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Title: The Role of Keratinocytic RXRα in Regulating Melanocyte Homeostasis and Carcinogen Induced Melanomagenesis

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Cutaneous melanoma remains the deadliest form of skin cancer, with a diagnosis of metastasis indicating a median survival rate of less than a year. Solar ultraviolet (UV) radiation, especially childhood sun exposure, is an important etiological risk factor of melanoma. Previous studies determined that mice selectively lacking the nuclear hormone receptor Retinoid X Receptor α in epidermal keratinocytes ($RXR\alpha^{ep-/-}$) developed a higher number of aggressive melanocytic tumors compared to wild type mice after two-step chemical carcinogenesis, suggesting a novel role of keratinocytic nuclear receptor signalling during melanoma progression. We then discovered a progressive loss of RXRα expression in epidermal keratinocytes during

melanoma progression in humans. We also investigated the contributions of $CDK4^{R24C/R24C}$ and keratinocytic RXRα to influence metastatic progression in a mouse model by generating $RXR\alpha^{ep-/-}/CDK4^{R24C/R24C}$ bigenic mice containing an activated cyclin dependent kinase 4 (CDK4), besides lacking RXRα in epidermal keratinocytes. Those bigenic mice developed malignant melanomas that metastasized to regional lymph nodes after carcinogen exposure. Expression of several keratinocyte-derived growth factors implicated in melanomagenesis were upregulated in the skin of bigenic mice, and recruitment of RXRα was shown on the promoters of endothelin-1 (*Edn1)* and hepatocyte growth factor (*Hgf)*. We then confirmed a downregulation of factors (FAS, E-cadherin and PTEN) implicated in apoptosis, invasion and survival within the melanocytic tumors.

To further evaluate the paracrine role that EDN1 has on melanocyte activation, we utilized a transgenic mouse model where the gene encoding *Edn1* was selectively ablated from epidermal keratinocytes using the Cre-LoxP strategy to create the $EDN1^{ep-/-}$ knockout mouse line. We discovered a direct *in vivo* transcriptional regulation of keratinocytic *Edn1* by the tumor-suppressor p53 in epidermal keratinocytes in response to UV irradiation. We also demonstrate that *in vivo* disruption of keratinocyte-derived EDN1 signaling alters melanocyte proliferation and decreases epidermal and dermal melanocyte populations in both normal and UV exposed mouse skin. EDN1

also has a protective role against UVR-induced DNA damage and apoptosis and similar effects on UV-induced melanocyte proliferation and DNA damage are observed in p53-null mice. Inhibition of EDN1 signaling by topical application of an EDNRB antagonist BQ788 on mouse skin also recapitulates epidermal EDN1 ablation. Furthermore, treatment of primary murine melanocytes with BQ788 abrogates signaling downstream of this receptor.

Taken together, these studies demonstrate the contribution of RXRα regulated keratinocytic paracrine signaling during the cellular transformation and malignant conversion of melanocytes. Also, they establish an essential role of EDN1 in epidermal keratinocytes to mediate UV-induced melanocyte homeostasis *in vivo*.

The Role of Keratinocytic RXRα in Regulating Melanocyte Homeostasis and Carcinogen Induced Melanomagenesis

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Stephen D. Hyter, Author

DEDICATION

To Stella and Emmet, everybody needs some motivation

CONTRIBUTION OF AUTHORS

Dr. Arup K. Indra helped design research experiments and Stephen D. Hyter primarily performed the research. Stephen D. Hyter and Dr. Arup K. Indra analyzed most of the data and wrote the manuscripts. Dr. Gaurav Bajaj and Xiaobo Liang assisted in western blot experiments. Gitali Indra provided critical analyses of manuscripts. Mariano Barbacid provided the $CDK4^{R24C/R24C}$ mice, Masashi Yanagisawa provided the $EDN1^{L2/L2}$ mice and Gary Merrill provided the $p53^{-/-}$ neonatal mice as well as genotyping strategies. Daniel Coleman and Steven Ma performed histopathological analysis and assisted with *in vitro* work.

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Chapter 1

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1.1 Abstract

Skin homeostasis is maintained, in part, through regulation of gene expression orchestrated by type II nuclear hormone receptors in a cell and context specific manner. This group of transcriptional regulators is implicated in various cellular processes including epidermal proliferation, differentiation, permeability barrier formation, follicular cycling and inflammatory responses. Endogenous ligands for the receptors regulate actions during skin development and maintenance of tissue homeostasis. Type II nuclear receptor signaling is also important for cellular crosstalk between multiple cell types in skin. Overall, type II nuclear receptors are critical players in keratinocyte and melanocyte biology and present targets for cutaneous disease management.

1.2 Mechanisms of action for type II nuclear receptors

Transcriptional control of gene expression is achieved, in part, through protein factors bound to regulatory elements present on the chromatin. The type II nuclear receptors (NR), belonging to the superfamily of steroid-thyroid hormone nuclear receptors, contribute to the cellular responses of physiological demands (Evans, 1988; Mangelsdorf et al., 1990; Mangelsdorf et al., 1995; Chambon, 1996). Transcriptional modulation is achieved by structural adjustments initiated through ligand binding. Present throughout the animal kingdom, this family of environmental sensors contributes both positively and negatively to gene expression. This differential regulation is useful in organismal development and homeostasis, though it is also implicated in a variety of pathological conditions. The present review will only detail the contributions of type II NRs towards epidermal and follicular development and homeostasis, and in skin diseases. Particular emphasis is given on melanocyte biology and in melanomagenesis arising from altered signaling between keratinocytes and melanocytes, while highlighting the potential therapeutic value of these pliable receptors.

Type II NRs belong to a larger family of steroid hormone receptors, all sharing similarities in domain structure (Figure 1.1) (Escriva et al., 1998; Kumar and Thompson, 1999). Distinct variations in domain sequence has

allowed for the diversification and specialization currently present within the family (Laudet, 1997). The DNA binding domain is highly conserved across the family and contains two zinc finger motifs. These domains recognize and bind short response elements, allowing for both homo- and hetero-dimerization combinations. Two activation domains called AF-1 and AF-2 assist the receptors in dimerization and DNA binding. Variability is more evident within the carboxyl terminal ligand binding domain, where individual receptors have evolved to bind a variety of signaling molecules (Bourguet et al., 2000). Receptors for which ligand specificity has yet to be determined are labeled as orphan receptors. Endogenous ligands for NRs known to be expressed in skin include: all-trans retinoic acid (RA) and 9-*cis* RA for retinoic acid receptor (RAR) (Giguere et al., 1987; Petkovich et al., 1987), 9-*cis* RA for retinoid-X-receptor (RXR) (Mangelsdorf et al., 1990; Heyman et al., 1992), 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) for vitamin D receptor (VDR) (Simpson and DeLuca, 1980), fatty acids/lipids for peroxisome proliferator-activated receptor (PPAR) (Forman et al., 1995; Kliewer et al., 1995; Kliewer et al., 1997; Forman et al., 1997), oxysterols for liver X receptor (LXR) (Janowski et al., 1996) and triiodothyronine for thyroid receptor (TR) (Samuels et al., 1974).

A distinguishing feature of type II NRs is the promiscuity displayed by RXR. All NRs from this class form heterodimers with an isoform of RXR ($\alpha/\beta/\gamma$) and regulate gene expression in a ligand dependent fashion. RXRα is able to

heterodimerize with some 15 NR family members and occupy direct repeat response elements present on the promoters of target genes (Leid et al., 1992; Kliewer et al., 1992; Willy et al., 1995; Chandra et al., 2008; Orlov et al., 2012). The nonsteroidal ligands of RXR/NR heterodimers dictate the organization of complexes associated with the receptors. Serial combinations of regulatory proteins allow chromatin remodeling and recruitment of basal factors to initiate and/or repress transactivation (Figure 1.2) (Glass and Rosenfeld, 2000; Perissi and Rosenfeld, 2005). Coactivators include ATP-dependent chromatin remodelers, histone acetyltrasferases and the Mediator complex (Dilworth et al., 2000; De Luca et al., 2003; Blanco et al., 1998; Fondell et al., 1996; Rachez et al., 1999). Corepressors comprise the N-CoR/SMRT assembly and histone deacetylases (Underhill et al., 2000; Yoon et al., 2003; Miyata et al., 1998; Lee and Wei, 1999). The large numbers of regulatory factors, as well as tissue specific localization, allow NRs to influence a diverse range of gene expression in a cell and tissue specific manner. For example, the PPARγ cofactor PGC-1 is present in adipose tissue but not fibroblasts, allowing a cell-type specific activation of genes related to adaptive thermogenesis (Puigserver et al., 1998). Post-translational modifications of co-factors such as phosphorylation, methylation, sumoylation and ubiquitination are also known to contribute to the extensive specificity of NR regulation (Hermanson et al., 2002).

1.3 Skin morphogenesis, epidermal homeostasis and hair cycling

Skin is the largest organ in the body and is comprised of multiple cell types such as epidermal keratinocytes, dermal fibroblasts and hypodermal adipocytes, besides Langerhans cells, melanocytes and endothelial cells. It utilizes both autocrine and paracrine signaling for development and maintenance of tissue homeostasis (Fuchs and Raghavan, 2002). The outermost epidermal layer provides a protective barrier to environmental and physical stresses and constantly progresses through cycles of proliferation and differentiation. Basal keratinocytes located on the innermost epidermal basement membrane (separating epidermis from the underlying dermis) generate daughter cells, which undergo committed differentiation that give rise to ordered layers of suprabasal early- and late-differentiated keratinocytes. Appendages such as hair follicles and sebaceous glands (SGs) are invaginated into the mesenchymal-derived dermal layer. Different resident multipotent skin stem cell (SC) niches contribute to the renewal, maintenance and repair of the epidermal tissues of the skin, including interfollicular epidermis (IFE), hair follicles and SGs (Fuchs, 2007). Epidermal SCs play a crucial role in maintaining tissue homeostasis by supplying new daughter cells to replace those constantly lost during turnover or following injury. Bulge SCs are known to maintain normal follicle homeostasis and can also contribute to

the formation of IFE following skin injury and during wound healing. Melanocytes are also located within the hair follicles where they primarily contribute pigmentation to coat color. In humans, IFE melanocyte populations rely on keratinocytic paracrine signaling for assistance in photoprotection from solar ultraviolet irradiation (Nishimura, 2011).

Type II nuclear receptor signaling contributes to the perpetual renewal of keratinocytic layers, maintenance of the epidermal permeability barrier (EPB) and follicular cycling. Impaired expression or function of these receptors is implicated in aberrant proliferation and/or differentiation of epidermal tissue and alopecia (hair loss). The promiscuous role that RXRs play in type II NR signaling hints to the impact that impaired RXR expression or activity influences receptor signal transduction. Although many of RXRs heterodimer partners are implicated in a wide variety of skin diseases, in many cases these pathologies could instead be associated with compromised RXR mediated gene regulation. Regardless, only studies that have specifically indicated a specific role for RXR function will be discussed here. RXRs play a critical and varied role in epidermal differentiation and EPB maintenance. RXRα, the primary isoform in skin and hair follicles, has stronger expression levels compared to RARs, and RXRα/RARγ heterodimer appears to be the major retinoid transducing element in epidermal biology (Fisher et al., 1994; Billoni et al., 1997; Feng et al., 1997). RXRα/RARγ heterodimerization is also critical in

the development and formation of epidermal lamellar granules by repression of target genes, as RARγ agonists promote lamellar granule defects in murine skin. Similarly, RXRα/PPARβ/δ heterodimers are equally important to stratum corneum homeostasis through activation of gene transcription (Calleja et al., 2006).

The importance of vitamin A signaling to cutaneous homeostasis has long been observed. Although, comprehensive studies have investigated multiple proteins along this pathway in skin (such as retinoic acid binding proteins), this review will focus primarily on RARs in human and rodent skin, particularly the role of RARγ (Fisher et al., 1994; Chapellier et al., 2002). RA is widely used as a therapeutic against various skin diseases due to its inhibitory effects on keratinocytic terminal differentiation, besides regulating keratinocyte proliferation and apoptosis, through modulation of RAR target genes (Lee et al., 2009). Management of keratin expression, which in turn dictates status of epidermal differentiation, is a known regulatory mechanism of cutaneous RARs (Tomic et al., 1990; Navarro et al., 1995; Virtanen et al., 2010). Unfortunately, the use of pan-retinoids is limited due to unwanted side effects such as skin irritation, hypertriglyceridemia, bone toxicity and teratogenicity (Chandraratna, 1998; Altucci and Gronemeyer, 2001). In order to utilize these efficacious compounds in a dermatological setting, receptor-selective retinoids

in parallel with prophylactic lipid management therapies may provide clinical benefits with decreased risk of toxicities.

Early studies have elucidated the synthesis of pre-hormone 25-hydroxycholecalciferol (calcifediol) by murine epidermal basal cells that acts in a paracrine manner for the CYP27B1 and VDR expressing differentiated keratinocytes (Rizk-Rabin et al., 1994). Typical of the type II nuclear receptors, $1,25(OH)_2D_3$-induced VDR activation inhibited proliferation and promoted differentiation of keratinocytes, though VDR/RXRα heterodimers can transactivate keratinocytic genes independent of $1,25(OH)_2D_3$ binding (Ellison et al., 2007). DeltaNp63alpha, a critical regulator of epidermal biology, is known to directly control VDR expression in murine skin and mediate its function in a ligand-independent manner (Kommagani et al., 2009). Mice bearing a targeted ablation of VDR present a large number of pathological properties in the skin, including progressive alopecia and dermal cysts as well as decreased expression of epidermal differentiation markers (Yoshizawa et al., 1997; Xie et al., 2002). By expressing in discrete epidermal locations, VDR-interacting coactivators contributed divergent roles towards keratinocyte proliferation and differentiation (Oda et al., 2009; Bikle et al., 2010). VDR and its associated cofactors also contributed to lipid production and epidermal barrier formation. Genetic network analysis has implicated VDR as a necessary regulator of skin barrier formation and predicts the phenotype

characterized in the VDR-null mice (Quigley et al., 2009). Many epidermal genes induced by WNT/β-catenin contain VDR response elements and were activated independently of TCF/LEF, implying that it is part of a TCF/LEF-independent aspect of WNT signaling (Palmer et al., 2008). Likewise, depletion of follicular keratinocyte populations in VDR-null mice was linked to aberration of the canonical WNT pathway (Cianferotti et al., 2007).

