for the multiple pairwise comparisons using the Bonferroni method $p<0.004$. A three-way ANOVA was used to compare the increase in epidermal or extrafollicular melanocytes following UVR. The hypothesised difference between treatments was expressed as a contrast of means and tested with the associated *t*-test. Pearson's Chi squared test was used to compare the number of melanoma cases per animal between cohorts.

**Acknowledgements**

We thank David Yarosh and AGI Dermatics for the T4 endonuclease V DNA repair enzyme (Dimericine) lotion. This work was funded by the Cancer Council of Queensland. Elke Hacker and Nicholas Hayward are supported by fellowships from the National Health and Medical Research Council (NHMRC) of Australia and Graeme Walker is supported by a fellowship from the Cancer Council Queensland.

**References**

ABDEL-MALEK, Z. A., KNITTEL, J., KADEKARO, A. L., SWOPE, V. B. & STARNER, R. (2008) The melanocortin 1 receptor and the UV response of human melanocytes--a shift in paradigm. *Photochem Photobiol,* 84**,** 501-8.

ACKERMANN, J., FRUTSCHI, M., KALOULIS, K., MCKEE, T., TRUMPP, A. & BEERMANN, F. (2005) Metastasizing melanoma formation caused by expression of activated N-RasQ61K on an INK4a-deficient background. *Cancer Res,* 65**,** 4005-11.

COTTER, M. A., THOMAS, J., CASSIDY, P., ROBINETTE, K., JENKINS, N., FLORELL, S. R., LEACHMAN, S., SAMLOWSKI, W. E. & GROSSMAN, D. (2007) N-acetylcysteine protects melanocytes against oxidative stress/damage and delays onset of ultraviolet-induced melanoma in mice. *Clin Cancer Res,* 13**,** 5952-8.

DE FABO, E. C., NOONAN, F. P., FEARS, T. & MERLINO, G. (2004) Ultraviolet B but not ultraviolet A radiation initiates melanoma. *Cancer Res,* 64**,** 6372-6.

HACKER, E., MULLER, H. K., IRWIN, N., GABRIELLI, B., LINCOLN, D., PAVEY, S., POWELL, M. B., MALUMBRES, M., BARBACID, M., HAYWARD, N. & WALKER, G. (2006) Spontaneous and UV radiation-induced multiple metastatic melanomas in Cdk4R24C/R24C/TPras mice. *Cancer Res,* 66**,** 2946-52.

KRAEMER, K. H., LEE, M. M., ANDREWS, A. D. & LAMBERT, W. C. (1994) The role of sunlight and DNA repair in melanoma and nonmelanoma skin cancer. The xeroderma pigmentosum paradigm. *Arch Dermatol,* 130**,** 1018-21.

LARUE, L. & BEERMANN, F. (2007) Cutaneous melanoma in genetically modified animals. *Pigment Cell Res,* 20**,** 485-97.

MORIKAWA, K. & SHIRAKAWA, M. (2000) Three-dimensional structural views of damaged-DNA recognition: T4 endonuclease V, E. coli Vsr protein, and human nucleotide excision repair factor XPA. *Mutat Res,* 460**,** 257-75.

RANE, S. G., DUBUS, P., METTUS, R. V., GALBREATH, E. J., BODEN, G., REDDY, E. P. & BARBACID, M. (1999) Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in beta-islet cell hyperplasia. *Nat Genet,* 22**,** 44-52.

TANAKA, K., SEKIGUCHI, M. & OKADA, Y. (1975) Restoration of ultraviolet-induced unscheduled DNA synthesis of xeroderma pigmentosum cells by the concomitant treatment with bacteriophage T4 endonuclease V and HVJ (Sendai virus). *Proc Natl Acad Sci U S A,* 72**,** 4071-5.

