Distinct Pigmentary and Melanocortin 1 Receptor–Dependent Components of Cutaneous Defense against Ultraviolet Radiation

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Genetic variation at the melanocortin 1 receptor (MC1R) is an important risk factor for developing ultraviolet (UV) radiation–induced skin cancer, the most common form of cancer in humans. The underlying mechanisms by which the MC1R defends against UV-induced skin cancer are not known. We used neonatal mouse skin (which, like human skin, contains a mixture of melanocytes and keratinocytes) to study how pigment cells and Mc1r genotype affect the genome-level response to UV radiation. Animals without viable melanocytes (KitW+KitW–) or animals lacking a functional Mc1r (Mc1r+Mc1r−) were exposed to sunburn-level doses of UBV radiation, and the patterns of large-scale gene expression in the basal epidermis were compared to each other and to nonmutant animals. Our analysis revealed discrete Kit– and Mc1r-dependent UVB transcriptional responses in the basal epidermis. The Kit-dependent UVB response was characterized largely by an enrichment of oxidative and endoplasmic reticulum stress genes, highlighting a distinctive role for pigmented melanocytes in mediating antioxidant defenses against genotoxic stresses within the basal epidermal environment. By contrast, the Mc1r-dependent UVB response contained an abundance of genes associated with regulating the cell cycle and oncogenesis. To test the clinical relevance of these observations, we analyzed publicly available data sets for primary melanoma and melanoma metastases and found that the set of genes specific for the Mc1r-dependent UVB response was able to differentiate between different clinical subtypes. Our analysis also revealed that the classes of genes induced by UVB differ from those repressed by UVB with regard to their biological functions, their overall number, and their size. The findings described here offer new insights into the transcriptional nature of the UV response in the skin and provide a molecular framework for the underlying mechanisms by which melanocytes and the Mc1r independently mediate and afford protection against UV radiation.

Introduction

One of the most important functions of cutaneous pigmentation in humans is protection against the damaging effects of ultraviolet (UV) radiation. Depending on wavelength and intensity, UV radiation can have broad-ranging effects on DNA damage, cell cycle arrest, and apoptosis [1] in virtually every one of the more than 25 differentiated cell types in the skin, as well as more specialized responses such as immunosuppression, vitamin D synthesis, and sunburn/tanning [2]. Inadequate protection against UV radiation is a major contributor to melanoma and nonmelanoma skin cancer and a significant public health concern in many populations [3].

Pigmentary defenses against UV radiation depend on both quantitative variation in the number, size, and arrangement of melanosomes—pigment granules transferred from melanocytes to surrounding keratinocytes—and qualitative variation in the type of pigment, eumelanin or pheomelanin, made within those granules. In general, increasing skin darkness correlates closely with an increased number and size and greater dispersal of melanosomes [4,5] and with increasing amounts of both eumelanin and pheomelanin (although eumelanin predominates) [6,7]; this quantitative variation is controlled by polygenic inheritance. By contrast, a single major gene, melanocortin 1 receptor (MC1R), can regulate the ratio of eumelanin to pheomelanin. Complete loss-of-function for MC1R, which encodes a seven-transmembrane receptor coupled to adenylate cyclase, produces a so-called red hair color phenotype in individuals of northern European ancestry—bright red hair, fair skin, freckling, inability to tan, and increased susceptibility to sunburn—and is associated with nearly exclusive production of pheomelanin [8–10]. Pedigree studies are generally consistent with Mendelian expectations for recessive inheritance of the red hair color phenotype [11,12], but in many populations there is considerable MC1R diversity with a range of hypomorphic
Skin cancer is the most common type of cancer in humans and annually accounts for approximately 60,000 deaths worldwide. The most important factors causally linked to skin cancer susceptibility are inadequate protection against ultraviolet (UV) B radiation, fair skin color, and variation of the melanocortin 1 receptor (MC1R) genotype. We used cDNA microarrays to measure the genome-wide transcriptional responses to UVB irradiation in the epidermis of neonatal mice (which approximates the human basal epidermis in its cellular composition and general physiology). To investigate how pigment cells (melanocytes) and MC1R afford protection against UVB radiation, we compared results from normal mice to those from mutant mice that lacked either melanocytes (Kit^W^/Kit^W^) or a functional Mc1r (Mc1r^+/Mc1r^+). We identified melanocyte- and Mc1r-dependent UVB gene expression profiles in the basal epidermis. Surprisingly, the melanocyte- and Mc1r-dependent UVB responses highlighted distinct functions, with the former largely mediating antioxidant defenses and the latter regulating the cell cycle and susceptibility to oncogenesis. We also demonstrated that a subset of Mc1r-dependent UVB-responsive genes could discriminate among human melanoma subtypes, thereby suggesting a mechanism by which MC1R gene variants may predispose toward skin cancer.

### Results

#### A UVB-Responsive Transcriptional Profile in the Basal Epidermis

Between postnatal day 1.5 (P1.5) and P3.5 when hair follicles are still developing, melanocytes are relatively abundant in the basal epidermis, which provides a useful model to study melanocyte–keratinocyte interactions. In control experiments to examine how the transcriptional response to UV radiation changes over time, we determined that 24 h captures both early and intermediate response genes and provides substantial information in terms of the underlying gene expression profiles (unpublished data). For the experiments described below, P1.5 mice were exposed to a single dose of 100 mJ/cm² UVB radiation, which corresponds to mild sunburn, approximately one minimal erythemal dose; control animals underwent the identical procedure but were shielded from UV radiation with a black Perspex box. At 24 h after exposure, we isolated basal epidermis and compared the patterns of gene expression among different experimental groups.