Alopecia is a commonplace in hereditary resistance to $1,25(OH)_2D_3$ and VDR expression is seen in the outer root sheath keratinocytes and dermal papilla of follicles, increasing during late anagen and catagen (Reichrath et al., 1994). The transcriptional repressor hairless (HR) co-localized with VDR in the outer root sheath of hair and has been shown to inhibit target genes leading to hair cycle progression (Haussler et al., 2010). Another VDR cofactor and critical subunit of the transcriptional coactivator complex Mediator (MED), MED1, has been recently linked to hair cycling and epidermal proliferation and differentiation. Selective ablation of MED1 in keratinocytes presents a similar phenotype to VDR or HR ablation (Oda et al., 2012). miR-125b is a known repressor of skin differentiation in the bulge region of the hair follicle and expressing miR-125b in SC progeny significantly downregulated VDR activity (Zhang et al., 2011).

PPARβ/δ expression was seen in both basal and suprabasal layers of the normal human epidermis, while PPARα and PPARγ expression was confined

to suprabasal epidermal cells and was linked to squamous differentiation (Westergaard et al., 2001). PPARs are transcriptionally regulated by AP-1, C/EBP family members and p63, the master regulator of epidermal morphogenesis and differentiation (Di-Poi et al., 2005; Pozzi et al., 2009). Mechanistically, inhibition of the PKC/MAPK pathway through PPAR-mediated PKCα-ubiqutitination has been implicated in reduction of epidermal proliferation (Kim et al., 2005). All PPAR isoforms were expressed in human hair follicle cells while PPARγ ablation in mice mimicked a human scarring alopecia phenotype (Karnik et al., 2009; Ruge et al., 2011).

The ability of LXRs to act as environmental cholesterol sensors enables them to contribute to consistent epidermal turnover which support a sound EPB. LXRα/β are expressed in all layers of the human epidermis, as well as in the outer root sheath and SG, and the LXR agonists oxysterols act on epidermal keratinocytes by reducing proliferation and inducing differentiation (Komuves et al., 2002). One proposed mechanism of LXR mediated induction of keratinocytic differentiation is influencing the binding of transcriptional complex AP-1 to target genes. ChIP-on-chip studies investigating LXRβ/RXRα binding in human keratinocytes revealed a strong correlation to AP-1 response elements, with up to 77% of all LXRβ/RXRα binding sites associated with AP-1 motifs (Shen et al., 2011). This could indicate LXR involvement in the

pathogenesis of certain diseases that present with abnormal keratinocytic differentiation and/or compromised barrier function.

Thyroid-stimulating hormone receptor signaling is commonly associated with cutaneous biology, though TR ligand binding is also known to contribute to epidermal homeostasis. Resistance to thyroid hormones is a syndrome with a wide variety of symptoms and is connected to TRβ mutations in in both mice and humans (Usala et al., 1990; Forrest et al., 1996). Follicular TRβ is the predominant isoform expressed within human skin and one phenotype of the disease linked to aberrant TRβ signaling is alopecia (Billoni et al., 2000; Guran et al., 2009). In support of a role for TRβ in hair cycle homeostasis, thyroid hormones and TRβ-selective thyromimetics are able to induce hair growth in both murine and simian models, as well as stimulate intrafollicular melanin production (van Beek et al., 2008; Li et al., 2010). TRs bind to conserved keratin response elements located in the promoter region of epidermal keratin genes and can directly control their transcription. One proposed mechanism of these genes involves transcriptional repression in the presence of a liganded TR receptor. In this scenario, proteins typically seen as components of a co-activator/histone acetylase complex display repressive tendencies in the presence of liganded receptors while co-repressors act as activators when unliganded TRs are present on the keratin response element (Jho et al., 2005). Transcriptome analyses from epidermal keratinocytes treated with

either thyroid hormone or vehicle display only a small number of genes that are differentially expressed. Among the suppressed genes (*integrin β4*, *plectin*, *collagen XVII*, *MMP1/3/14*) there was significant association to the blistering skin disease epidermolysis bullosa, implying an inhibitory role of thyroid signaling to extracellular matrix maintenance (Tomic-Canic et al., 2008).

1.4 Inflammatory skin diseases

Cutaneous inflammatory disorders are characterized by irritation and inflammation of the skin that can lead to patient discomfort or disfigurement. Psoriasis is a genetic-based cutaneous syndrome that involves epidermal hyperproliferation, compromised EPB and an infiltrative immune response. It affects around 5% of the population and is associated with additional maladies such as cardiovascular disease and non-melanoma skin cancer. It affects both sexes equally and tends to manifest within the first two decades of life (Benoit and Hamm, 2007). As it is currently incurable, primary treatment options include prevention of infection to minimize trauma. Control of symptoms is also important and includes the use of corticosteroids, phototherapy and moisturizing creams (Menter et al., 2008). Atopic dermatitis (AD) is another chronic inflammatory skin disease that is characterized by itchy skin, persistent infections and very early onset (Leung et al., 2004). AD is linked to other atopic diseases such as asthma and allergic rhinitis, and defective EPB function or environmental insults are predisposing factors for this heterogeneous disease (Kapoor et al., 2008; Cork et al., 2009). Treatment options are similar to those for psoriasis, while preventing future allergic reactions is also important. Unfortunately, the roots of these diseases may be too deeply embedded in immune functions to be eradicated. Due to early

onset and lifelong activity, the use of chronic corticosteroid therapy is discouraged. Knowledge regarding transcriptional regulation by key factors of EPB and epidermal proliferation gene networks enhances our retinue of therapeutic strategies in these diseases. NR ligands may be useful to modulate multiple pathways in order to lessen side effects and/or concentrations of the more efficacious compounds.

Mice with an epidermal-specific ablation of RXRα (RXRα$^{ep-/-}$) presented epidermal hyperplasia, alopecia, dermal cysts and a cutaneous inflammatory response (Li et al., 2000; Li et al., 2001). Likewise, mice lacking both RXRα and RXRβ in keratinocytes (RXRαβ$^{ep-/-}$) developed a chronic dermatitis similar to human AD patients, elevated serum IgE/IgG and cytokine production associated with Th2-type response. Importantly, thymic stromal lymphopoietin (TSLP) was strongly upregulated from the basal keratinocytes, potentially influencing the systemic AD phenotype in these mice (Li et al., 2005). To further support the hypothesis that loss of RXRα contributed to a derepressive mechanism on gene expression leading to inflammatory responses, expression of RXRα has been reported to decrease in human psoriatic lesions, with levels in progressive disease further reduced compared to stable stages (Feng et al., 2006).

Use of the anti-proliferative, pro-differentiative properties of 1,25$(OH)_2D_3$ is not ideal due to harmful calcemic effects. Therefore, synthetic deltanoids

represent the best way to initiate VDR activation for therapeutic or investigative purposes. It was previously discussed that keratinocytic RXRα/β ablation upregulates production of TSLP leading to an AD-like phenotype in the mouse. Interestingly, topical application of deltanoids also induces TSLP in epidermal keratinocytes, suggesting the role of RXR/VDR heterodimers in regulating *TSLP* expression in this cell type. That evidence suggests a role for VDR antagonists in the treatment of human AD patients (Li et al., 2006). Conversely, deltanoids were shown to significantly improve allergen-triggered eczema in a mouse model through increasing populations of FOXP3-expressing regulatory T cells (Hartmann et al., 2012). Further evidences are needed to determine how VDR transactivation can initiate anti-inflammatory mechanisms if deltanoids are to become accepted therapy for hyperproliferative epidermal disorders in the clinic.

The anti-inflammatory actions of PPARα offer additional treatment modalities for reoccurring skin conditions such as AD and psoriasis. Stimulation of epidermal differentiation and reduction of proliferation can potentially treat these conditions without severe side effects brought on by glucocorticoids (GC). PPAR$\alpha^{-/-}$ mice (with a germline deletion of the *Pparα* gene) subjected to antigen sensitization exhibit increased epidermal thickening and inflammatory responses compared to wild-type controls, potentially through the loss of IL-2-mediated induction of Treg populations (Dubrac et al., 2011). PPAR$\alpha^{-/-}$ skin

also display heightened levels of both Th2- and Th1-related responses, as well as enhanced NF-κB signaling. Furthermore, PPARα expression is downregulated in lesional skin of human AD patients compared to non-atopic individuals (Staumont-Salle et al., 2008). Topical application of PPARα agonists reduces the hyperplastic response in mouse skin brought on by TPA treatment, in part through the reduction of inflammatory cytokines. Similar results were seen in oxazolone (OX)-induced allergic dermatitis mouse models, and importantly, combination therapy of PPARα agonists and GCs for severe dermatitis prevented GC-induced side effects and inhibited rebound flares (Sheu et al., 2002; Hatano et al., 2011). Conversely, activation of PPARβ/δ may trigger psoriasis pathogenesis within the skin and treatment strategies may include antagonism of this signaling pathway. PPARβ/δ is upregulated at both the mRNA and protein level in psoriatic lesions compared to nonlesional skin in human patients and eicosanoid accumulations within the lesions can activate PPARβ/δ (Westergaard et al., 2003; Romanowska et al., 2010). Also, topical antagonists targeted to cutaneous PPARβ/δ demonstrated efficacy in a psoriatic mouse model (Hack et al., 2012). Alongside other physiological defects, PPARβ/δ-null mice exhibit an elevated epidermal hyperplastic response after TPA administration and delayed barrier recovery following acute barrier disruption (Peters et al., 2000; Man et al., 2008). An increase in the release of inflammatory cytokines after wound healing in

murine skin requires upregulation of PPARβ/δ that is otherwise undetected in normal skin. Interestingly, this pro-inflammatory cascade initiates production of endogenous PPARβ/δ ligands, reinforcing the activation of PPARβ/δ after skin injury (Tan et al., 2001; Tan et al., 2005). PPARβ/δ is also linked to UV-induced premature senescence via upregulation of PTEN that attenuates reactive oxygen species in keratinocytes (Ham et al., 2012). Likewise, epidermal PPARγ signaling is also a target for the UV-induced inflammatory response. UV irradiation of human keratinocytes produces potent PPARγ agonistic activity and enhances COX-2 expression in these cells. Furthermore, PPARγ activation was seen as a general consequence to various oxidative stressors in human sebocytes (Zhang et al., 2005; Zhang et al., 2006).

An increased accessibility of environmental antigens through epidermal tissue can activate cutaneous immune responses that potentially drive these syndromes. Creation and maintenance of the EPB is a multistep process involving lipid production and lamellar body formation and LXR activation appears to hold key functions in many of these stages (Fluhr et al., 2005; Man et al., 2006). EPB function is tightly linked to cholesterol levels in the epidermis and LXR has been shown to regulate the storage and efflux of lipid species. Members of the ATP-binding cassette transporter family, as well as glycerol channels, are transcriptionally regulated by oxysterols (Jiang et al., 2006; Jiang et al., 2008; Jiang et al., 2010; Jiang et al., 2011). Environmental toxins

present in cigarette smoke have been linked to several skin diseases such as psoriasis and melanoma. Translocation of LXR to the nucleus in HaCaT cells after exposure to cigarette smoke facilitated an increase in cholesterol trafficking as a result of increased ABCA1 expression (Sticozzi et al., 2010). The ability of the synthetic LXR agonist T1317 to prevent physical changes inherent to photoaging and chronological skin aging has been investigated. T1317 inhibited the expression of cytokines and metalloproteinases in cell-based models of skin aging. Furthermore, LXRβ-null murine skin mimics some of the characteristics seen in chronologically aged human skin and the topically applied agonist reduced UV-induced skin thickness and wrinkle formation in a mouse model (Chang et al., 2008).

The anti-inflammatory actions of LXR activators have been demonstrated in both irritant and oxazolone (OX)-induced allergic dermatitis mouse models, in part through the inhibition of proinflammatory cytokines that are specific to LXRβ (Fowler et al., 2003; Hatano et al., 2010). Similarly, global gene expression studies using primary human keratinocytes from psoriatic lesions, non-affected skin of the same patients and healthy control subjects, indicates a role for LXRα in regulating inflammatory response. Along the same line, knocking down LXRα in human keratinocytes lead to a genomic profile similar to that seen in psoriatic skin lesions (Gupta et al., 2010). Even if LXRs are not directly involved in disease pathogenesis, the receptors might still be utilized

as therapeutic tools to modulate cellular lipid levels. For example, a human sebaceous cell line treated with LXRα agonists increased lipid synthesis and induced the lipogenic gene SREBP-1, potentially useful for the treatment of seborrhea and acne (Hong et al., 2008). Selective LXR agonists that are isoform and tissue-specific could provide localized and specific cutaneous effects that do not influence other LXR receptor pools (Bookout et al., 2006; Viennois et al., 2012).

1.5 Epidermal carcinogenesis

Non-melanoma skin cancers arise from both the basal and squamous cells of the epidermis. Basal cell carcinoma (BCC) is the most common neoplasm related to the Caucasian population and its incidence rate is increasing yearly (Diepgen and Mahler, 2002). Though considered malignant due to its ability to invade deep into tissue, it rarely metastasizes from the primary location. Risk factors include fair skin disposed to freckling, family history of BCC and exposure to ultraviolet radiation. Patients with BCC also tend to be prone to other types of skin cancer including malignant melanoma (Wong et al., 2003). Disease progression typically arises from UV-induced actinic keratosis or mutations in the hedgehog signaling pathway. Surgical excision is the treatment of choice though pathway specific compounds such as vismodegib provide additional therapy options (Sekulic et al., 2012). Cutaneous squamous cell carcinoma (SCC) is the second leading cause of skin cancer, yet unlike BCC it tends to exhibit metastatic behavior (Johnson et al., 1992). Again, exposure to ultraviolet radiation is the predominant risk factor for this disease, though immunosuppressed patients are at increased risk for metastatic spread (Rowe et al., 1992). Treatment options include surgical removal, radiation therapy and in certain cases chemotherapeutics such as 5-fluorouracil or imiquimod (Love et al., 2009). Investigations into pathways regulating

keratinocytic proliferation and differentiation may lead to additional treatment options useful in treating these disfiguring diseases.