VAN SCHANKE, A., VAN VENROOIJ, G. M., JONGSMA, M. J., BANUS, H. A., MULLENDERS, L. H., VAN KRANEN, H. J. & DE GRUIJL, F. R. (2006) Induction of nevi and skin tumors in Ink4a/Arf Xpa knockout mice by neonatal, intermittent, or chronic UVB exposures. *Cancer Res,* 66**,** 2608-15.

WALKER, G. J., M, G. K., HACKER, E., RAVISHANKAR, S., MULLER, H. K., BEERMANN, F. & HAYWARD, N. K. (2008) Murine Neonatal Melanocytes Exhibit a Heightened Proliferative Response to Ultraviolet Radiation and Migrate to the Epidermal Basal Layer. *J Invest Dermatol*.

WOLF, P., MAIER, H., MULLEGGER, R. R., CHADWICK, C. A., HOFMANN-WELLENHOF, R., SOYER, H. P., HOFER, A., SMOLLE, J., HORN, M., CERRONI, L., YAROSH, D., KLEIN, J., BUCANA, C., DUNNER, K., JR., POTTEN, C. S., HONIGSMANN, H., KERL, H. & KRIPKE, M. L. (2000) Topical treatment with liposomes containing T4 endonuclease V protects human skin in vivo from ultraviolet-induced upregulation of interleukin-10 and tumor necrosis factor-alpha. *J Invest Dermatol,* 114**,** 149-56.

WOLNICKA-GLUBISZ, A., DAMSKER, J., CONSTANT, S., CORN, S., DE FABO, E. & NOONAN, F. (2007) Deficient inflammatory response to UV radiation in neonatal mice. *J Leukoc Biol,* 81**,** 1352-61.

YANG, G., CURLEY, D., BOSENBERG, M. W. & TSAO, H. (2007) Loss of xeroderma pigmentosum C (Xpc) enhances melanoma photocarcinogenesis in Ink4a-Arf-deficient mice. *Cancer Res,* 67**,** 5649-57.

YAROSH, D., ALAS, L. G., YEE, V., OBERYSZYN, A., KIBITEL, J. T., MITCHELL, D., ROSENSTEIN, R., SPINOWITZ, A. & CITRON, M. (1992) Pyrimidine dimer removal enhanced by DNA repair liposomes reduces the incidence of UV skin cancer in mice. *Cancer Res,* 52**,** 4227-31.

YAROSH, D., BUCANA, C., COX, P., ALAS, L., KIBITEL, J. & KRIPKE, M. (1994) Localization of liposomes containing a DNA repair enzyme in murine skin. *J Invest Dermatol,* 103**,** 461-8.

YAROSH, D., KLEIN, J., O'CONNOR, A., HAWK, J., RAFAL, E. & WOLF, P. (2001) Effect of topically applied T4 endonuclease V in liposomes on skin cancer in xeroderma pigmentosum: a randomised study. Xeroderma Pigmentosum Study Group. *Lancet,* 357**,** 926-9.

YAROSH, D. B. & KLEIN, J. (1996) DNA repair enzymes in prevention of photocarcinogenesis. *Photochem Photobiol,* 63**,** 445-7.

**Table 1. Melanoma penetrance in *Cdk4*$^{R24C/R24C}$/*Nras*$^{Q61K}$ mice.**

| Cohort | n= | Melanoma penetrance at 250 days | Mean age-of-onset | Log Rank Test p value^ | Log Rank Test p value^ |
|---|---|---|---|---|---|
| **NON-UV** | **33** | 33% | 259 Days | ref | p<0.001* |
| **+UV** | **19** | 100% | 142 Days | p<0.001* | 0.1178 |
| **Placebo +UV** | **20** | 100% | 178 Days | p<0.001* | 0.0115 |
| **Dimericine +UV** | **13** | 100% | 155 Days | p<0.001* | ref |

*Cdk4*$^{R24C/R24C}$/*Nras*$^{Q61K}$ animals on a FVB background were used in this study.