Our preparation of basal epidermis is based on a combination of enzymatic and physical dissociation steps that separates whole epidermal sheets, basal epidermal cells, and whole skins; in previous studies we have shown that this approach yields skin layer–specific gene expression signatures that correspond to the known biological functions of these compartments and in which the basal epidermal compartment is composed of mostly keratinocytes and melanocytes. All experiments were carried out as triplicate biological replicates (each replicate consisted of a separate pool containing two to four animals) with a common reference as described previously.

To establish a baseline, we first determined how UVB affects gene expression in C57BL/6J animals. From spotted cDNA arrays containing 31,756 features, we obtained a data...
set of 13,508 cDNAs that passed quality control filters for signal intensity and replication. At a false discovery rate (FDR) of 0.05 [45], 1,434 of the 13,508 cDNAs were differentially regulated by UVB (Figure 1A and Table S1). The distribution of quantitative changes in expression was similar for induction compared to repression (Figure 1B); however, there was a striking difference in the number of cDNAs affected, with many more repressed than induced by UVB: 1,065 versus 369. Large-scale transcriptional repression has also been observed in other microarray studies of UV radiation. Indeed, the cDNA whose UVB-induced repression exhibited the most significant $p$-value in our data set was Polr2e, one of a dozen subunits of RNA polymerase II.

Annotation using UniGene (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene) Cluster IDs (assigned for the majority of the UVB-responsive features on the array) revealed that 770 of 1,065 of the UVB-repressed (UVBr) and 332 of 369 of the UVB-induced (UVBi) cDNAs were unique. Readily apparent are several representatives of gene classes that are well known to participate in the response to UV radiation. Examples include those involved in cell cycle arrest (Cdk2 and Cyclin E1), DNA repair (Ercc1, Fanc1, and Rad51), and antiapoptosis (Aatf and Dad1), all of which were induced by UVB. The Tp53 pathway is known to play an important role in mediating many of these stress responses, and indeed, we found several Tp53-dependent genes in our UVB-
Figure 2. UVB Response and Gene Size
Gene (A) and intron (B) size data were retrieved for 305 of 332 UVB-induced, 705 of 770 UVB-repressed, and 10,316 of 14,605 35,000 unique mouse genes (by UniGene Cluster ID criteria) using the Ensembl (National Center for Biotechnology Information m34 Assembly) and National Institute on Aging Gene Index 5.0 databases. The size data were averaged for each UVB response class and were then compared, using a Student's t-test, to the gene and intron size averages for 10,316 unique genes on the 35,000 array. Significance values are shown above each UVB response class, and error bars are ±SEM. Gene and intron sizes are represented here on a linear scale, but were log2-transformed prior to t-test analyses, due to the non-normal distribution of the data. Non-parametric Mann-Whitney U tests on the untransformed data yielded similar results.

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responsive gene list, including Mem2, Hif1a, and Bcl3, a finding that, in general, is further supported by a recent microarray study reporting a set of p53-dependent UVB-responsive genes in primary human melanocyte cultures [46].

In a recent study of UV-sensitive and p53-dependent gene expression profiles in a colorectal carcinoma cell line, McKay et al. [47] noted that genes induced by UV tended to be more compact and to have smaller introns and proposed that a gene size constraint on UV-induced mRNA expression played a key role in the evolution of UV-response pathways. Remarkably, we found a similar bias in our system, with the mean gene size for UVBi genes (approximately 30 kb) approximately 1.8 times smaller than that of the UVBr genes (approximately 54 kb) and a mean intron size for the UVBi genes (approximately 4,000 bp) approximately 1.5 times smaller than that of the UVBr genes (approximately 5,900 bp) (Figure 2). The mean number of introns per gene was also significantly smaller in the UVBi compared to the UVBr gene class (unpublished data). There were no differences in gene size, intron size, and the number of introns per gene between the set of UVBr genes and the entire set of approximately 35,000 genes on the array.

Functional Annotation of UV-Responsive Genes by Over-Representation Analysis
To better characterize the UVB transcriptional skin response in normal C57BL/6J mice, we extracted Gene Ontology (GO) terms from all UVBr or UVBi genes and compared the relative frequencies of these GO terms to those for all of the genes on the 35,000 array. Using EASE software [48], we observed 220 and 199 GO categories that were significantly enriched ($p = 0.05$) for the UVBr and UVBi genes, respectively. Of these, 119 UVBr and 64 UVBi categories were supported by at least three gene hits and were enriched by at least 2-fold over the 35,000 array. We focused on non-overlapping biological process, molecular function, or cellular component terms for further study; results are presented in Figure 3.

For the 1,065 UVBr genes (Figure 3A), over-represented GO terms were largely consistent with those which we had observed in a previous study using basal epidermis [49]. Biological processes over-represented by UVBr genes in the basal epidermis included many metabolic and signaling functions: sterol and fatty acid metabolism, ATP metabolism, purine ribonucleoside triphosphate biosynthesis, regulation of Wnt receptor signaling pathway, antigen processing, endogenous antigen via MHC class I, and ribosome biogenesis. Molecular functions over-represented by UVBr genes in the basal epidermis largely corresponded to the aforementioned processes: MHC class I receptor activity, acetylcysteine A C-acetyltransferase activity, clathrin binding, protein disulfide isomerase activity, selenium binding, and iron binding.