The transcription factor KLF4 is required for proper EPB formation in mice and dysregulated KLF4 activity is shown to be oncogenic (Segre et al., 1999). KLF4 regulates expression of RXRα and KLF4-induced malignant transformation is sensitive to retinoids *in vitro*. Importantly, rexinoid application drastically prevented formation of SCC in a KLF4-activated transgenic mouse line (Jiang et al., 2009). These results suggest existence of a crosstalk between RXR and KLF4 signaling, where KLF4-mediated expression of RXRα contributes to tumor suppressor activity within the epidermis. RXRα$^{ep-/-}$ mice, selectively lacking RXRα in epidermal keratinocytes, subjected to a 7,12-dimethylbenz(a)anthracene (DMBA)/12-*O*-tetradecanolyphoral-13-acetate (TPA) induced two-step chemical carcinogenic protocol developed higher numbers of epidermal tumors compared to control mice (Indra et al., 2007). The RXRα$^{ep-/-}$ papillomas progressed towards SCC in a murine model, further validating the role of RXRα as a cutaneous tumor suppressor.

Both keratinocytes and melanocytes downregulated expression of RARs when subjected to UV exposure, potentially initiating cellular responses to ionizing radiation through the removal of repressive transcriptional controls (Wang et al., 1999; Boudjelal et al., 2000; Andersson et al., 2003). A similar downregulation of RAR in mouse skin was seen after application of TPA, as

well as when normal human skin progresses through premalignant actinic keratosis to invasive SCC (Kumar et al., 1994; Darwiche et al., 1995; Xu et al., 2001). A loss of RARγ expression exacerbated carcinogenic effects of tumor promoters and enhances malignant transformation (Rudd et al., 1993; Chen et al., 2004). Topical application of RA during the promotion stage of carcinogen treatment in mice reduced the yield of papilloma and carcinoma formation (Tennenbaum et al., 1998). Likewise, the RARβ/γ retinoid tazarotene displayed anti-tumoral activity when topically applied to BCC and may inhibit psoriatic proinflammatory gene networks (Nagpal et al., 1996; Orlandi et al., 2004; So et al., 2008). Above results indicate important roles of retinoids and retinoid receptors in the control of epidermal homeostasis and prevention of hyper-proliferative diseases and skin cancer.

The regulatory nature of VDR in hair cycle progression and barrier formation is critical to maintain skin homeostasis, therefore defects in the transactivating function of the receptor allow pathological conditions to manifest. Multiple VDR polymorphisms have been associated with a broad spectrum of cutaneous disease, including SCC, BCC and vitiligo (Han et al., 2007; Carless et al., 2008; Raimondi et al., 2009; Lesiak et al., 2011; Kostner et al., 2012; Aydingoz et al., 2012). Indeed, inactivation of VDR in mice enhanced sensitivity to both chemical and solar-induced skin carcinogenesis, potentially through activation of the hedgehog pathway (Ellison et al., 2008; Teichert et al., 2011).

Due to evidence of PPAR signaling in the development of aberrant growths in differing tissue types, studies have investigated the role of these receptors in cutaneous neoplasms. Tumor formation and size were increased after chemical carcinogen application in PPAR$\beta/\delta^{-/-}$ mice, while those selectively lacking PPARγ in epidermal keratinocytes show an increase in benign tumors, SCC and BCCs (Indra et al., 2007; Kim et al., 2004). Ligand activation of PPARβ/δ inhibits chemically induced skin tumorigenesis, most likely through induction of keratinocytic differentiation, and the addition of COX-2 inhibitors may result in synergistic efficacy (Bility et al., 2008; Zhu et al., 2010).

In mice, TRβ expression is detected in normal and TPA-treated hyperplastic skin. Loss of TRβ expression is seen in benign papillomas generated by the DMBA/TPA protocol and completely abrogated in subsequent SCC formation. Importantly, skin tumors from mice lacking both TRα and TRβ were more likely to develop *in situ* carcinoma and SCC than those from wildtype mice, supporting TRs role as a tumor suppressor (Martinez-Iglesias et al., 2009). In order to determine the role TRs have in the hyperproliferative response, TRα/β-double null mice were subjected to both TPA and RA treatments (Contreras-Jurado et al., 2011; Garcia-Serrano et al., 2011). Reduced hyperplasia with a decreased expression of cyclin D1 was seen in the TR$\alpha/\beta^{-/-}$ skin after TPA treatment. That profile was correlated with increases in cell cycle inhibitors p19 and p27, as well as induction of proinflammatory cytokines

and phosphorylation of p65/NF-κB and STAT3. This phenotype is opposite to that seen in PPARβ/δ$^{-/-}$ mice and elucidates the combinatorial mechanisms RXR/NR heterodimers regulate within the skin. Similarly, a typical retinoid response requires the presence of ligand-bound TR in mouse skin. Decreases in skin hyperplasia and expression of keratins 5/6, alongside increased transcription of inflammatory and chemotactic cytokines, are also seen in TRα/β$^{-/-}$ mice treated with 9-*cis* RA. Since TPA and retinoids are modulating separate signaling cascades, it is possible that TRs are involved in the cellular responses to both these compounds.

1.6 Melanocyte homeostasis and melanomagenesis

Melanocytes are neural crest-derived pigment producing cells that contribute photoprotective properties to the skin. Cutaneous melanoma is the deadliest form of skin cancer, with a diagnosis of metastasis indicating a median survival rate of less than a year (Chin et al., 2006). Solar ultraviolet irradiation, especially childhood sun exposure, is an important etiological risk factor of melanoma (Hodis et al., 2012). Surgical excision prevents growth of the primary lesion, yet once transformed melanocytes spread to distal organs and this disease is refractory to current therapeutics. Recent evidence supports the use of MAPK inhibitors and immunomodulatory treatments with the goal of increasing lifespan (Flaherty et al., 2010, Hodi et al., 2010). Therefore, any research directed towards key regulators of melanocytic activity could potentially open up new avenues for disease management.

Type II NR-mediated signaling via RXR dimerization is essential to melanocyte biology. RXRα/β expression has been detected in B16 and S91 murine melanoma cells, with RXRβ being the predominant isoform (Desai and Niles, 1995; Spanjaard et al., 1995). Interestingly, loss of melanocytic RXRα expression was seen in human primary and metastatic melanoma compared to benign nevi, indicating the importance of this signaling pathway to the differentiation of these cell types (Chakravarti et al., 2007). RARβ expression

was also downregulated in melanoma and its RA-induced expression was linked to growth inhibition and differentiation in these cells. Sequential occupation of the RA response element located on the *Rarβ* promoter by RXR/RXR and RXR/RAR combinations is believed to be a molecular switch responsible for *Rarβ* transcriptional activation in these cells. Since ligand activation of RXR heterodimeric partners regulates transcriptional activity of target genes involved with differentiation and growth arrest, it is possible that combinatorial activation of both dimer partners may assist in melanoma therapy. Rexinoid treatment alongside glitazones (PPARγ agonists) have displayed efficacy against melanomagenesis in *in vitro* and xenograft models, potentially through the inhibition of matrix metalloproteases and increases in S100A2 calcium binding activity (Papi et al., 2009; Klopper et al., 2009; Klopper et al., 2010).

Our own work has demonstrated the role of keratinocytic RXRα in manipulating melanocyte activation and proliferation through modulation of the cutaneous microenvironment (Wang et al., 2011). RXRα/NR heterodimer binding regulates transcriptional repression of soluble mitogens and cytokines, making it a critical factor in the ultraviolet radiation (UVR) induced cellular responses. The loss of keratinocytic RXRα in RXRα$^{ep-/-}$ mice relieves transcriptional repression and allows overexpression of keratinocytic soluble factors that act on melanocytic cell-surface receptors. Higher numbers of

epidermal melanocytes were seen after UVR exposure in RXRα$^{ep-/-}$ mice compared to controls, suggesting contribution of soluble factors in the cellular microenvironment for melanocyte activation and migration out of the hair follicle. Indeed, increased expression of *Edn1*, *Fgf2* and *Kitlg*, that are known to be upregulated by keratinocytes and influence melanocyte activation, migration and proliferation, were seen in RXRα$^{ep-/-}$ skin post-UVR (Wang et al., 2011). Interestingly, in a two-step carcinogenesis model, RXRα$^{ep-/-}$ mice developed a higher number of dermal melanocytic growths (nevi) compared to control mice, implicating contribution of keratinocyte-derived factors in melanomagenesis. Only nevi from RXRα mutant mice progressed to melanoma-like tumors, suggesting that RXRα-mediated distinct non-cell autonomous actions suppressed nevi formation and melanoma progression in mice (Indra et al., 2007). Similarly, VDR$^{-/-}$ mice undergoing identical treatments also developed higher numbers of melanocytic lesions, indicating RXRα/VDR heterodimerization may be the causative factors, at least in part, in these non-cell autonomous events (Indra et al., 2007). Finally, the loss of keratinocytic RXRα alongside an activated-CDK4 mutation enhanced the metastatic transformation of cutaneous melanoma after chemical carcinogenesis. Expression of *Edn1*, *Hgf*, *Fgf2*, *Pomc* and *Kitlg* were all upregulated in the skin of RXRα$^{ep-/-}$/CDK4$^{R24C/R24C}$ bigenic mice. Direct binding of RXRα on the promoter of *Edn1* and *Hgf* was also seen in *ex vivo* primary murine

keratinocytes. Gene expression analyses on mRNA isolated from melanocytic lesions from the bigenic skin using laser-capture microdissection demonstrated reduced apoptotic and enhanced invasive responses within bigenic melanomas. Loss of epidermal RXRα was also seen in human melanoma progression and could potentially be utilized as a therapeutic biomarker (Hyter et al., 2010). These results indicated a crucial role of RXRα/NR signaling in melanocytic homeostasis and in cellular responses to tumor promotion.

All RAR isoforms were expressed in melanocytes and treatment of retinoids, particularly RARγ-specific agonists, inhibits growth and initiates apoptosis in melanoma cells (Schadendorf et al., 1994; Zhang et al., 2003; Pan et al., 2009). RA treatment increases expression of RARβ and PKCα while stimulating AP-1 transcriptional activity in melanoma cells. RARα specific agonists were most effective in upregulating RARβ levels and combinatorial RAR/RXR activation demonstrate the strongest induction of both PKCα and AP-1 function in murine melanoma cells (Desai et al., 2000; Boskovic et al., 2002; Huang et al., 2003). Loss of RARs, especially RARβ and its tumor suppressor activities, was observed during melanomagenesis and could account for RA resistance in this disease (Chakravarti et al., 2007; Boehm et al., 2004; Fan et al., 2010). That disruption of RARβ or RARγ signaling in benign nevi may predispose a lesion towards malignant transformation. Co-

treatment of retinoids with histone deacetylases also counteracted the epigenetic silencing of RARβ often seen in RA resistant melanoma cell lines and provides increased antitumor activity (Kato et al., 2007). Another mechanism implicated in RA-resistant melanoma cells is an increase in intracellular reactive oxygen species (ROS) that is inversely related to RAR activity. RA-sensitivity was restored when ROS levels were lowered, potentially through improved RXR/RAR promoter binding activity (Demary et al., 2001). RA treatment in combination with polyI:C synergistically increased the toll-like receptor 3 chemokine responses in human melanoma cells and induced migration of antigen-presenting cells (Szabo et al., 2012). Decreased expression of collagenolytic-enzymes and alterations in mobility-associated cell surface receptors were consequences of RAR activation in melanoma cells (Hendrix et al., 1990; Lee et al., 2010). Further evidence of RARγ-mediated inhibition of melanoma invasion came from microarray analysis of murine melanoma cells treated with RARγ agonists, which identified the sulfotransferase CHST10 as being directly regulated by RARγ through RA response elements located in its promoter region. CHST10 synthesizes a HNK-1 carbohydrate that was involved with cell adhesion in the nervous system and RA-mediated embryonic plasticity (Zhao et al., 2009). Altogether, RAR-specific agonists present a potential therapeutic candidate for the

treatment of metastatic melanoma if mechanisms of RA resistance can be elucidated and exploited.

Skin is both a target and producer of $1,25(OH)_2D_3$, the secosteroid ligand of VDR and regulation of melanocyte homeostasis is a physiological role that is attributed to VDR signaling. The use of vitamin D analogs to combat vitiligo depigmentation has been well reviewed (Birlea et al., 2008). It is hoped that Vitamin D agonists might protect melanocyte loss in this disease through inhibition of immune response and modulating calcium flux. VDR activation may also promote re-pigmentation processes by upregulating melanogenic cytokines that drive pigment production. Due to insufficient knowledge on what drives the etiology of this disease, any research implicating driver mutations is important for developing effective therapeutics. Genomic DNA isolated from vitiligo patients, as well as age and sex-matched controls, demonstrated the TaqI VDR polymorphism is a risk factor for the disease. Haplotype BsmI/ApaI/TaqI/FokI/Cdx2 was also overrepresented in those patients (Aydingoz et al., 2012).

The use of low-calcemic deltanoids for treatment of melanocytic lesions is another potential candidate for future therapies. Sunlight generates DNA damage within melanocytes yet also produces anti-proliferative $1,25(OH)_2D_3$ ligands. This physiological dichotomy provides endogenous regulation of melanomagenesis and may offer directions on how to manage cutaneous

melanoma. Human melanoma cell lines have been shown to posses different expression levels of VDR and different growth inhibitory responses to $1,25(OH)_2D_3$. Importantly, melanoma cells demonstrate increased sensitivity to deltanoids compared to normal melanocytes (Slominski et al., 2011). Similar to some other type II NRs, a loss of VDR expression was seen during the progression of melanocytic lesions and that attenuated VDR signaling was linked to decreased overall survival time (Brozyna et al., 2011). Likewise, a retrospective cohort study of large numbers of melanoma patients associated higher serum $1,25(OH)_2D_3$ levels with thinner presenting melanomas and improved survival from melanoma (Newton-Bishop et al., 2009). In addition to investigations associating VDR polymorphisms to keratinocytic pathologies, extensive studies have shown VDR mutations as a critical contributing factor towards melanoma susceptibility and outcome, in particular the BsmI, FokI and TaqI polymorphisms (Denzer et al., 2011; Mandelcorn-Monson et al., 2011; Orlow et al., 2012). Not all melanoma cell lines were sensitive to $1,25(OH)_2D_3$ treatment though. Resistance to $1,25(OH)_2D_3$ treatment may be attributed to epigenetic mechanisms that abrogate VDR signaling in those cell lines. An inverse relationship has been shown to exist between VDR mRNA expression and level of microRNA miR-125b, most likely through posttranscriptional regulation of VDR by miR-125b interacting with a recognition element in the 3'-UTR of human VDR mRNA. Treatment of these

cells with a DNA methyltransferase inhibitor reduces expression of miR-125b and may prove efficacious alongside other chemical therapies (Essa et al., 2010; Essa et al., 2012). In short, aberrant VDR signaling appears to contribute significantly to the development of melanoma in humans and $1,25(OH)_2D_3$ analogs may provide additional targeted chemotherapeutics in the treatment of that disease.