^Corrected for multiply pair wise comparisons Bonferroni method p<0.004 for significants

* denotes statistically significant comparisons.

All melanoma cases were confirmed by pathologist (HKM).

**Table 2 Number of Melanoma cases per Animal**

| Cohort | 1 melanoma per animal | >1 melanoma per animal | Total number of animals |
|---|---|---|---|
| **NON-UV** | 8 (80%) | 2 (20%) | 10 |
| **+UV** | 14 (74%) | 5 (26%) | 19 |
| **Placebo +UV** | 13 (65%) | 7 (35%) | 20 |
| **Dimericine +UV** | 5 (39%) | 8 (61%) | 13 |

**Figure legends**

**Figure 1. Dimericine treatment reduced the level of DNA damage present in the skin following UVR exposure.**

A) Graph displaying the average proportion of epidermal cells that contain 6-4 photoproducts 3 h post UVR (Mann Whitney p=0.014). B) Line graph displaying the average precentage of epidermal cells that contain CPD DNA damage (Mann Whitney 3 hrs post-UVR, p=0.004). At least three animals were used to generate each point and all animals used in these figures were genotyped *Cdk4*$^{R24C/R24C}$/*Nras*$^{Q61K}$. * denotes statistically significant comparisons.(error bar= SEM).

**Figure 2. Immunohistochemical images of 6-4 photoproduct, CPD, Ki67 and Tyrp-1 staining in mouse skin following UVR exposure.**

A) Placebo treated skin 3 h post UVR with nuclear staining positive (green) for 6-4 photoproducts in the epidermis, no melanocytes were identified by Tyrp1 staining (centre image), DAPI (right). B) CPD damage was identified by green staining in the nucleus of placebo treated skin. Melanocytes were identified by Tyrp1 staining (centre image), note the damaged epidermal melanocyte staining above the dotted line for both CPD and Tyrp-1 in the overlay image (right). C) Proliferating cell's nucleus are stained with Ki67 (green) and melanocytes identified by cytoplasmic staining with Tyrp1 (centre image). Note the proliferating epidermal melanocyte staining above the dotted line in the overlay image (right). D) Placebo treated skin 72 h post UVR with cytoplamsic Tyrp1 staining (red) for melanocytes, note the large number of epidermal melanocytes (staining above the dotted line). E) Dimericine treated skin 72 h post UVR with cytoplamsic Tyrp1 staining (red) for melanocytes, note the lack of epidermal melanocytes (staining above the dotted line). All sections were counterstained with DAPI (blue). (Scale bar for all images = 10 μm).

**Figure 3. Dimericine treatment and melanocyte proliferation.**

A) Proportion of proliferating epidermal cells at 72 h post UVR exposure. All animals used in this figure were genotyped *Cdk4*$^{R24C/R24C}$/*Nras*$^{Q61K}$ (Placebo +UV, n= 3; Dimericine +UV, n= 4; non-UVR, n= 3) (ANOVA, p<0.001). B) Line graph displaying the average number of epidermal melanocytes at time points following UVR exposure in *Cdk4*$^{R24C/R24C}$/*Nras*$^{Q61K}$ mice (Placebo +UV, 3 h, n= 4; 24 h, n= 3; 72 h, n= 3; Dimericine +UV, 3 h, n= 3; 24 h, n= 4; 72 h, n= 4; non-UVR, 3 h, n= 3; 24 h, n= 3; 72 h, n= 5) (ANOVA, contrast *t* test p<0.01). C) Line graph displaying the average number of extra-follicular dermal melanocytes at time points following UVR exposure (ANOVA p<0.01). Figure C used the same number of *Cdk4*$^{R24C/R24C}$/*Nras*$^{Q61K}$ animals as in Figure B with the addition of *Cdk4*$^{R24C/R24C}$ Placebo +UV, 3 h, n= 3; 24 h, n= 3; 72 h, n= 3; *Cdk4*$^{R24C/R24C}$ Dimericine +UV, 3 h, n= 2; 24 h, n= 1; 72 h, n= 1; non-UVR, 3 h, n= 2; 24 h, n= 3; 72 h, n= 2 animals. * denotes statistically significant comparisons. (error bar= SEM).