The pattern of GO terms over-represented for the 369 UVBi genes in the basal epidermis was very different from that of the UVBr genes (Figure 3B versus Figure 3A). Biological processes over-represented by UVBi genes included DNA repair (nucleotide-excision repair) and response to DNA damage stimulus, S-phase and G2/M transition of mitotic cell cycle, DNA replication initiation, rRNA metabolism, and nuclear mRNA splicing. Molecular functions over-represented by UVBi genes included nuclelease and helicase activities, damaged DNA binding, pre-mRNA splicing factor activity, DNA-directed DNA polymerase activity, and cyclin-dependent protein kinase activity.

As described further below, UVB-responsive genes identified in our study and others exhibit only a partial overlap when considered from the perspective of individual genes, in part due to differences in array platforms and in part due to different patterns of gene expression between skin in vivo and cells in culture. However, large-scale transcriptional repression, downregulation of basal metabolic functions, upregulation of DNA repair functions, and bias in gene and
intron size between these functional classes are general themes emerging from our analysis of the UVB response in vivo and are concordant with earlier work carried out on a variety of cultured cells and UV exposures.

Genetic Deconvolution of Gene Expression in the Basal Epidermis: Melanocytes (Kit-Dependent) and Mc1r Signaling

To investigate how melanocytes and Mc1r signaling contribute to epidermal gene expression, we made use of two classic pigmentation mutants, viable dominant spotting (KitW-v/KitW-v) and recessive yellow (Mc1re/Mc1re). The KitW-v allele is a missense mutation that severely impairs tyrosine kinase activity [50], yielding animals that, post embryonic day 12.5, are almost completely bereft of skin and hair melanoblasts [51]; the Mc1re allele is a complete loss-of-function that causes almost exclusive production of pheomelanin instead of eumelanin [52]. Using KitW-v and KitW-v alleles on a congenic C57BL/6 background, we measured gene expression profiles in basal epidermis of P2.5 mutant animals and compared the results to those obtained from nonmutant C57BL/6 animals. Kit signaling is also required for development of germ cells, erythrocytes, and mast cells. However, pigment cell development is, in general, more sensitive to Kit gene dosage than mast cell development ([KitW-v/KitW-v] mutant mice retain approximately 40% of the normal number of mast cells), and mast cells are normally found in the dermis rather than the epidermis; therefore, we expected that epidermal gene expression in KitW-v/KitW-v mutant mice would be influenced mostly by deficiency of pigment cells.

We first characterized patterns of gene expression in KitW-v/KitW-v and Mc1r- and Kit-Dependent UV Gene Profiles
addition to pigmentation, melanocytes influence the development and maintenance of the extracellular environment in the basal epidermis.

We also identified 284 genes whose expression was altered in Mc1r\(^{-/-}\)/Mc1r\(^{-/-}\) compared to nonmutant animals (in the absence of UV radiation): 184 downregulated and 100 upregulated (Figure 4B). Approximately 10% of the genes downregulated in Mc1r\(^{-/-}\)/Mc1r\(^{-/-}\) animals were also downregulated in Kit\(^{W+}/Kit^{W+}\) animals (Figure 4C), including two genes required for eumelanogenesis, *Dopachrome tautomerase* and *Silver*. However, there was virtually no overlap between Mc1r- and Kit-dependent genes when conditioning on UVB exposure (described further below); thus, Mc1r signaling plays a specific and largely independent role from Kit (and the presence of melanocytes) in the transcriptional response to UV radiation.

We compared the profiles of Mc1r- and Kit-dependent genes in the basal epidermis with the 1,102 unique genes that were either induced or repressed by UVB in nonmutant skin as depicted in Figure 1. The proportion of UVB-sensitive genes in each of the four groups (induced or repressed in Kit\(^{W+}/Kit^{W+}\) or Mc1r\(^{-/-}\)/Mc1r\(^{-/-}\) animals) varied from 10% (induced in Mc1r\(^{-/-}\)/Mc1r\(^{-/-}\) animals) to 24% (repressed in Mc1r\(^{-/-}\)/Mc1r\(^{-/-}\) animals), with the same general theme described earlier—large-scale transcriptional repression of basal metabolic functions—apparent in each of the four groups (right side, Figure 4A and 4B).
Figure 5. Heatmap of UVB × Pigmentation Genotype Interaction Genes in the Basal Epidermis of P2.5 Mice

Two-way ANOVA tests, using 13,508 genes, were performed on Mc1r+/Mc1r and Mc1r/Mc1r samples (–UVB and +UVB) and Kit+/Kit+ and Kit−/Kit− (–UVB and +UVB) samples, yielding 120 Mc1r-dependent and 147 Kit-dependent UVB interacting genes. Post hoc analyses, using pairwise Student’s t-tests, were used to reveal interactions between specific genotypes and UVB treatment. The ordered columns of the heatmap represent replicates of sham-irradiated (blue) and UVB-irradiated (blue) basal epidermal samples derived from Mc1r+/Mc1r (+/+, Mc1r+/Mc1r, and Kit−/Kit−) and Mc1r−/Mc1r (Mc1r−/Mc1r) P2.5 mice. The genes are represented by rows and are arranged to illustrate interactions in the basal epidermis between UVB and Mc1r (A) and UVB and Kit (B). Red, green, and black indicate induction, repression, and no change, respectively, relative to the centered median. Gray represents missing data. The ratio fold-change is indicated by a scale bar.