Due to importance of lipid metabolism in skin homeostasis, PPAR signaling in skin has been thoroughly investigated, including its role in melanocyte homeostasis. All PPAR isoforms are expressed in human melanocytes, though PPARγ signaling appears to be a primary possible drug target for melanoma. Two common PPARγ polymorphisms implicated in susceptibility of the malignant disease were evaluated as influences in melanoma risk, though it appeared that inactivating mutations do not appear to be a significant risk factor for the disease (Mossner et al., 2007). Recently a link was shown between α-MSH signaling and PPARγ nuclear translocation that reduced proliferation rates and increased melanogenesis through the $Pi(4,5)P_2$/Plcb pathway (Maresca et al., 2012). Glitazones are known to activate PPARγ and promote melanocyte differentiation. One mechanism to induce melanocyte differentiation is through b-catenin mediated upregulation of MITF levels. Phenotypic changes that occured in melanoma cells post-ciglitazone treatment include dendritic morphology and increased tyrosinase functions, possibly

linked to large increases of *Mitf* promoter activity. β-catenin protein levels in ciglitazone-treated murine melanoma cells followed the same trend of transient increase followed by gradual decrease pattern as seen with MITF, suggesting that depleted levels of nuclear β-catenin was influencing the downregulation of MITF activity in these cells (Grabacka et al., 2008). In addition to its pro-differentiative properties, inhibition of proliferation was seen in melanoma cells after ciglitazone treatment and is also associated with induction of apoptosis (Liu et al., 2006). PPARγ agonists regulate the WNT3A/β-catenin signaling pathway and inhibits human melanoma cell proliferation through direct inhibition of β-catenin activity (Smith et al., 2009). Though PPAR functions as a tumor suppressor at times, two studies screening large numbers of melanocytic lesions determined that both PPARγ and COX-2 expression was increased during progression from benign nevi to metastatic melanoma and may indicate therapy response levels of this disease (Lee et al., 2008; Meyer et al., 2009). That conflicting role of PPARγ in tumorigenesis has yet to be elucidated but provides an exciting investigative target in the treatment of metastatic melanoma.

Few reports demonstrate a role of LXR activation in melanocytes, though known LXR target genes are important in melanocyte biology. LXRα appears to be the predominant isoform and could provide LXR-oriented strategies of melanoma therapy. LXRα expression was also localized adjacent to the

follicular dermal papilla, suggesting a contribution to hair follicle melanocyte activity (Russell et al., 2007). LXRα expression was seen in both human melanocytes and in melanoma cells. Moreover, LXRα mRNA and protein levels were increased in melanocytes present in the skin surrounding vitiligo lesions compared to normal skin, suggesting LXRα is modulating the melanocytic response to this disorder (Chen et al., 2005; Kumar et al., 2010). Interestingly, LXRα has been linked to immunoevasion of melanoma through inhibition of dendritic cell (DC) migration to lymphoid organs. Production of LXR ligands from both human and mouse tumors were shown to hinder CCR7 expression on DCs that is required for lymphoid homing. Conditioned media from the human melanoma cell line MSR3 was shown to inhibit CCR7 expression in DCs, though not affecting other aspects of DC activation. Also, the media from MSR3, as well as from another melanoma cell line MR255, was able to activate LXRα reporter constructs, suggesting the presence of ligands expressed in the media. Use of the sulfotransferase enzyme SULT2B1b, which inactivates natural oxysterols, was able to protect CCR7 inhibition from the conditioned media. Similarly, mice receiving bone marrow transplants from an LXRα-null line demonstrated an enhanced ability of tumor rejection (Villablanca et al., 2010). Altogether, studies indicate an important immuno-modulatory role of LXR signaling in melanocytic lesions and in melanoma primarily mediated by LXRα. Additional studies are required to

establish the receptor functions in melanocyte homeostasis and in melanomagenesis.

Evidence for a role of TR in melanocyte biology is limited, though thyroid-stimulating hormone expression is seen in epidermal melanocytes. Of note, one study involving patients with uveal melanoma demonstrated that 60% of the cohort contained a loss of heterozygosity in the TRβ alleles in both the ciliary body and choroidal melanomas (Sisley et al., 1993). Further *in vivo* studies are needed to determine how TR signaling is impaired in epidermal diseases and in skin cancer, as well as the severity of any non-autonomous actions that affect the malignant transformation of melanocytic cells.

1.7 Conclusion

RXR/NR signaling makes important contributions to both the development and homeostasis of keratinocyte and melanocyte biology. The ability of epidermal tissue to maintain a rigorous cycle of proliferation and differentiation utilizes complex transduction pathways that rely on tight transcriptional control of key target genes. Ligand activated regulation of gene transcription and/or repression by type II NR heterodimers is one example of how skin is able to continually replenish itself while at the same time inhibiting neoplastic transformation. Extensive research presents a scenario where type II NRs are critical tumor suppressors that regulate the sequential differentiation of maturing keratinocytes. With the exception of TR, which does not appear to play a major role in skin biology, loss of RXR/NR activity through inactivating mutations or epigenetic silencing leads to hyperproliferation and immune responses. Likewise, activation of NR heterodimers through endogenous ligand binding, or exogenous topical application of synthetic agonists, typically provides anti-inflammatory actions. Modulation of these receptors could provide supplemental therapy for inflammatory skin disease that is usually treated with potent GCs. The development of tissue specific NR ligands may provide additional relief for diseases such as psoriasis and AD that are not currently curable. RXR/VDR regulation of genes such as *TSLP* provides strong

drug targets that could significantly alter the micro-environmental milieu that drives skin inflammation. Abolishing keratinocytic derived paracrine signaling utilized by melanocytes and immune cells may provide additional options in curative techniques for skin diseases including melanoma. Overall, RXR/NR signaling has evolved as extensive and dominant signaling networks within skin, the largest organ and most important barrier to environmental damages and insults. Utilizing these regulatory checkpoints through the use of synthetic ligands will be an important focus of cutaneous research in the immediate future.

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Figure 1.1 Schematic representation of functional domains in type II nuclear receptors. Transcriptional activation function 1 (AF-1) domain initiates at the amino terminus, followed by the DNA-binding domain (DBD). A flexible hinge region (H) assists in DNA binding, dimerization and transactivation functions. Variable ligand-binding domains (LBD) and a second activation function (AF-2) are present at the carboxyl terminus.

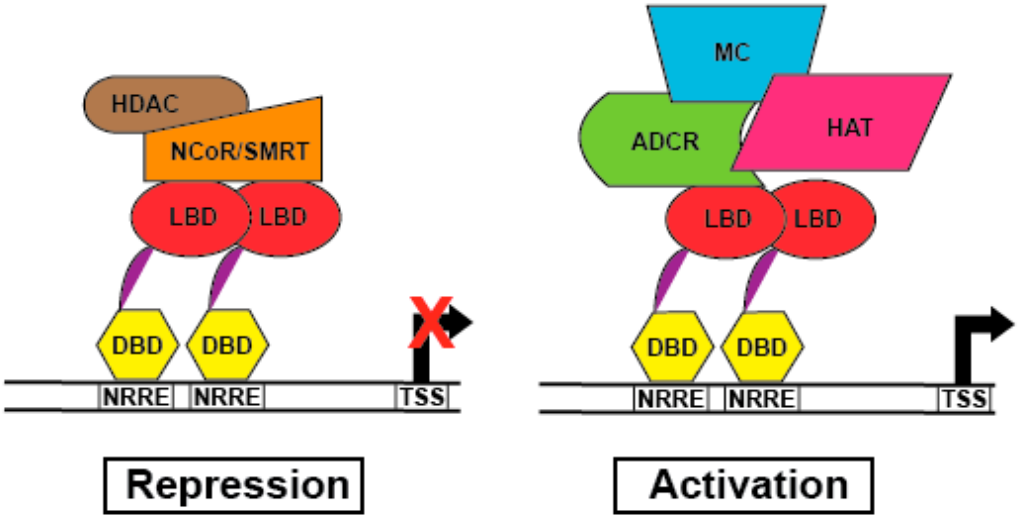

Figure 1.2 Putative mechanisms of transcriptional regulation by type II nuclear receptors. Repression of gene expression by nuclear receptor heterodimers involves association with co-repressor protein complexes, including NCoR/SMRT and histone deacetylases (HDAC). Positive transactivation occurs when co-repressor complexes are replaced by co-activator proteins such as ATP-dependant chromatin remodelers (ADCR), histone acetyltransferases (HAT) and the Mediator complex (MC). DBD, DNA-binding domain; LBD, ligand-binding domain; NRRE, nuclear receptor response element; TSS, transcriptional start site.

Loss of Nuclear Receptor RXRα in Epidermal Keratinocytes Promotes the Formation of CDK4-Activated Invasive Melanomas

Chapter 2

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2.1 Abstract

Keratinocytes contribute to melanocyte transformation by affecting their microenvironment, in part, through the secretion of paracrine factors. Here we report a loss of expression of nuclear receptor RXRα in epidermal keratinocytes during human melanoma progression. We show that absence of keratinocytic RXRα in combination with activated CDK4 generated cutaneous melanoma that metastasized to lymph nodes in a bigenic mouse model. Expression of several keratinocyte-derived mitogenic growth factors (*Edn1, Hgf, Kitlg, α-Msh* and *Fgf2*) were elevated in skin of bigenic mice, while FAS, E-cadherin and PTEN, implicated in apoptosis, cellular invasion and melanomagenesis, respectively, were downregulated within the microdissected melanocytic tumors. We demonstrated that RXRα is recruited on the proximal promoter of both *Edn1* and *Hgf*, possibly directly regulating their transcription in keratinocytes. These studies demonstrate the contribution of keratinocytic paracrine signaling during the cellular transformation and malignant conversion of melanocytes.

2.2 Introduction

Cutaneous melanoma remains the deadliest form of skin cancer. Early detection can lead to effective treatment, but metastatic melanoma is typically non-responsive to current therapies and the median time of survival for patients is less than a year (Chin et al., 2006). Melanoma originates from pigment-producing cells in the skin called melanocytes that play a role in the epidermal response to UV radiation. In recent years, animal models that mimic genetic aberrations linked to melanomagenesis in humans have led toward a better understanding of underlying molecular mechanisms (Larue and Beermann, 2007).

Keratinocytes encompass and support the skin epidermal layer through a constant cycle of proliferation and differentiation. Epidermal proliferation is regulated through secretion of keratinocytic autocrine growth factors that influence receptor-mediated signals within the cell (Cook et al., 1991). The transfer of melanin from melanocytes to keratinocytes aids in the prevention of UV-induced genetic mutations and requires interaction between these two cell types (Scott, 2006). Communications between melanocytes and keratinocytes rely upon paracrine signaling that utilize soluble factors such as endothelin 1 (EDN1), fibroblast growth factor 2 (FGF2), Kit ligand (KITLG), alpha-melanocyte stimulating hormone (α-MSH) and hepatocyte growth factor (HGF); all of which have been shown to influence melanocyte mitogenesis,

melanogenesis, and melanomagenesis (Cui et al., 2007; Halaban et al., 1988; Halaban et al., 1992; Hirobe, 2005; Imokawa et al., 1992; Kunisada et al., 1998). Aberrant paracrine signaling might result in the transformation of melanocytes to malignant cells (Mangahas et al., 2004; Otsuka et al., 1998).

The steroid hormone receptor protein retinoid-X-receptor α (RXRα), a member of the type II nuclear receptor (NR) superfamily, mediates biological effects in part through the regulation of gene transcription. RXRα is able to heterodimerize with some 15 NR family members and occupy corresponding response elements present in the promoters of target genes (Chambon, 1996; Mangelsdorf et al., 1995). RXR/NR heterodimerization modulates gene transcription (activation and/or repression), and acts on multiple developmental and differentiation processes (Altucci and Gronemeyer, 2001).

Three subtypes of the RXR protein are present in mammals, with RXRα being the predominant form expressed in murine epidermis (Fisher and Voorhees, 1996; Mangelsdorf et al., 1992). The RXRα-null mutation is embryonic lethal in mice, thereby preventing analysis of *in vivo* function of this protein in skin homeostasis and diseases (Sucov et al., 1994; Kastner et al., 1994). To circumvent this problem, Cre/loxP technology was utilized to selectively delete the RXRα protein in epidermal keratinocytes (Metzger et al., 2003). The RXR$\alpha^{ep-/-}$ mouse line displayed alopecia, epidermal keratinocytic hyperproliferation, aberrant terminal differentiation and an inflammatory

reaction within the skin (Li et al., 2001). In a two-step chemical carcinogen model using topical applications of the tumor initiator 7, 12-dimethyl-benz[a]antracene (DMBA) and tumor promoter 12-O-tetradecanoylphorbol-13 acetate (TPA), RXR$\alpha^{ep-/-}$ mice developed a higher number of dermal melanocytic growths (nevi) compared to control mice. Only nevi from RXRα mutant mice progressed to human-melanoma-like tumors, suggesting that RXRα-mediated distinct non-cell autonomous molecular events appear to regulate suppression of nevi formation and melanoma progression (Indra et al., 2007). Of note, the tumors that formed in RXR$\alpha^{ep-/-}$ mice after DMBA/TPA treatment rarely invaded or metastasized to distal organs.

Cyclin-dependent kinase 4 (CDK4) protein, a product of the Ink4a locus, has been implicated in the development of cutaneous melanoma (Curtin et al., 2005; Kamb et al., 1994). An arginine to a cysteine point mutation in the CDK4 protein found in familial melanoma (CDK4$^{R24C/R24C}$) prevents the kinase activity of CDK4 from being inhibited by the G1/S phase regulator p16, leading to an increase in cell-cycle activity (Wolfel et al., 1995; Zuo et al., 1996). CDK4$^{R24C/R24C}$ knock-in mice harboring this activated form of CDK4 demonstrated increased susceptibility to melanoma formation after DMBA/TPA treatment (Rane et al., 1999; Sotillo et al., 2001). Mice containing the CDK4$^{R24C/R24C}$ mutation in cooperation with deregulated receptor tyrosine kinase signaling, or activated RAS, has been shown to promote development

of spontaneous and carcinogen-induced metastatic melanoma (Hacker et al., 2006; Tormo et al., 2006).