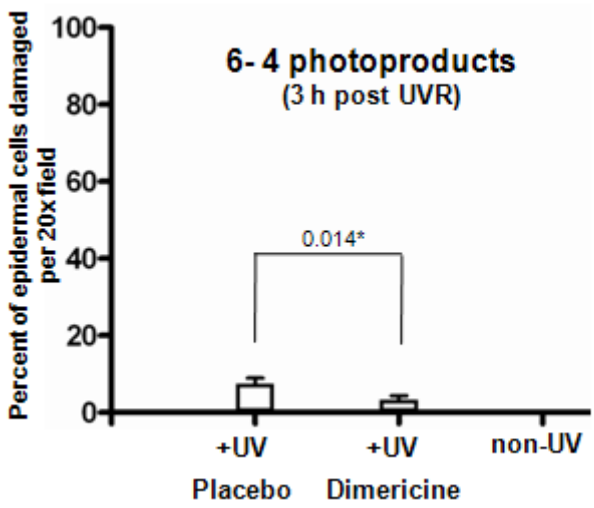
**Figure 4. Melanoma phenotypic characteristics and histological staining of melanoma sections.**

A) Macro image of melanoma (scale bar= 0.5 cm). B) H&E stained section of a cutaneous melanoma (scale bar= 100 μm). C) Dermal lesion stained for Tyrp1 (red) (scale bar= 100 μm). D) Tyrp1 stained lesion

showing melanocytic clusters along the superficial dermis (scale bar= 100 μm). E) High power image of lesion stained for Tyrp1 with melanocytic cells (red) along the basal layer and dermis (scale bar = 50 μm). F) Bar graph displaying the average size of all lesions $cm^2$ (error bar= SEM), (n=number of melanoma cases per cohort), (ANOVA p=0.37).

**Figure 1**

**A**


6- 4 photoproducts
(3 h post UVR)
Percent of epidermal cells damaged per 20x field
100
80
60
40
20
0
0.014*
+UV
Placebo
+UV
Dimericine
non-UV

**B**

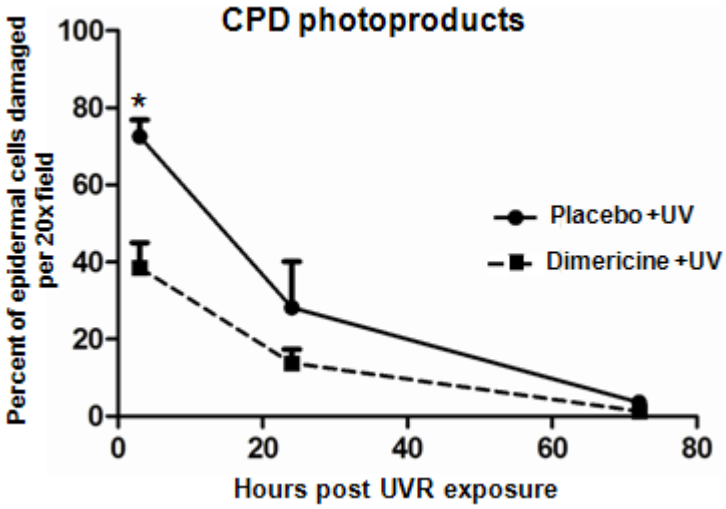
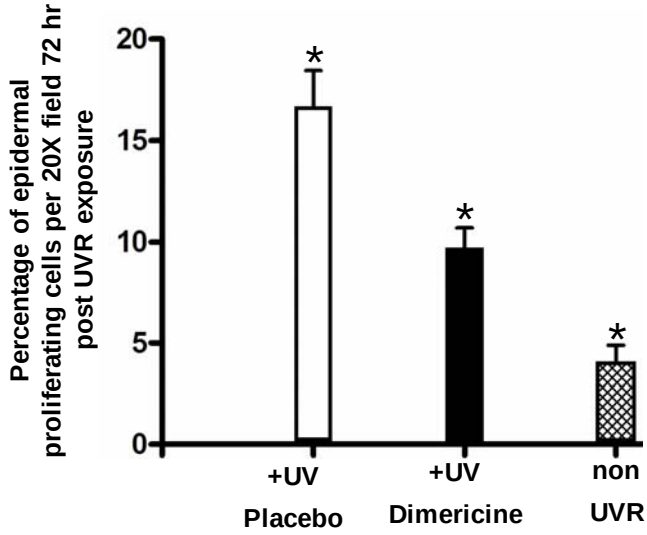