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Distinct Signatures for Mc1r- and Melanocyte (Kit)-Dependent Gene Expression in the Response to UV Radiation

To investigate if melanocytes and Mc1r signaling are required specifically for the cutaneous response to UV radiation, we used the same UV exposure paradigm described earlier and compared gene expression profiles in the basal epidermis of P2.5 Kit+/Kit− and Mc1r+/Mc1r to that obtained from nonmutant animals. The total data set consisted of 18 arrays with three biological replicates per sample (three genotypes, either exposed or not exposed to UV radiation).

We used two-way analysis of variance (ANOVA) to characterize the interaction between genotype and UVB exposure and identified 120 and 147 genes that showed a significant interaction (p = 0.05) between UVB and Mc1r and between UVB and Kit, respectively. Post hoc analyses performed separately on each of the 120 and 147 gene sets yielded three patterns, depending on whether a gene responded to UVB only in the mutant, only in the nonmutant, or in both mutant and nonmutant genotypes (Figure 5). Collectively, we refer to the 120 UVB × Mc1r genes as the Mc1r-dependent UVB-responsive genes (Table S4) and the 147 UVB × Kit genes as the Kit-dependent UVB-responsive genes (Table S5).

Surprisingly, we found very little overlap between the Mc1r- and the Kit-dependent UVB-responsive genes (only five in common of a total of 267), suggesting that the biological processes engaged by Mc1r signaling in the response to UVB are distinct from the ability of pigment cells to protect against UV radiation by providing a simple physical barrier. To further explore this idea, we extracted and compared GO terms derived from the Mc1r- and the Kit-dependent UVB-responsive gene sets using the same approach described earlier (Figure 3). Significant (p = 0.05) GO terms that contained at least three hits and that were enriched by at least 2-fold over the 35,000 array are depicted in Figure 6 for both sets of genes. The Mc1r-dependent UVB response was characterized largely by genes involved in the cell cycle (Cdc211, Fos, Hspa8, Mtcp1, Top3a, and 2810406C15Rik) and oncogenesis (Cdc211, Fg3f1, and Mtcp1) and included functions and components relating to GTPase-mediated signal transduction (Grb2, Rab7, Rab12, Rab35, and Rabbp1), chromatin (H2faz, Sema1, and 5430405G24Rik), ribosomes (Rpl8, Rpl13, and Rps16), and mitochondria (Atpjfl, C1qbp, Cndp2, Mtcp1, Pdha1, and Phb).

By contrast, the Kit-dependent UVB response was characterized largely by genes involved in glycoprotein biosynthesis (Ciga1atc1, Itml1, Man1a2, and Prksh) and the response to oxidative stress (Pdrx5, Seph1, and Sth25) and included functions and components relating to protease activity (Adcy7, Col6a1, Klk5, Hspb8, and Mhps1) and the Golgi transport system (Inpp5e, Man1a2, Rer1, Etx1, Grb2, Inpp5e, Man1a2, Rer1, and Surf4).

To better understand how the set of Mc1r-dependent UVB-responsive genes in mice might be related to MC1R genotype as a risk factor for human skin cancer, we considered Mc1r-dependent UVB-responsive genes known to have GO-anno-
tated roles in the cell cycle (Table 1). In nearly every case, the direction in which expression of these genes was altered suggested that Mc1r signaling might facilitate UV-induced cell cycle arrest. For example, Phb, a negative regulator of the cell cycle, was induced by UVB in Mc1r<sup>+</sup>/Mc1r<sup>+</sup> animals but not in Mc1r<sup>e</sup>/Mc1r<sup>e</sup> animals. Similarly, Bop1, another negative regulator of the cell cycle, was repressed by UVB in Mc1r<sup>e</sup>/Mc1r<sup>e</sup> animals but not in Mc1r<sup>+</sup>/Mc1r<sup>+</sup> animals, while Mark3 and Cdc2l1, both positive cell cycle regulators, were induced by UVB in Mc1r<sup>e</sup>/Mc1r<sup>e</sup> animals but not in Mc1r<sup>+</sup>/Mc1r<sup>+</sup> animals.

We also considered whether any of the Mc1r-dependent UVB-responsive genes had previously been implicated in cancer biology by virtue of their aberrant regulation. Of 20 genes for which roles in cancer susceptibility or progression had previously been suggested (Table 2), 75% exhibited the direction of change expected for a protective Mc1r-dependent UVB response in the basal epidermis. For example, whereas Arpc1b is normally induced in the Mc1r<sup>+</sup>/Mc1r<sup>+</sup> basal epidermis, it remained unresponsive in Mc1r<sup>e</sup>/Mc1r<sup>e</sup> animals and is frequently silenced in gastric cancers [53]. In contrast, while Mapkapk2 was normally unresponsive to UVB in the basal epidermis of Mc1r<sup>+</sup>/Mc1r<sup>+</sup> animals, it was induced in Mc1r<sup>e</sup>/Mc1r<sup>e</sup> animals and has been reported to be activated in some breast cancers [54].