In this study, we evaluated expression of RXRα protein in normal human skin, tumor adjacent normal, benign nevi, *in situ* and malignant melanoma. We investigated the contributions of $CDK4^{R24C/R24C}$ and keratinocytic RXRα to influence metastatic progression in a mouse model. Expression of several keratinocyte-derived growth factors, implicated in melanomagenesis, were upregulated in the skin of bigenic mice, and recruitment of RXRα was shown on the promoters of *Edn1* and *Hgf*. We also confirmed a downregulation of factors (FAS, E-cadherin and PTEN) implicated in apoptosis, invasion and survival within the melanocytic tumors.

2.3 Results

Downregulation of RXRα protein in epidermal keratinocytes during human melanoma progression

Our earlier studies demonstrated that loss of keratinocytic RXRα in a non-cell autonomous manner regulated melanocyte homeostasis during DMBA/TPA-induced melanomagenesis (Indra et al., 2007). It has been previously shown that a loss of nuclear RXRα protein in melanocytes correlates with melanoma progression in human patients (Chakravarti et al., 2007). We therefore investigated expression levels of RXRα protein in epidermal keratinocytes by immunohistochemistry (IHC) of normal human skin, tumor adjacent normal (TAN) epithelium, benign nevi, *in situ* melanoma and malignant melanoma samples from tissue microarray and de-identified human tissues. Normal and TAN epidermis showed strong nuclear RXRα expression in most, if not all, basal keratinocytes, as well as in the suprabasal layers (Figures 2.1A-D). The epidermis adjacent to benign nevi displayed a hyperplastic appearance that was also present in the *in situ* and malignant melanoma samples. Most keratinocytes, as well as nests of nevus cells, exhibited strong nuclear RXRα expression in the benign nevi (Figures 2.1E and 1F). A general trend of reduced expression (~50%) of RXRα protein was seen in suprabasal keratinocytes for *in situ* melanomas (Figures 2.1G and

2.1H, Table S1), and a marked absence of RXRα protein was seen in all layers of epidermis from malignant melanoma samples (Figures 2.1I and 2.1J, Table S1). A Fisher's Exact test showed a significantly low probability ($p < 0.001$) that loss of keratinocytic RXRα expression would be seen only in these two tissues, suggesting an association with metastatic progression in humans.

Loss of keratinocytic RXRα in cooperation with activated CDK4 leads to larger melanocytic tumors in mice

Involvement of the p16/CDK4 pathway has been implicated during melanomagenesis in humans (Curtin et al., 2005; Kamb et al., 1994; Wolfel et al., 1995; Zuo et al., 1996). We hypothesized that the increased melanocyte proliferation reported in our previous study (Indra et al., 2007) could be due to deregulated cell cycle control. Additionally, the use of activated CDK4, paired alongside a secondary genetic alteration of melanocytic signaling pathways, has been shown to promote formation of metastatic melanomas in mouse models (Hacker et al., 2006; Tormo et al., 2006). We therefore investigated the cooperative effects of activated CDK4, in parallel with loss of keratinocytic RXRα, towards melanoma metastasis. To that end, we have bred the $RXR\alpha^{ep-/-}$ and $CDK4^{R24C/R24C}$ mice to generate $RXR\alpha^{ep-/-}/CDK4^{R24C/R24C}$ bigenic mice and utilized the two step carcinogenesis (DMBA/TPA) protocol (DiGiovanni, 1992). $RXR\alpha^{L2/L2}$ (floxed RXRα mice containing LoxP sequences flanking exon

4) mice were used as wild-type controls, and those with single mutation (RXRα$^{ep-/-}$ and CDK4$^{R24C/R24C}$) served as additional controls. Although all mice in each cohort developed melanocytic tumors (MT) within the timecourse of the study, a greater number of MTs grew to sizes larger than 2mm in diameter in both the RXRα$^{ep-/-}$ and RXRα$^{ep-/-}$/CDK4$^{R24C/R24C}$ mice compared to the RXRα$^{L2/L2}$ or CDK4$^{R24C/R24C}$ mice. Strikingly, the bigenic mice developed a significantly higher number of MTs larger than 4mm in size compared to the RXRα$^{ep-/-}$ mice (Figure 2.2A, Figure S2.1)**.** The appearance of melanocytic tumors arose within a similar timeframe of 7-8 weeks in all treatment groups, however decreased tumor latency was observed in mice lacking RXRα in epidermal keratinocytes (Figures S2.1B and S2.1C). All subsequent comparative studies were performed on RXRα$^{ep-/-}$/CDK4$^{R24C/R24C}$ and CDK4$^{R24C/R24C}$ mice to determine the contribution of keratinocytic RXRα signaling on melanomagenesis.

To further characterize the MTs formed in the dorsal skin of the CDK4$^{R24C/R24C}$ and RXRα$^{ep-/-}$/CDK4$^{R24C/R24C}$ mice, we performed hematoxylin and eosin (H&E) staining of 5μm thick paraffin sections of melanocytic tumors from both genotypes **(**Figure 2.2B**)**. A significant increase in epidermal thickness was observed in the skin from RXRα$^{ep-/-}$/CDK4$^{R24C/R24C}$ skin compared to CDK4$^{R24C/R24C}$ alone **(**Figure 2.2C**)**. This phenotype is a hallmark of mice bearing an epidermal ablation of RXRα, as the basal layer

keratinocytes maintain a hyperproliferative state (Li et al., 2001). Importantly, both the radial growth phase (RGP) and vertical growth phase (VGP) of the MTs in the dermis were significantly increased in the bigenic mice compared to the control mice **(**Figure 2.2D**)**. Similar results were seen when comparing the bigenic mice to both RXR$\alpha^{L2/L2}$ and RXR$\alpha^{ep-/-}$ mice (data not shown). Altogether the above results suggest a synergistic effect of RXRα ablation and activated CDK4 in the development of cutaneous melanoma.

Immunohistochemical analyses of melanocytic tumors from RXR$\alpha^{ep-/-}$ /CDK4$^{R24C/R24C}$ bigenic mice

We performed IHC using specific antibodies for markers of proliferation, malignant conversion, and vascularization, to further characterize the MTs from the bigenic mice. Immunohistochemical staining was performed on paraffin sections of tumors taken from CDK4$^{R24C/R24C}$ and RXR$\alpha^{ep-/-}$ /CDK4$^{R24C/R24C}$ mice after DMBA/TPA treatment. To investigate the mitogenicity of the MTs, we co-labeled with antibodies directed against the proliferation marker PCNA and the melanocyte-specific enzyme tyrosinase related protein 1 (TRP1) (Jimenez et al., 1988; Waseem and Lane, 1990). A significantly higher percentage (67% vs 21%) of TRP1+ cells were co-labeled with PCNA (TRP1+/PCNA+) in the RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ skin compared to the CDK4$^{R24C/R24C}$ skin [($p < 0.01$), Figures 2.3A and 2.3B]. In order to

determine the malignant nature of the MTs from the bigenic mice, IHC was performed using an antibody cocktail directed against melanoma antigens MART-1 and HMB45 (Yamazaki et al., 2005). That combination showed increased staining in all of the MTs from the RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ compared to the CDK4$^{R24C/R24C}$ mice, suggesting a larger population of malignant cells in these tumors (Figures 2.3C and 2.3D). Similarly, an increased immunoreactivity for the endothelial cell-specific marker CD31 was also detected in the bigenic mice (17% of DAPI positive cells) compared to CDK4$^{R24C/R24C}$ mice (5% of DAPI stained cells), suggesting enhanced vascularization in the MTs from those mice (Wang et al., 2008) (Figures 2.3E and 2.3F). Altogether, these results (histopathological and IHC) confirm that the MTs from the bigenic mice have greater malignant tendencies compared to those from RXR$\alpha^{ep-/-}$ or CDK4$^{R24C/R24C}$ mice.

Cutaneous melanoma formed in the RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ bigenic mice metastasized to distal lymph nodes

In order to determine if combinatorial alterations of multiple molecular pathways could activate a malignant neoplastic process, we analyzed draining lymph nodes and internal organs from RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ mice for the presence of metastatic melanocytes and compared them with lymph nodes from CDK4$^{R24C/R24C}$ and RXR$\alpha^{ep-/-}$ mice. Both the subiliac lymph nodes from

each of the six surviving bigenic mice were enlarged and hyperpigmented compared to only a single lymph node belonging to one of the eight control CDK4$^{R24C/R24C}$ mice (Figures 2.4A and 2.4B). Additional distal organs did not exhibit sign of metastasis in the bigenic or control mice (data not shown). Fontana Masson staining for detecting melanin granules on paraffin sections identified the presence of numerous pigmented cell types in the bigenic lymph nodes, compared to an occasional pigmented cell found in the control mice (Figures 2.4C and 2.4D and data not shown). IHC analyses of lymph nodes using an antibody raised against the melanocyte-specific enzyme TRP1 confirmed the presence of melanocytes in the draining lymph nodes from all the bigenic mice compared to only two of the control mice (Figures 2.4E and 4F)**.** Similarly, IHC analyses using a melanoma cocktail for detecting malignant melanocytes confirmed the presence of multiple malignant melanocytes in the bigenic mice compared to very few cells in the control lymph nodes (Figures 2.4G and 2.4H), thus corroborating the results obtained from the Fontana Masson staining. These results demonstrate that the RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ line represents a melanoma mouse model with a tendency for metastatic progression.

Upregulation of mitogenic factors and regulatory proteins in the skin of bigenic mice

It has previously been shown that various mitogenic growth factors secreted by keratinocytes exert both autocrine and paracrine effects in the microenvironment of the skin and contribute to melanoma formation. We therefore investigated if expression of specific soluble factors are altered in the skin of our bigenic mice. We also examined activation of regulatory proteins that lie downstream of those signaling pathways in both CDK4$^{R24C/R24C}$ and RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ skin (Recio and Merlino, 2002; Tada et al., 2002).

To determine if expression of genes encoding soluble growth factors are altered at the transcriptional level, we utilized quantitative RT-PCR (qRT-PCR) to examine the relative mRNA levels in skin of RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ mice compared to the CDK4$^{R24C/R24C}$ mice. Significant increases in mRNA expression level were seen for the paracrine factor *Edn1* (~3.5 fold) in the skin of bigenic mice (Figure 2.5A). Similarly, expression of other factors such as *Hgf* (~1.5 fold), *Fgf2* (~1.5 fold), *Pomc* (the precursor peptide of α-MSH, ~2 fold) and *Kitlg* (~2 fold) were also upregulated in RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ mice. We then evaluated the transcription factor MITF that regulates expression of several genes implicated in proliferation, survival and apoptosis of melanocytes (Levy et al., 2006), since amplification of MITF has been linked to malignant conversion of MTs (Garraway et al., 2005). Interestingly, relative mRNA levels of *Mitf-M*, a melanocyte specific isoform, were also found to be increased (~1.5 fold) in the skin of RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ mice compared to

the control group (Figure 2.5A). Similarly, relative mRNA levels of *Il1α* and *Cox2* expression were significantly upregulated (~2 fold and ~1.5 fold, respectively), and a ~1.4 fold increase was seen in *HbEgf* transcript levels in the skin of bigenic mice (Luger and Schwarz, 1990; Tripp et al., 2003) (Figure 2.5B). Protein levels and phosphorylation status of key MAPK protein family members were measured to determine if altered MAPK signaling is responsible, at least in part, for increased melanomagenesis in the bigenic mice. A modest increase in expression level of p38, ERK and JNK proteins, and an increased phosphorylation of p38 and JNK, was seen in the skin of RXRα$^{ep-/-}$/CDK4$^{R24C/R24C}$ compared to CDK4$^{R24C/R24C}$ mice (Figure 2.5C, and data not shown).

Nuclear receptor RXRα is recruited to the promoter of genes encoding paracrine factors EDN1 and HGF in keratinocytes

It is possible that absence of RXRα in keratinocytes is increasing the basal rate of transcription for specific soluble communication factors in the skin of RXRα$^{ep-/-}$/CDK4$^{R24C/R24C}$ mice through a derepression mechanism. In order to verify if keratinocytic RXRα plays a direct or indirect role in regulating the expression of soluble growth factors at the transcriptional level, we performed *ex vivo* chromatin immunoprecipitation (ChIP) experiments on extracts from

primary mouse keratinocytes and determined if RXRα protein is recruited onto the promoter of target genes.

The promoters of numerous keratinocyte-derived soluble growth factors were selected as potential candidates of RXRα-mediated cell signaling. A region of ~10kb upstream of the transcriptional start site was screened for possible binding motifs of RXRα/NR heterodimers. Sequences on the promoters of *Edn1*, endothelin 3 (*Edn3*), fibroblast growth factor 7 (*Fgf7*), *Hgf*, *p53*, proopiomelanocortin (*Pomc*), nerve growth factor (*Ngf*), nitric oxide synthase (*Nos*) and *Kitlg* were targeted for further investigation and primers were designed to capture these regions (Figure 2.6A, data not shown). Amplification of isolated chromatin using specific primers was performed on wild-type primary keratinocytes, as well as on those collected from mutant RXRα$^{ep-/-}$ mice. Increased promoter occupancy by RXRα protein was detected by ChIP assay 1.4kb and 9.1kb upstream of the transcriptional start site of the *Edn1* and *Hgf* gene promoters, respectively (Figure 2.6B). These results suggest that RXRα is recruited to the promoter of paracrine mitogenic factors *Edn1* and *Hgf* in murine keratinocytes.