CPD photoproducts
Percent of epidermal cells damaged per 20x field
100
80
60
40
20
0
*
Placebo +UV
Dimericine +UV
0
20
40
60
80
Hours post UVR exposure

**Figure 2**

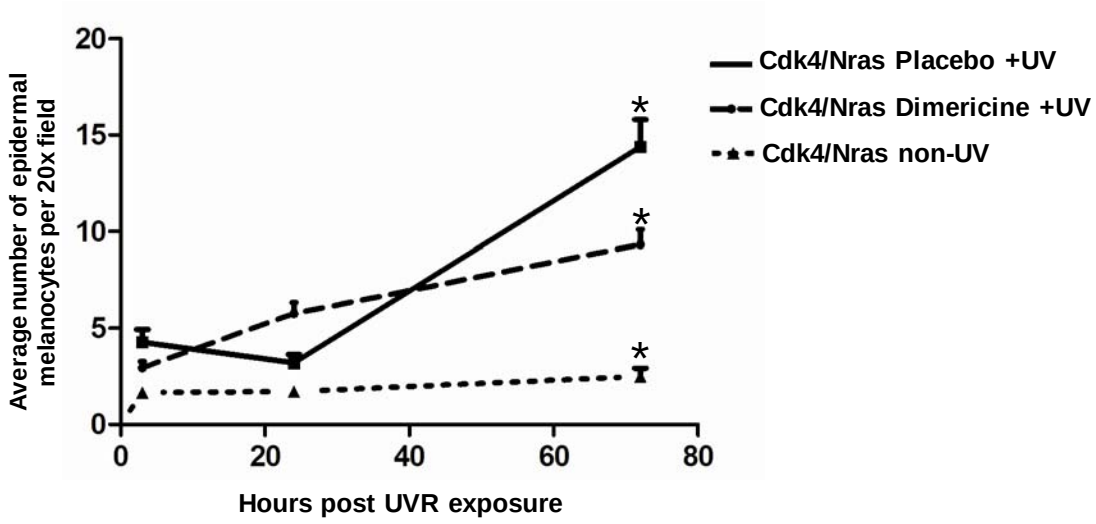
**Jpeg attached**

**Figure 3**

**A**


Percentage of epidermal proliferating cells per 20X field 72 hr post UVR exposure
20
15
10
5
0
*
*
*
+UV Placebo
+UV Dimericine
non UVR

**B**


Average number of epidermal melanocytes per 20x field
20
15
10
5
0
0
20
40
60
80
Hours post UVR exposure
Cdk4/Nras Placebo +UV
Cdk4/Nras Dimericine +UV
Cdk4/Nras non-UV
*
*
*