**Figure 6.** Over-Representation Analysis of Functional GO Terms for Mc1r- and Kit-Dependent UVB-Responsive Genes in the Basal Epidermis of P2.5 Mice

GO terms, using EASE software [48], were mapped to (A) 120 Mc1r-dependent UVB-responsive genes (red bars) and (B) 147 Kit-dependent UVB-responsive genes (blue bars). GO terms that were significantly enriched (p = 0.05), by 2-fold, relative to all of the assayed genes on the 35,000 array (35,328 genes) (black bars) and that contained at least three gene hits, are displayed. GO terms are ranked, within high level GO branches, by fold-enrichment relative to the 35,000 array. A thumbnail image provides a reference for each of the analyzed UVB response classes.
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**Table 1.** Mc1r-Dependent UVB-Responsive Genes Previously Reported to Regulate the Cell Cycle

| Gene       | UVB Response | Activity in Cell Cycle | Reference |
|------------|--------------|------------------------|-----------|
| Mapkapk2   | Mc1r<sup>e</sup>/Mc1r<sup>e</sup> | +                       | [80]      |
| Lglh       | Mc1r<sup>e</sup>/Mc1r<sup>e</sup> | –                       | [81]      |
| Rac1       | Both<sup>+</sup> | –                       | [82]      |
| If3        | Mc1r<sup>e</sup>/Mc1r<sup>e</sup> | +                       | [83]      |
| Ppp3ca     | Mc1r<sup>e</sup>/Mc1r<sup>e</sup> | +                       | [84]      |
| Phb        | Mc1r<sup>+</sup>/Mc1r<sup>+</sup> | –                       | [85]      |
| 2810406C15Rik | Mc1r<sup>e</sup>/Mc1r<sup>e</sup> | +                       | [86]      |
| Mtcp1      | Mc1r<sup>+</sup>/Mc1r<sup>+</sup> | +                       | [87]      |
| Hsp8       | Mc1r<sup>e</sup>/Mc1r<sup>e</sup> | +                       | [88]      |
| Fox        | Mc1r<sup>+</sup>/Mc1r<sup>+</sup> | +                       | [89]      |
| Mark3      | Mc1r<sup>e</sup>/Mc1r<sup>e</sup> | +                       | [90]      |
| Cdc2l1     | Mc1r<sup>e</sup>/Mc1r<sup>e</sup> | +                       | [91]      |
| Bop1       | Mc1r<sup>e</sup>/Mc1r<sup>e</sup> | –                       | [92]      |

<sup>+</sup> (Induction) or | (repression) in the indicated genotype.
<sup>+</sup> (Positive) or – (negative) regulator of the cell cycle.
UVB-responsive in both genotypes.
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We also considered whether any of the Mc1r-dependent UVB-responsive genes had previously been implicated in cancer biology by virtue of their aberrant regulation. Of 20 genes for which roles in cancer susceptibility or progression had previously been suggested (Table 2), 75% exhibited the direction of change expected for a protective Mc1r-dependent UVB response in the basal epidermis. For example, whereas Arpc1b is normally induced in the Mc1r<sup>+</sup>/Mc1r<sup>+</sup> basal epidermis, it remained unresponsive in Mc1r<sup>e</sup>/Mc1r<sup>e</sup> animals and is frequently silenced in gastric cancers [53]. In contrast, while Mapkapk2 was normally unresponsive to UVB in the basal epidermis of Mc1r<sup>+</sup>/Mc1r<sup>+</sup> animals, it was induced in Mc1r<sup>e</sup>/Mc1r<sup>e</sup> animals and has been reported to be activated in some breast cancers [54].

**MC1R-Dependent UVB-Responsive Genes, Melanoma Profiling, and Cancer Biology**

Given the over-representation of cell cycle and oncogenesis genes in our Mc1r-dependent UVB response, we sought to determine whether the expression patterns of these genes might be informative in a clinical setting. Among a number of previous microarray studies of human melanoma or melanoma cell lines, we identified two large data sets based on tissue samples from either primary melanoma [55] or melanoma metastases [56].

Starting with our 120 Mc1r-dependent UVB-responsive genes, we were able to retrieve gene expression data for 86 and 53 orthologous human genes from the primary melanoma and the melanoma metastases data sets, respectively (see Materials and Methods for further details), of which approximately one third (47) were shared between the two data sets. Hierarchical clustering revealed that our Mc1r-dependent
**Validation, Comparison, and Context**

The microarray platform and design used here are identical to those of a previous study in which we compared the primary melanoma subclusters obtained here were nearly identical to those originally identified, with the primary melanoma subtypes [55] corresponding to TNM stage (I-II versus III-IV), and the melanoma metastases subtypes [56] corresponding to growth phase (type I radial, in situ and minimally invasive tumors, versus type II vertical, aggressive metastatic tumors). Of the 86 genes tested on the primary melanoma data set, expression levels for 19 distinguished the different stages, with all of the 19 underexpressed in the stage III-IV samples relative to the stage I-II samples (Figure 7A). Of the 53 genes tested on the melanoma metastases data set, expression levels for 33 distinguished the different growth phases, with 10 and 23 genes overexpressed and underexpressed in the type I radial growth relative to the type II vertical growth samples, respectively (Figure 7B). Closer inspection of the overlap between the 19 and 33 genes used to distinguish the primary melanoma and melanoma metastases data sets revealed nine genes in common: ACTN1, ARPC1B, IMP3, KIAA0553, MGC3731, MTCPI, RAC1, RALBP1, and SDFR1.