Altered gene expression in the melanocytic tumors of RXRα$^{ep-/-}$ /CDK4$^{R24C/R24C}$ mice

Laser-capture microdissection (LCM) was performed on frozen sections of melanocytic tumors from CDK4$^{R24C/R24C}$ and RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ mice to investigate the influence of RXRα epidermal ablation on melanoma progression. Seventy percent of the pigmented melanocytic tumors were extracted from the surrounding tissue (Figures S2.2A and S2.2B). Total RNA was isolated, amplified and converted to cDNA to evaluate the differential expression of genes commonly implicated in neoplastic processes by using a qPCR-based gene array (Cancer PathwayFinder Array, SABiosciences). Approximately 30 genes were differentially regulated more than 2-fold in the array, pertaining to several distinct biochemical processes, and data was analyzed using the ddCT method (Figure 2.7A and Table S2.2). The death receptor *Fas*, which is involved in the extracellular response to apoptosis, was downregulated more than 7-fold. Other genes involved in apoptosis inhibition and downregulated in the mutant melanocytic tumors were *caspase 8* (~5 fold), *Apaf1* (~3 fold), *Bcl2l1* (~4 fold), *Tnfrsf10b* (~2 fold), *Tnfrsf1* (~2 fold), *E2f1* (~3 fold), *Chek2* (~3 fold), *Cdkn1a* (~2 fold), while *Birc5/survivin* was upregulated (~3 fold). Elements of the plasminogen activation pathway, *Plaur* and *Serpinb2*, were up- and down-regulated (~2 and ~3 fold, respectively) in the RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ melanomas compared to those in CDK4$^{R24C/R24C}$ tumors. Additionally, members of signal transduction and adhesion pathways which were upregulated included *Cdkn2a* (~5 fold), *Itga4* (~2 fold), and *Ffg1*

(~2 fold), while *Syk*, *Nfkbia*, *Cdh1/E-cadherin*, *Pten* and *Ctnnb1* were downregulated (~3, ~4, ~3, ~2 and ~2 folds, respectively) (Figure 2.7A). We performed qRT-PCR validation on LCM samples from control and bigenic mice for some of the interesting candidates implicated in melanomagenesis such as *Fas* and *Birc5/survivin* (apoptosis), *Plaur* and *Cdh1/E-cadherin* (invasion), and the tumor suppressor *Pten*. The results obtained further confirmed our initial PCR array data (Figure 2.7B). Furthermore, western blot validation was used to determine if similar changes were present at the protein level. A downregulation in FAS, CDH1/E-cadherin and PTEN protein levels, and a mild upregulation for PLAUR expression, were observed in the bigenic MTs. BIRC5/survivin was not detectable by either western blot or IHC in either group (Figure 2.7C and data not shown). Altogether, these results demonstrate a differential regulation of apoptotic and invasive mechanisms within the transformed melanocytes from the bigenic MTs.

2.4 Discussion

Formation of metastatic melanoma in RXRα$^{ep-/-}$/CDK4$^{R24C/R24C}$ mice

We have shown that deletion of RXRα in keratinocytes contributes to the development of cutaneous melanoma after carcinogen treatment (Indra et al., 2007). Since melanocytes in the previous study were not genetically modified, it suggests an influence of paracrine signal(s) from RXRα-ablated epidermal keratinocytes on melanocyte biology during melanomagenesis. Our present study demonstrated that the addition of activated CDK4 contributes to the malignant transformation and metastatic potential of proximal melanocytes. IHC studies on human tissues confirmed decreased expression of nuclear receptor RXRα in melanocytes during melanoma progression, thereby corroborating the results reported earlier (Chakravarti et al., 2007). In addition, we showed downregulation of RXRα expression in tumor adjacent keratinocytes from human *in situ* and malignant melanoma samples. Our results suggest a protective role of keratinocytic RXRα against aggressive tumor formation, the lack of which resulted in a significantly increased RGP and VGP in the bigenic RXRα$^{ep-/-}$/CDK4$^{R24C/R24C}$ tumors. In order to achieve vertical growth, the nevus must acquire characteristics of both tumorigenicity and mitogenicity, properties that enable cellular proliferation within a foreign matrix (Hearing and Leong, 2006). The increase in PCNA^{+}/TRP1^{+} cells within

the RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ melanocytic tumors verified the presence of a large population of proliferating melanocytes. Increased staining against melanoma antibody cocktail HMB45 and MART-1 detected in the RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ tumors correlated well with the higher VGP in those mice. Similarly, CD31 staining in the bigenic skin implied that the larger MTs formed in those mice would necessitate additional vascular formation for nutritional support. Altogether, these data confirm that melanocytic tumors formed in the absence of keratinocytic RXRα, and in the presence of activated CDK4, have increased metastatic capabilities compared to the control mice. It has been previously shown that the loss of the transcriptional regulator TAF4 in epidermal keratinocytes can lead to melanocytic tumors with invasion of lymph nodes after carcinogen treatment (Fadloun et al., 2007). It remains to be determined whether TAF4 and RXRα act through the same or different paracrine/juxtacrine pathways to regulate melanocyte homeostasis. Activating mutations in N-RAS and its downstream effector B-RAF are frequent events in human nevi and melanomas (Papp et al., 1999; Demunter et al., 2001; Garnett and Marais, 2004). Importantly, N-RAS and B-RAF mutations are mutually exclusive, strongly suggesting that both oncogenic activities are in the same linear pathway deregulating the mitogen activated protein kinase (MAPK) pathway. Mice bearing oncogenic N-RAS^{Q16K} or a B-RAF^{V600E} have been shown to develop melanomas mimicking the genetics and pathology of the

human disease (Ackermann et al., 2005; Dhomen et al., 2009). It is possible that keratinocytic RXRα mediated NR pathway(s) also cooperate with the N-RAS^{Q16K} or B-RAF^{V600E} driven MAPK pathway towards melanoma progression in humans.

Loss of keratinocytic RXRα and increased melanomagenesis are associated with altered expression of paracrine growth factors

Previous *in vitro* studies have confirmed the role of numerous mitogenic factors produced in and released from keratinocytes that are involved in regulating the proliferation and differentiation of mammalian epidermal melanocytes through receptor-mediated signaling pathways. Among those factors are EDN1, α-MSH (the mature cleavage product of POMC), HGF, KITLG and FGF2 all of which have profound mitogenic and melanogenic effects on cultured mouse and human melanocytes (Abdel-Malek et al., 1995; Imokawa et al., 1992; Hirobe, 1992; Swope et al., 1995; Hirobe, 2001; Hirobe, 2003; Hirobe, 2004). Enhanced secretion of most of these paracrine factors upon loss of keratinocytic RXRα could potentially mediate increased mitogenesis and melanomagenesis in the bigenic mouse model. Interestingly, increased secretion of most of these paracrine factors have been seen in the skin of neonatal RXRα$^{ep-/-}$ mice after UV exposure (Wang et al., 2011)

Downstream effectors that propagate signaling cascades initiated by these ligand-receptor interactions are not mutually exclusive of each other, but instead interact through crosstalk at multiple levels, such as the activation of RAS-MAPK pathway (van Biesen et al., 1995). It is noteworthy that the stress activated kinases p38 and JNK, both of whose expression and subsequent phosphorylation were enhanced in the skin of RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ mice, have been shown to lie downstream of HGF/MET signaling pathways and activate proliferation of melanoma cells (Recio and Merlino, 2002). We showed an enhanced expression of the melanocyte master regulator MITF in the mutant skin, whose expression is shown to be regulated by both MAPK and p38 signaling (Vance and Goding, 2004 and references therein). Although independence from other cell types is a hallmark of metastatic potential, it is possible that upregulation of mitogenic factors from keratinocytes into the extracellular locale provides a pro-carcinogenic environment for melanocytic transformation (Haass and Herlyn, 2005).

Our *ex vivo* ChIP data demonstrated that RXRα/NR dimer was recruited on the promoter of both *Edn1* and *Hgf* genes in murine keratinocytes. It has been previously shown that absence of RXR/NR dimers from the regulatory regions of the soluble cytokine *Tslp* lead to a dramatic increase in transcript levels for *Tslp* (Li et al., 2006). We propose a similar model of derepression on the promoters of specific soluble growth factors such as *Edn1* and *Hgf* in our

RXR$\alpha^{ep-/-}$ mice that increases the basal transcriptional activity due to loss of RXRα/NR. It has been reported that UV induction of POMC is directly controlled by p53 (Cui et al., 2007). Although we have identified potential RXR/NR response elements on the promoters of both *p53* and *Pomc*, our ChIP data did not confirm recruitment of RXRα to the promoters of either gene, suggesting an alternative mechanism(s) of *Pomc* regulation by RXRα.

Molecular alterations of apoptosis, invasion and mitogenic signaling in the malignant melanomas from RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ mice

We used LCM technology to show the inherent functional differences between the melanocytic tumors from CDK4$^{R24C/R24C}$ and RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ mice. A significant downregulation of the death receptor *Fas*, which has been linked to apoptotic resistance in melanocytic lesions and melanoma cell lines (Bullani et al., 2002), was observed in the MTs from bigenic mice. The ability to disregard pro-apoptotic external stimuli in these transformed melanocytes would confer a selective advantage for both malignant transformation and therapeutic resistance (Ivanov et al., 2003; Niedojadlo et al., 2006). There was also an upregulation of the caspase activation inhibitor *Birc5/Survivin*, which is shown to be highly expressed in malignant melanoma but absent in normal melanocytes (Grossman et al., 1999; Ryu et al., 2007), suggesting that melanomas from RXR$\alpha^{ep-/-}$

/CDK4$^{R24C/R24C}$ mice might utilize multiple pathways to attenuate apoptotic signals.

The invasion-linked gene *Plaur* was expressed at a higher level in the microdissected RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ melanomas. Although the process is poorly understood, the plasminogen-activation system has been shown to play an important role in hypoxic-induced metastasis through the upregulation of the receptor PLAUR (Rofstad et al., 2002). Changes in expression levels for genes related to adhesion would be expected between tumor types with differing metastatic potential. In the MTs of bigenic mice, we showed a downregulation of CDH1/E-cadherin expression, loss of which is a hallmark transition for metastatic progression (Hsu et al., 1996, Ryu et al., 2007). It is noteworthy that UV-induced production of EDN1 by keratinocytes is known to downregulate E-cadherin expression in both melanocytes and melanoma cells (Jamal and Schneider, 2002). Finally, a downregulation of the tumor suppressor PTEN was shown in melanomas from bigenic mice, as has been previously reported in certain cases of primary melanomas. Loss of PTEN favors melanoma formation by reducing apoptosis and promoting cell survival (Stahl et al., 2003; Tsao, et al., 2003).

Presently, there are concerted efforts toward producing mouse models that recapitulate the processes of human cutaneous melanoma with a goal of providing effective therapeutic strategies (Ibrahim and Haluska, 2009).

Findings have led to recent advances in targeted therapy against factors implicated in melanoma progression (Solit et al, 2006; Bedikian et al, 2006), but have been less effective in preventing metastases. Further studies of mouse models investigating the individual contribution of paracrine factors during melanomagenesis are necessary. Additionally, investigations using sequentially timed ChIP and re-ChIP experiments are necessary to determine both the temporal events and recruitment cascades of regulatory proteins specific to transcriptional regulation of growth factors such as *Edn1* and *Hgf*. Screening for genetic polymorphisms for RXRα in keratinocytes, in addition to melanocytes, is warranted in human melanoma patients, as we demonstrate keratinocytic contributions to both melanomagenesis and metastatic spread.

2.5 Materials and Methods

Mice

Generation of RXR$\alpha^{ep-/-}$ (Li et al., 2001) and CDK4$^{R24C/R24C}$ (Rane et al., 1999) mice have previously been described. Cohorts of 8-10 sex and age-matched mice were shaved and treated with 50μg DMBA in 100μl of acetone. Five days after DMBA application, 5μg TPA in 200μl acetone was applied topically twice a week for up to 25 weeks. All mice were shaved weekly, and the number and size of melanocytic tumors were recorded. Mice were housed in our approved University Animal Facility with 12h light cycles, food and water were provided ad libitum, and institutional approval was granted for all animal experiments.

Tissue Samples

Keratinocytic RXRα expression was evaluated using normal (n=30), tumor adjacent normal (n=18), benign nevi (n=27), *in situ* melanoma (n=3) and malignant melanoma (n=128) skin sections from de-identified human samples (Oregon Health and Sciences University) and a malignant melanoma array (Biomax, ME482). Skin samples and melanocytic tumors were collected from each group of mice 25 weeks after DMBA/TPA application, fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin.

Histological analyses

Skin and tumor sections (5μm thick) from mouse tissue samples were stained with hematoxylin and eosin (H&E) as previously described (Indra et al., 2007). Fontana-Masson staining was performed according to manufactures instructions (American MasterTech). All microscopic studies were performed using a Leica DME light microscope and analyzed using the Leica Application Suite software, version 3.3.1.

Immunohistochemistry

For DAB color reactions, human tissue sections were deparaffinized in xylene, dehydrated through graded alcohols, and high temperature (20 minute, hot water bath) antigen retrieval was performed on 5μm sections using citrate buffer, then placed in 0.3% hydrogen peroxide to quench any endogenous peroxidase activity. Sections were blocked with 10% normal goat serum in PBS-Tween (0.05%) for 30 minutes to block nonspecific antibody binding and then incubated overnight with rabbit anti-RXRα polyclonal antibody (Santa Cruz, 1/300 dilution). Secondary antibody staining was carried out with a biotin-labeled goat anti-rabbit antibody (Jackson ImmunoResearch, 1/1500 dilution) for 2hrs at room temperature, followed by incubation with a streptavidin-biotin horseradish peroxidase complex (Vector Laboratories). Detection was done using DAB+ substrate (Vector Laboratories) for 5 minutes,

then counterstained with Harris hematoxylin, followed by dehydration and mounting.

For immunofluorescence staining studies, paraffin sections from mouse skin and tumor samples were rehydrated and processed as above. Antigen retrieval was performed in hot water bath either using citrate buffer pH 6.0 (TRP1, PCNA and CD31 staining) or tris-EDTA buffer pH 9.0 (HMB45 staining), and then bleached at 60°C with 10% H_20_2 for 30-60 minutes. Primary antibody incubation was followed by three washes with PBS-Tween (0.05%) before addition of the secondary antibodies. Nuclei were counterstained with DAPI. Finally, sections were rinsed with PBS-Tween (0.05%), dehydrated through sequential alcohol washes and then cleared in xylene. Slides were mounted with DPX mounting media and allowed to dry overnight. The following antibodies were used for immunohistochemistry: anti-Pep1 (kindly provided by V. Hearing, NIH), anti-PCNA (Abcam), anti-HMB45 + DT101 + BC199 cocktail (Abcam), anti-CD31 (Abcam). The secondary antibodies used were goat anti-rabbit CY2 and goat anti-mouse CY3 (Jackson ImmunoResearch). All images were captured using either a Zeiss AXIO Imager.Z1 with a digital AxioCam HRm, and processed using AxioVision 4.7 and Gimp 2.6. Sections stained without primary antibody was used as a negative control, and all experiments were performed in triplicates.