**C**

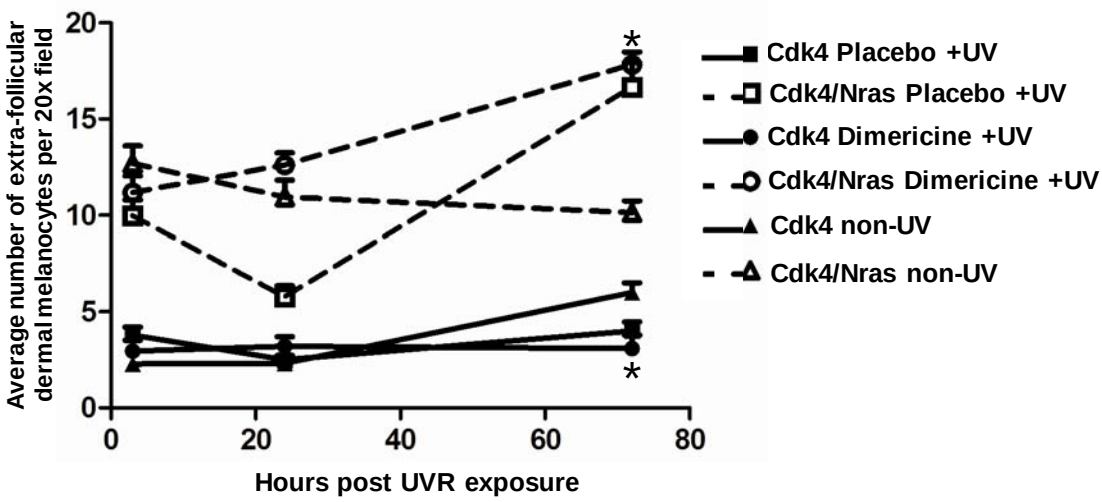
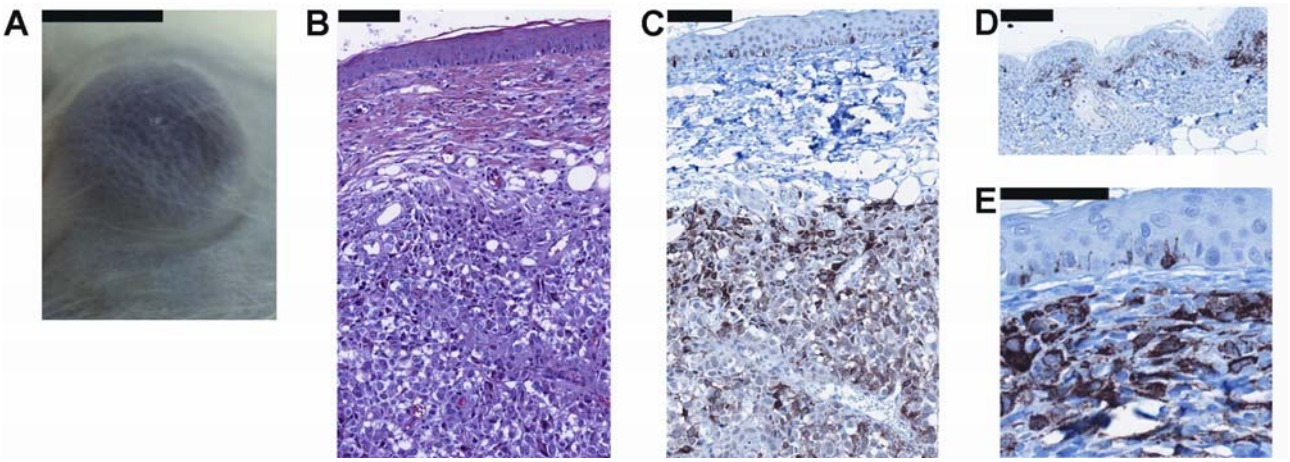
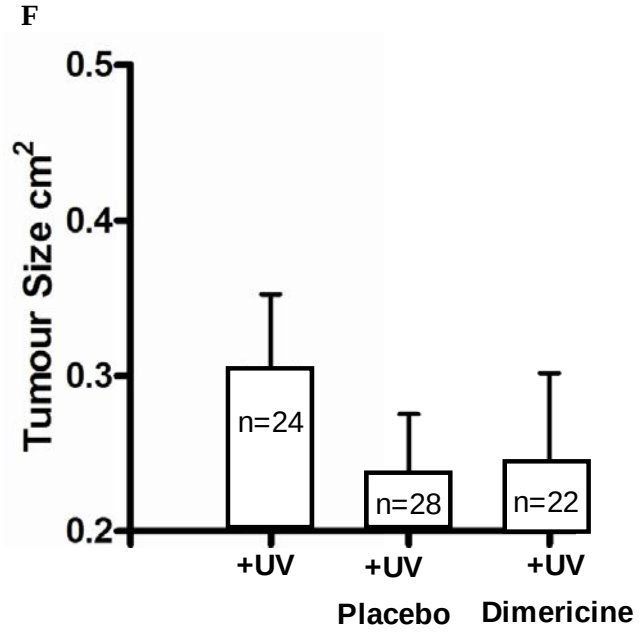