**Discussion**

Of 28 previous studies designed to study the large-scale transcriptional response to UV radiation, only three examined tissues or tissue compartments; the remainder focused on cultured cells. In two studies from Hochberg and colleagues [57,58] where the UV response of human suprabasal epidermis (obtained from suction blisters) was compared directly to that of cultured human keratinocytes using the same platform, only 207 of 1,931 genes found to be UV responsive were themselves less likely to sustain UV-induced DNA lesions. Very genes required for a critical response to UV radiation, including DNA repair and cell cycle; moreover, the pattern in which specific characteristics of the UV response are characterized by small changes in expression that affect a large number of genes. All of these features may be linked to the observation made here and by McKay et al. [47] that UV-induced and UV-repressed genes differ not only in their biological function but also in their size, suggesting evolutionary pressure to ensure that the very genes required for a critical response to UV radiation are themselves less likely to sustain UV-induced DNA lesions.

In addition to providing a broader perspective on the transcriptional response to UV radiation, our work helps to explain the epidemiologic relationship between MC1R genotype and skin cancer susceptibility. The set of Mc1r-dependent UVB-responsive genes we identified is greatly enriched for processes and functions involved in oncogenesis and the cell cycle; moreover, the pattern in which specific genes change suggests that a functional Mc1r contributes to the ability of UVB to induce cell cycle arrest in the basal epidermis (Figure 8). Our work on the genome-level response to UV radiation was necessarily carried out in laboratory mice, where the availability of specific mutations on a defined genetic background allows a substantial degree of experi-
mental control. However, our conclusions about the general relationship between MC1R genotype and cancer susceptibility in the mouse are supported by previous studies of two specific melanoma genes in humans: CDKN2A (a tumor suppressor) and BRAF (a proto-oncogene). In families carrying a germline CDKN2A mutation, the risk of melanoma was substantially increased by the presence of an MC1R variant [59–63]. In recent work from Landi et al. [64], germline MC1R variation was found to be a risk factor for developing melanomas with BRAF mutations. Because the association between MC1R variation and BRAF was most apparent in melanomas without evidence of chronic sun damage, Landi et al. hypothesized that UV radiation acted indirectly to promote BRAF-mutant melanoma, which is also consistent with our findings.

The nature of our experimental system—an integrated tissue collected from whole animals—makes it difficult to assess whether Mc1r signaling might affect UVB-induced cell cycle arrest in keratinocytes, melanocytes, or both. However, in recent work from Yamaguchi et al. [65] where the effects of UV radiation on cutaneous DNA damage and cell death were examined in individuals with different pigmented phenotypes, light skin was found to exhibit a surprising lack of UVB-induced apoptosis in many cells of the basal epidermis, as well as their chromosomal location. Genes highlighted in red are common to both primary and metastatic melanoma gene clusters. Red, green, and black indicate induction, repression, and no change, respectively, relative to the centered median. Gray represents missing data. The ratio fold-change is indicated by a scale bar.

Figure 7. Unsupervised Hierarchical Clustering of Mc1r-Dependent UVB-Responsive Genes in Human Melanoma Samples
Orthologous human gene expression data, derived from 120 mouse genes that were identified as Mc1r-dependent and UVB-responsive in the basal epidermis of P2.5 mice, were clustered in (A) a primary melanoma data set [55], here consisting of 86 genes and 45 samples, and (B) a melanoma metastases data set [56], here consisting of 53 genes and 19 samples. Melanoma subclusters correspond to the original subtypes, TNM stage I-II (blue) versus III-IV (orange) in (A), first identified by Talantov et al. [55], and growth phase type I radial (orange) versus type II vertical (blue) in (B), first identified by Haqq et al. [56]. Genes driving the observed melanoma clustering patterns are displayed to the right of each large cluster, along with their corresponding UVB response (↑, induction; ↓, repression; and Both, regulation in both Mc1r+/Mc1r+ and Mc1re/Mc1re) in the Mc1r+/Mc1r+ or Mc1re/Mc1re basal epidermis, as well as their chromosomal location. Genes highlighted in red are common to both primary and metastatic melanoma gene clusters. Red, green, and black indicate induction, repression, and no change, respectively, relative to the centered median. Gray represents missing data. The ratio fold-change is indicated by a scale bar.
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autonomous perspective, phosphorylation of p53 Ser389 is a critical and major step in triggering apoptosis in response to UV-induced DNA damage [66], and it is possible that MC1R activation (either constitutive or in response to UV radiation) plays a permissive role. However, recent studies by Abdel-Malek and colleagues [67] indicate that MC1R deficiency causes increased UV-induced apoptosis in cultured melanocytes. Thus, a more likely scenario is that decreased MC1R signaling in melanocytes exposed to UV radiation in vivo impairs the ability of surrounding cells to undergo p53-mediated cell cycle arrest and apoptosis. Indeed, previous work from our group and others indicates that while Mc1r expression is confined mainly to melanocytes, Me1r deficiency leads to substantial changes in gene expression throughout the skin and suggests that melanocytes influence the behavior of surrounding cells via paracrine mechanisms. For example, L-type prostaglandin D2 synthase (Pgdels) is expressed at high levels in pigment cells [68] and is repressed in Mc1r+/Me1r+ skin (current data, [49]); prostaglandin D2 and its derivatives could be one of several mediators released from Mc1r-