Real-time PCR

Total RNA was extracted from either dorsal skin after DMBA/TPA treatment using Trizol (Invitrogen), or PicoPure RNA isolation kit (Arcturus) for LCM validation studies, and cDNA was created using SuperScript II RT (Invitrogen). Amplification was performed on an ABI Real Time PCR machine using a QuantiTect SYBR Green PCR kit (Invitrogen), and all targets were normalized to *Hprt*, which was used as an internal control. All reactions were performed in triplicates using a minimum of three biological replicates from each group of mice. Melting curve analyses were performed to ensure the specificity of amplification. Statistical analysis was done with GraphPad Prism software.

Western blot

The skin biopsy or melanocytic tumor of each mouse was homogenized, and proteins were extracted with a lysis buffer (150mM NaCl, 50mM Tris pH 7.5, 5mM EDTA, and 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate) containing protease inhibitors (2μg/ml Aprotinin, 2μg/ml Leupeptin, 100μg/ml Pefabloc and 1μg/ml Pepstatin). Equal amounts of protein extract (25μg) from each lysate were resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The blots were blocked overnight with 5% nonfat dry milk and incubated with specific antibodies. The antibodies used were mouse anti-JNK (cat. no. sc-571, Santa

Cruz Biotechnology, Santa Cruz, CA), anti p-JNK (cat. no. sc-6254, Santa Cruz Biotechnology, Santa Cruz, CA), anti-p38 MAPkinase (cat. No. 9212, Cell Signaling Technology, Danvers, MA) and anti-phospho p38 MAPkinase (cat. No. 9211, Cell Signaling Technology, Danvers, MA), anti-FAS (cat. no. sc-716, Santa Cruz Biotechnology, Santa Cruz, CA), anti-uPAR (cat. no. sc-10815, Santa Cruz Biotechnology, Santa Cruz, CA), anti-E-Cadherin (cat. no. 4065, Cell Signaling Technology, Danvers, MA) and anti-PTEN (cat. no. 9559, Cell Signaling Technology, Danvers, MA). After incubation with the appropriate secondary antibody, signals were detected using immunochemiluminescent reagents (GE Healthcare, Piscataway, NJ**)**. Equal protein loading in each lane was confirmed with anti-actin antibody (catalog no. A5060, Sigma-Aldrich, St. Louis, MO).

Chromatin immunoprecipitation

Briefly, primary keratinocytes from wild-type and RXR$\alpha^{ep-/-}$ mice were fixed in 1% formaldehyde, washed and sonicated. A 10% input sample was removed and the remaining chromatin was immunoprecipitated with and without 3μg RXRα (Santa Cruz Biotechnology) antibody and incubated overnight. Protein G Dynabeads (Invitrogen) were added and the chromatin complexes were washed and subjected to reversal of protein-DNA crosslinks. DNA was recovered using phenol-chloroform extraction and ethanol precipitation,

amplification was performed on an ABI Real Time PCR machine using a QuantiTect SYBR Green PCR kit (Invitrogen). Experiments were performed a minimum of three times.

LCM and PCR Array Cancer PathwayFinder

Melanocytic tumors from RXR$\alpha^{L2/L2}$, RXR$\alpha^{ep-/-}$, CDK4$^{R24C/R24C}$ and RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ skin was collected after 25 weeks of DMBA/TPA treatment and embedded in OCT using isopentane-dry ice. Eight micron thick frozen sections were prepared and kept at -80°C to preserve RNA integrity. Sections were dehydrated and melanocytic tumors were microdissected using a PixCell II (Arcturus). Total RNA was then extracted using a PicoPure RNA isolation kit (Arcturus) according to manufacturer instructions. Isolated RNA was amplified using the RT^2 Nano PreAMP cDNA Synthesis Kit (SABiosciences) according to manufacturers instructions. The cDNA was evaluated using a Mouse Cancer PathwayFinder PCR Array System (SABiosciences) consisting of 84 primer sets, as well as 5 housekeeping genes and multiple internal controls, according to manufactures instructions and evaluated using the $\Delta\Delta C_t$ analysis method.

Statistical analysis

To determine the probability that loss of epidermal RXRα should not differ between the five types of tissue, a 5x2 table was analyzed using a Fisher's Exact test. Quantification of TRP1+/PCNA+ double-positive cells within the melanocytic tumors was determined by counting total number of TRP1 and PCNA positive cells, and expressed as a percentage of TRP1+ cells that also stained positive for PCNA. Multiple sections were analyzed and significance was determined using a Student's t-test. Similarly, the number of CD31-positive cells was counted within the tumors and expressed as a percentage of total DAPI stained cells. The analyses of RXRα, HMB45/MART-1, PCNA/TRP1 and CD31 expression were independently performed by two investigators.

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Conflict of interest

The authors declare that no conflicting interests exist

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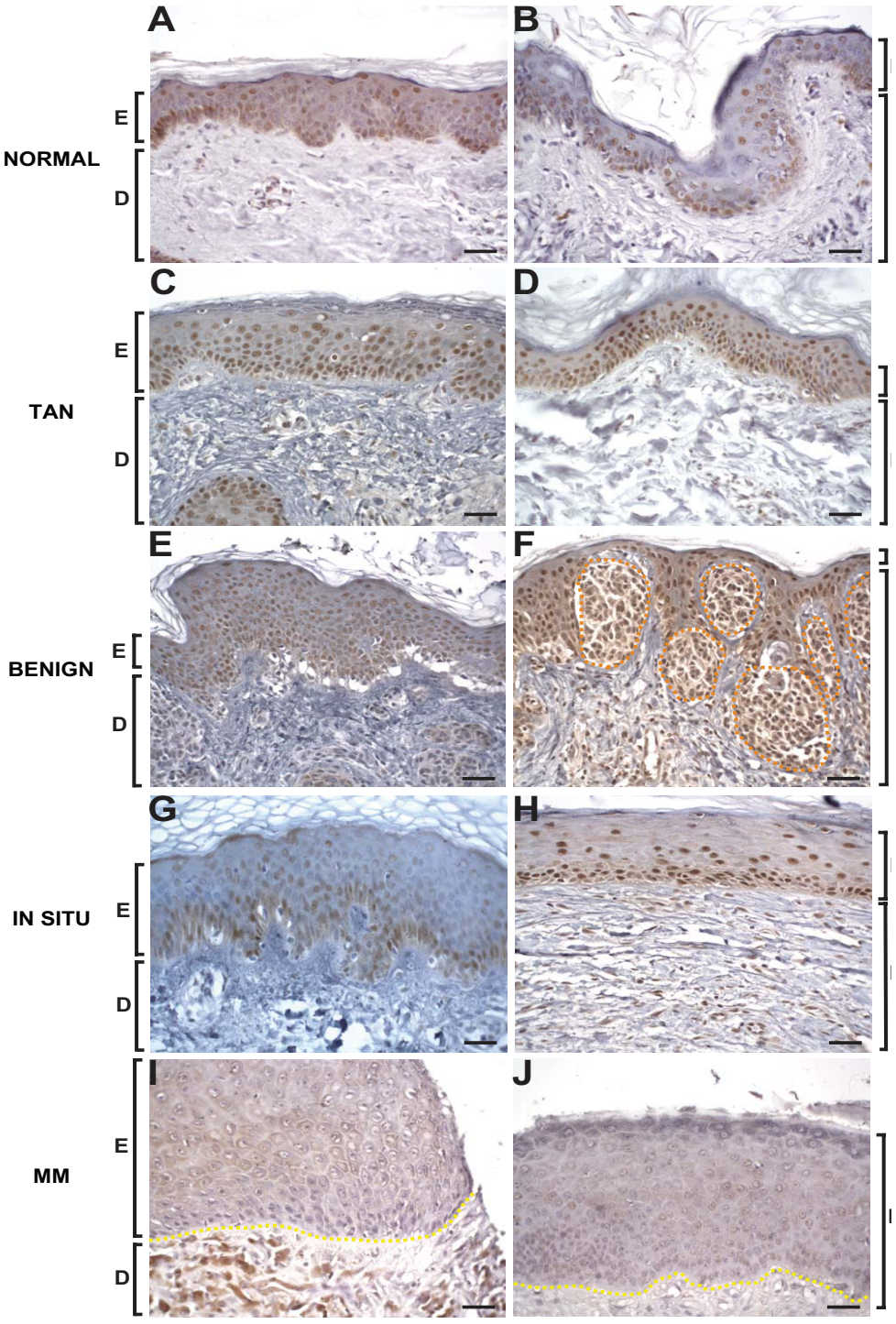


Figure 2.1

Figure 2.1. Reduced expression of keratinocytic RXRα during melanoma progression in human skin. Representative localization of RXRα protein within the epidermis of normal (A,B), tumor adjacent normal [TAN] (C,D), benign melanocytic nevi (E,F), *in situ* melanoma (G,H) and malignant melanoma [MM] sections (I, J). Brown staining represents RXRα expression (except for the dermal pigmentation present in malignant melanoma sections), counterstained with Harris hematoxylin. E, epidermis; D, dermis. Orange dashed line encompass nests of nevus cells, yellow dashed lines indicates epidermal-dermal junction. Scale bar = 33μm.

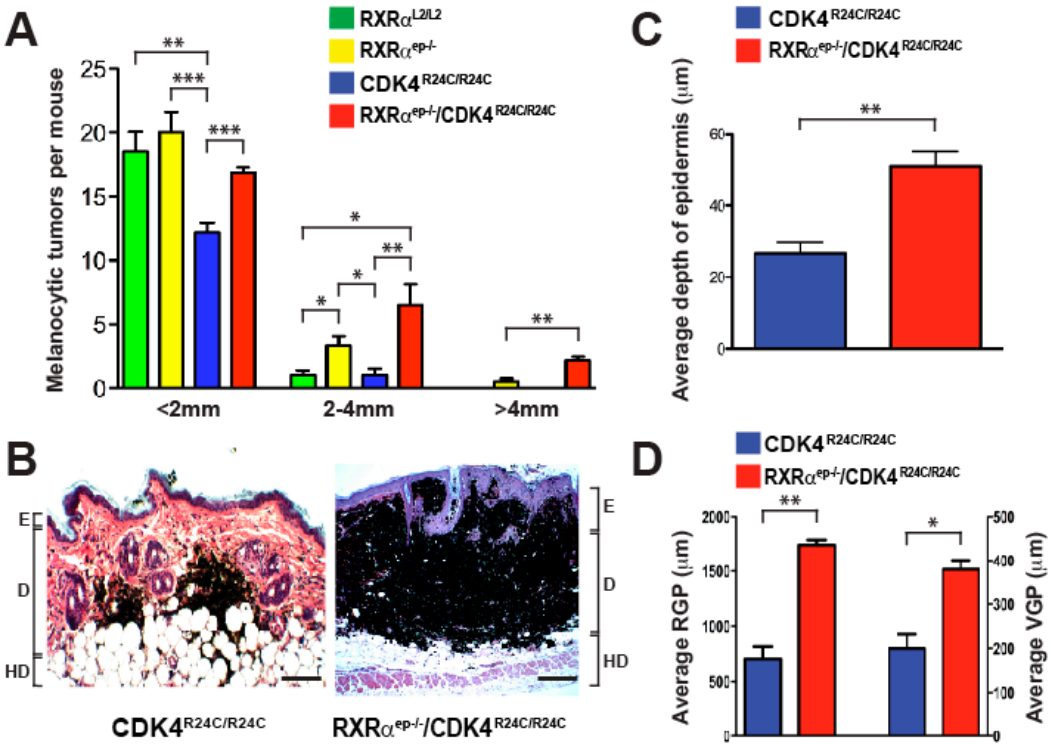

A
RXRα L2/L2
RXRα ep-/-
CDK4 R24C/R24C
RXRα ep-/-/CDK4 R24C/R24C
Melanocytic tumors per mouse
<2mm
2-4mm
>4mm
B
E
D
HD
CDK4 R24C/R24C
RXRα ep-/-/CDK4 R24C/R24C
C
CDK4 R24C/R24C
RXRα ep-/-/CDK4 R24C/R24C
Average depth of epidermis (μm)
D
CDK4 R24C/R24C
RXRα ep-/-/CDK4 R24C/R24C
Average RGP (μm)
Average VGP (μm)

Figure 2.2

Figure 2.2. Formation of larger melanomas in RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ bigenic mice. (A) Size distribution of melanocytic tumors in RXR$\alpha^{L2/L2}$, RXR$\alpha^{ep-/-}$, CDK4$^{R24C/R24C}$ and RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ mice after DMBA/TPA treatment. The number of tumors, less than 2mm, 2-4mm and greater than 4mm in diameter, were determined in the four groups of mice, as indicated. Values are expressed as mean +/- SEM. (B) Histological analyses of melanocytic tumors from CDK4$^{R24C/R24C}$ and RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ mice. Hematoxylin and eosin (H&E)-stained 5μm thick paraffin skin sections of biopsies taken after DMBA/TPA treatment. E, epidermis; D, dermis; HD, hypodermis. Scale bar = 62μm**.** (C, D) Increased epidermal thickness, and higher radial growth phase (RGP) and vertical growth phase (VGP) of melanocytic tumors in RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ mice. Statistical significance was calculated using unpaired Student's t-test with GraphPad Prism software. Statistical relevance indicated as follows; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