Average number of extra-follicular dermal melanocytes per 20x field
20
15
10
5
0
0
20
40
60
80
Hours post UVR exposure
*
*
Cdk4 Placebo +UV
Cdk4/Nras Placebo +UV
Cdk4 Dimericine +UV
Cdk4/Nras Dimericine +UV
Cdk4 non-UV
Cdk4/Nras non-UV

**Figure 4**


A
B
C
D
E


F
Tumour Size cm2
0.5
0.4
0.3
0.2
n=24
n=28
n=22
+UV
+UV
+UV
Placebo
Dimericine

**Supplementary Materials**

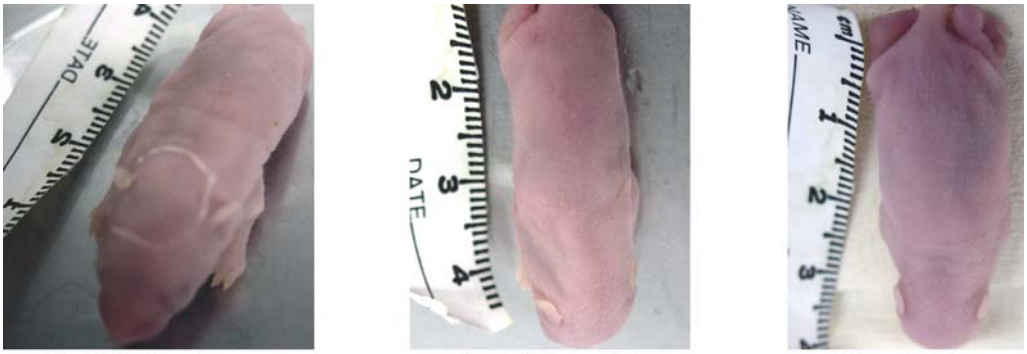



**Figure S1. Dimericine treatment reduced the level of erythema present in mouse skin following UVR exposure.** Skin peeling was observed in animals treated with placebo lotion (left) 72 h post UVR exposure, while Dimericine application prior to UVR (centre) protected the skin from peeling. No erythema was observed in non-UVR treated animals (right).

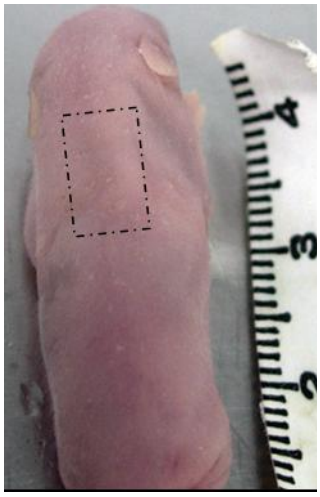


**Figure S2. Dorsal region of mouse skin used in the proliferation and DNA damage assays.** The same regional area was collected from all animals and sections were cut 50 μm in from the tissue edge for immunostaining.