Several known antioxidant genes, including Glo1, Prdx5, Sepp1, and Sh25, were all repressed in basal epidermis of Kit+/Kit+ animals but not KitW-Wo/KitW-Wo animals. These results are consistent with reports in which UVB, in inducing oxidative stress, was reported to deplete the antioxidant defense system in the epidermis of mice [70,71] and humans [72]. We also identified additional genes, within the Kit-dependent UVB response, that were not annotated in the GO database as oxidative or endoplasmic reticulum (ER) stress response genes, including Cdk5, Egr1, Egln1, Egr1, Gpr175, Gpt2, Ilm1, Nar1, Pahp1, Pecam1, Prdx4, Ptkp1i, Rhl, S3glb1, Tbc1d20, and Tub3, for which roles in oxidative stress/hypoxia and the ER stress/unfolded protein response have been reported [73–76]. Several other genes involved in the ER/Golgi trafficking or folding of proteins, but not previously reported to be regulated during oxidative stress/hypoxia or the unfolded protein response, were also identified, including Exd1, Ergc3, Gbr2, Inpp5e, Manal2, Prkcsb, Rer1, and Surf4, raising the possibility that these genes may represent new skin stress response genes. Given the absence of this oxidative/ER stress response signature in our Mc1r-dependent UVB response, we interpret our findings to suggest that melanocytes, despite constituting a small fraction of the skin cell population, play a major role in oxidative and ER stress in the basal epidermis and, significantly, that this role is independent of Mc1r signaling.

Taken together, our results provide a molecular framework for the underlying mechanisms by which melanocytes and the Mc1r independently mediate, and afford protection against, UVB signals within the normal skin environment. This latter finding is of particular significance as it suggests insights into the molecular mechanisms by which MC1R mutations predispose toward melanoma susceptibility.

Materials and Methods

Mice and UVB irradiation. C57BL/6J mice of three different pigmentation genotypes—Mc1r+/Mc1r+, Kit+/Kit+, Mc1r+/Me1r+; Kit+/Kit+—were obtained from The Jackson Laboratory (Bar Harbor, Maine, United States). P1.5 pups were immobilized (ventrum side down) with double-sided adhesive tape on an inverted plastic mouse cage. The dorsums of the pups were irradiated with a single dose of 10 mJ/cm² UVB, using a Stratagene 2400 UV crosslinker (Stratagene, http://www.stratagene.com), equipped with five 15-W bulbs with a peak emission wavelength of 312 nm. The irradiance of the bulbs was measured with an International Light 1400 Radiometer (International Light, http://www.int-light.com). The administered UVB dose corresponded to a mild sunburn dose, approximately one minimal erythemal dose, for the C57BL/6J strain [77]. Control pups from all three genotypes were shielded from UV irradiation with a black Perspex box. Immediately postirradiation, all pups were returned to their mothers for 24 h, after which they were killed. Because Kit+/Kit+ mouse is sterile, homozygous neonates were obtained by heterozygous matings followed by a polymerase chain reaction and NciI restriction digest–based diagnostic genotyping assay for the KitW-Wo mutation [78]. Dorsal trunk skins were dissected and basal epidermal cells were prepared and stored as previously described [49].

Experimental design. The methodology described here conforms

![Figure 8. Model of MC1R-Dependent Protective Mechanisms against UVB Radiation in the Basal Epidermis](image-url)
Gene expression patterns were studied in the basal epidermis of neonatal mice by comparing three groups of animals (Mc1r+/Mc1r+; Kit+/Kit+, Mc1r+/Mc1r+; Kit−/Kit−, and Mc1r+/Mc1r+; Kit−/Kit−) under two conditions (+UVB and +KitW−/KitW−). We thus studied six groups altogether, with three biological replicates each. Each epidermal replicate was derived from a separate pool containing between two and four neonatal litters. We used a common reference experimental design in which each basal epidermal sample was hybridized together with a common RNA reference pool. This reference pool has been previously described [49] and allowed us to easily perform comparisons across all of our basal epidermal samples.

**Microarray design.** The cDNA microarrays used in this study were generated at Stanford University, Stanford Functional Genomics Facility (www.stanford.edu/group/microarray), using standard protocols (http://cmgm.stanford.edu/pbrown/protocols/index.html). Briefly, 35,328 cDNA clones, representing 14,605 unique mouse transcripts (UniGene Build 148), were obtained from the following five sources: 16,896 clones from RIKEN (http://fantom.gsc.riken.jp), 15,264 clones from the University of California, Institute on Aging (http://uqun.grc.nia.nih.gov/cDNA/15k.html), 1,032 LMA.G.E. clones from Invitrogen/Research Genetics (http://www.invitrogen.com), 960 clones from the Brain Molecular Anatomy Project (http://trans.nih.gov/bmap/resources/resources.htm), and 576 hand-selected clones from Stanford University.

**RNA labeling.** Total RNAs were extracted from the basal epidermal cells using TRIzol Reagent (Invitrogen) followed by further purification using a RNeasy cleanup protocol (Qiagen, http://www.qiagen.com). Experimental and reference RNA samples (20 μg each) were reverse-transcribed and then directly labeled using an anchored oligo-dT-primer (Qiagen) and a Cy3/Cy5-labeled First-Strand cDNA Labeling Kit (Amersham Biosciences, http://www.amersham-biosciences.com), as per the manufacturer’s instructions. The RNA was degraded in 1N NaOH for 30 min at 70°C followed by neutralization in 1N HCl. The Cy5-Cy3 labeled basal epidermal and Cy3-Cy5-labeled reference samples were combined, concentrated using Centriprep YM-30 micro-concentrators (Millipore/Amicon, http://www.millipore.com), and then competitively hybridized to a 35,000 spotted glass mouse microarray slide.

**Microarray hybridizations.** Hybridizations were performed in 32-μl reaction volumes, as previously described [49] in 3.4× standard sodium citrate (SSC), 0.3% sodium dodecyl sulfate, containing 1 μg/ml mouse Cot-1 DNA (Invitrogen), 10 μg/ml yeast tRNA (Invitrogen), and 10 μg/ml synthetic poly(A)+ RNA (Sigma-Aldrich, http://www.sigmaaldrich.com), at 65°C for 16 h, using custom-designed humidified hybridization chambers (Die-Tech, http://www.die-technology.com). Posthybridization washes were done for 2 min in each of 2× SSC, 0.03% sodium dodecyl sulfate, 2× SSC, 1× SSC, and 0.1× SSC. All washes were performed at room temperature. Slides were dried by centrifugation for 10 min at 900 rpm immediately prior to scanning.

**Data processing and statistical analyses.** Images were acquired with a GenePix 4000A scanner and feature measurements were extracted using GenePix Pro 3.0 software (Axon Instruments, http://www.axon.com). Raw data were uploaded into the Stanford Microarray Database (publicly available at http://smld.stanford.edu) where background-subtracted fluorescence ratios were linear-normalized so that the mean Cy5/Cy3 ratio on each array was 1. Good-quality spots were selected by manually excluding spots that were irregularly shaped or of small diameter or contained excessive background intensities. We next selected spots whose median signal intensity was 1.5-fold above the median background for both Cy5 and Cy3 channels. Our normalized, background-subtracted, raw data set totaled 31,756 spots. For our statistical analyses, the raw data set was filtered such that measurements were present in at least two of three replicates per experimental condition. This filter reduced our data set to 13,508 cDNAs (9,110 unique genes). Student’s t- and two-way ANOVA tests were performed using GeneSpring Software (Agilent, Palo Alto, California, United States) on centered data sets, in which the median log2 ratio for each gene equaled 0. Over-representation analysis was performed using EASE [48], which reports functional gene categories as statistically significant GO terms. Significance values were adjusted for multiple hypothesis testing by the FDR, and values with an FDR of 0.05 were selected for further analyses.

**Unsupervised hierarchical clustering of melanoma data sets.** Using our list of 120 Mc1r-dependent UVB-responsive mouse genes, together with NanoString Center for Biotechnology Information’s HomoSapiens and BLASTn algorithms, we retrieved gene expression data for 147 and 74 orthologous human genes from the primary melanoma and melanoma metastases data sets of Talantov et al. [55] and Haqq et al. [56], respectively. The log2 ratios for both data sets were median-centered separately, and nonredundant human gene lists for each of the data sets were generated by averaging expression data for gene replicates. The resulting primary melanoma data set, now consisting of 86 genes and 45 samples, and the melanoma metastases data set, now consisting of 53 genes and 19 samples, were clustered separately using an unsupervised algorithm in Cluster [79], in which we centered correlation coefficients (similarity metric) for both samples and genes underwent average linkage clustering. The results are displayed as heatmaps using Treeview software [79].

## Supporting Information

### Table S1. UVB-Responsive Genes in the +/- Basal Epidermis of P2.5 Mice

| Found at doi:10.1371/journal.pgen.0030095.st001 (684 KB PDF). |

### Table S2. Enriched GO Terms for Downregulated Genes in the Basal Epidermis of P2.5 KitW+/- KitW−/KitW− Mice

| Found at doi:10.1371/journal.pgen.0030095.st002 (67 KB PDF). |

### Table S3. Enriched GO Terms for Upregulated Genes in the Basal Epidermis of P2.5 KitW+/KitW− Mice

| Found at doi:10.1371/journal.pgen.0030095.st003 (68 KB PDF). |

### Table S4. Mc1r-Dependent UVB-Responsive Genes in the Basal Epidermis of P2.5 Mice

| Found at doi:10.1371/journal.pgen.0030095.st004 (50 KB PDF). |

### Table S5. Kit-Dependent UVB-Responsive Genes in the Basal Epidermis of P2.5 Mice

| Found at doi:10.1371/journal.pgen.0030095.st005 (58 KB PDF). |

### Table S6. Summary of Large-Scale Gene Expression Studies that Have Identified UV-Responsive Genes

| Found at doi:10.1371/journal.pgen.0030095.st006 (89 KB PDF). |

### Table S7. Nonoverlapping UVB-Responsive Genes in the +/- Basal Epidermis of P2.5 Mice

| Found at doi:10.1371/journal.pgen.0030095.st007 (212 KB PDF). |

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**Author contributions.** Both authors conceived and designed the experiments. CSA performed the experiments. Both authors analyzed the data and contributed to writing the paper.

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