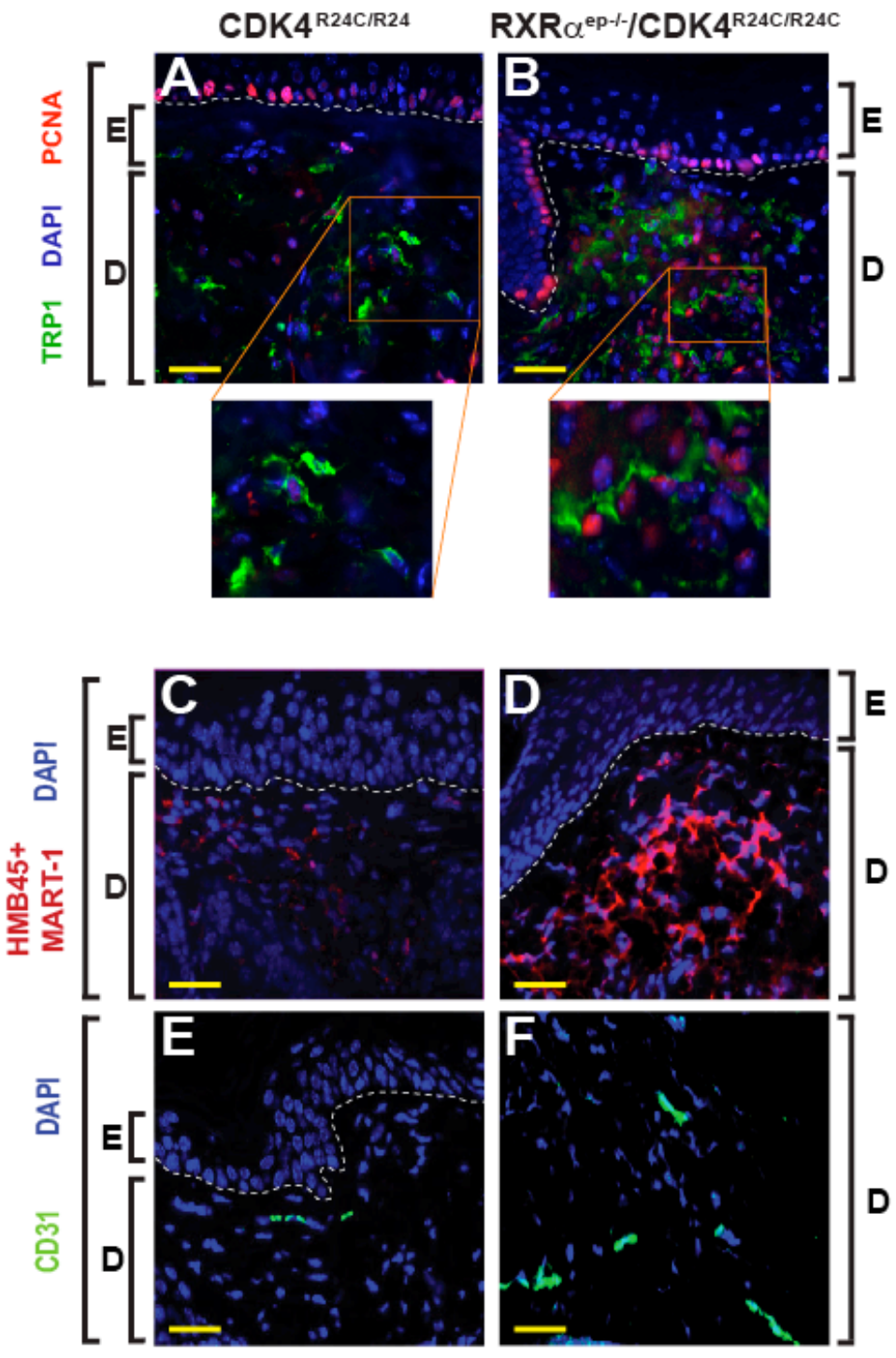

CDK4R24C/R24
RXRαep-/-/CDK4R24C/R24C
A
B
C
D
E
F
PCNA
DAPI
TRP1
HMB45+
MART-1
CD31
E
D

Figure 2.3

Figure 2.3. Immunohistochemical (IHC) characterization of melanocytic tumors in CDK4$^{R24C/R24C}$ and RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ bigenic mice. (A, B) Enhanced melanocyte proliferation in melanocytic tumors from RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ mice as determined by co-labeling with anti-TRP1 [green] and anti-PCNA [red] antibodies. (C, D) IHC staining for malignant melanocytes using a cocktail of antibodies directed against melanoma antigens HMB45 and MART-1 [red]. (E, F) Increased vascularization was detected by anti-CD31 antibody [green] in melanocytic tumors from bigenic mice compared to CDK4$^{R24C/R24}$ alone. E, epidermis; D, dermis. Scale bar (in yellow) = 33μm. White dashed lines, artificially added, indicate epidermal-dermal junction. Blue color corresponds to DAPI staining of the nuclei.

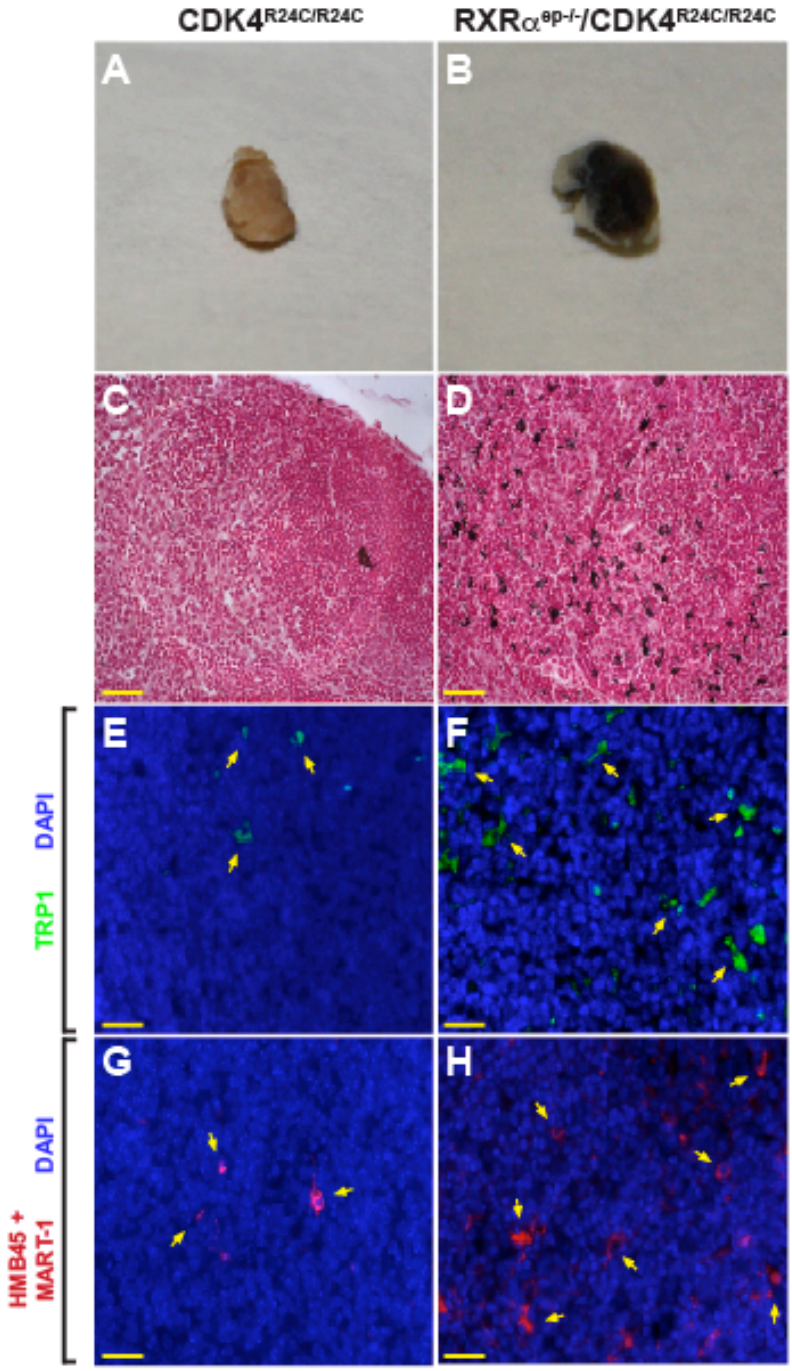

CDK4R24C/R24C
RXRαep-/-/CDK4R24C/R24C
A
B
C
D
E
F
G
H
TRP1
DAPI
HMB45 +
MART-1
DAPI

Figure 2.4

Figure 2.4. Lymph node metastasis in RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ mice. (A, B) Excised draining lymph nodes from CDK4$^{R24C/R24C}$ and RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ mice after DMBA/TPA treatment. (C, D) Fontana-Masson staining of draining lymph nodes from CDK4$^{R24C/R24C}$ and RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ mice after carcinogenic treatment. Melanin granules stain black and red corresponds to Nuclear Fast Stain. (E, F) IHC staining was performed using antibodies directed against TRP1 [green]. (G, H) IHC staining was performed using antibodies directed against HMB45 and MART-1 [red]. Scale bar (in yellow) = 33μm. Blue corresponds to DAPI staining of the nuclei.

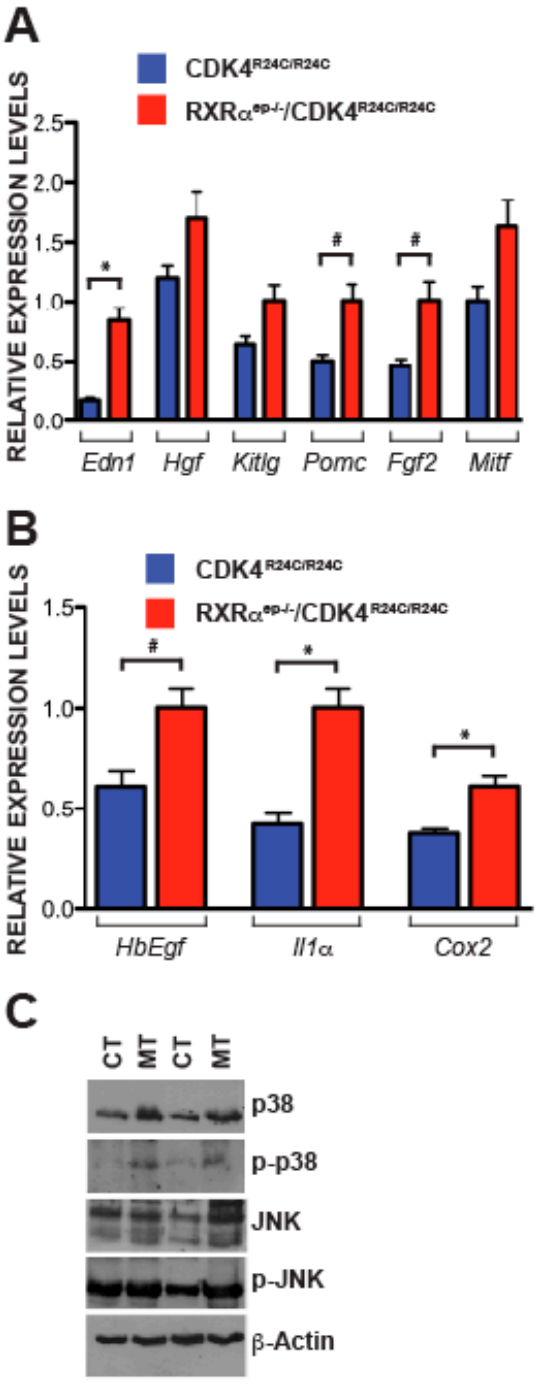
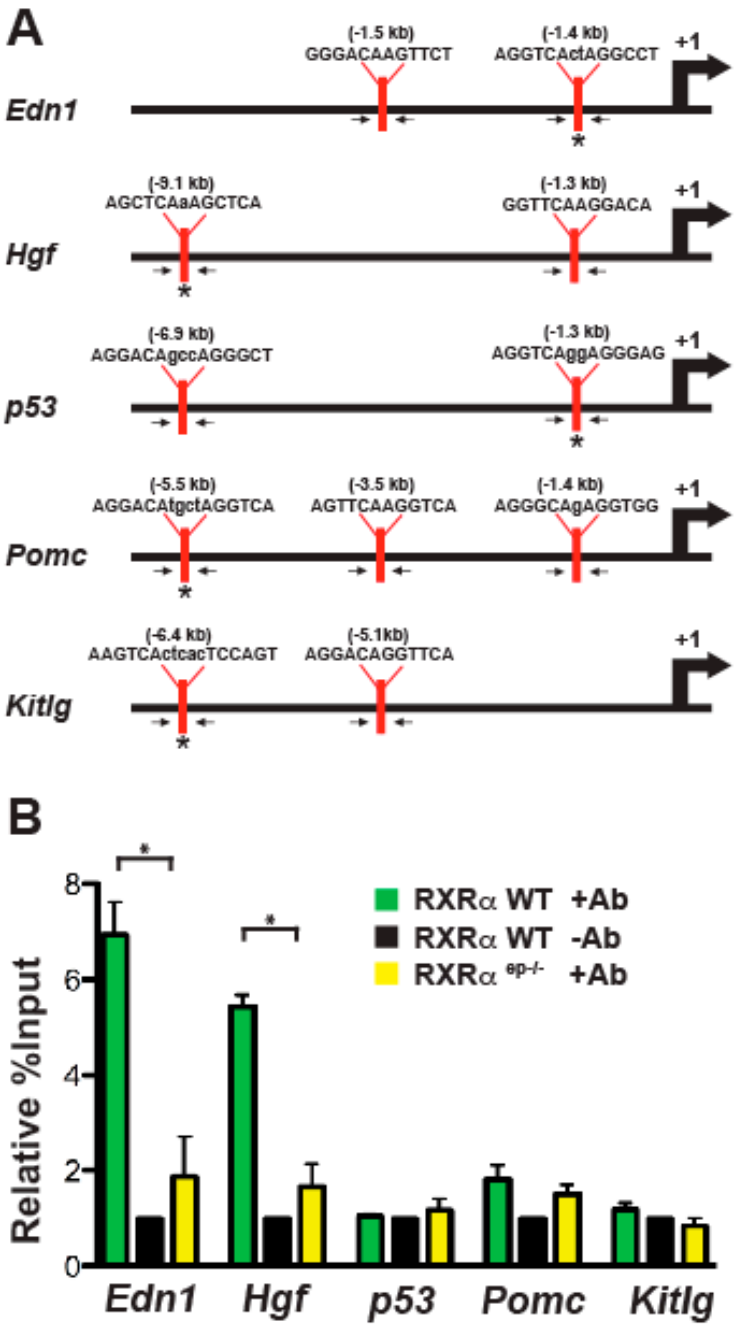

A
CDK4R24C/R24C
RXRαep-/-/CDK4R24C/R24C
RELATIVE EXPRESSION LEVELS
2.5
2.0
1.5
1.0
0.5
0.0
*
#
#
Edn1
Hgf
Kitlg
Pomc
Fgf2
Mitf
B
CDK4R24C/R24C
RXRαep-/-/CDK4R24C/R24C
RELATIVE EXPRESSION LEVELS
1.5
1.0
0.5
0.0
#
*
*
HbEgf
Il1α
Cox2
C
CT
MT
CT
MT
p38
p-p38
JNK
p-JNK
β-Actin

Figure 2.5

Figure 2.5. Increased expression of paracrine factors and regulatory proteins in the skin of RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ mice. (A) Enhanced expression of mitogenic paracrine factors and regulatory proteins in skin of RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ mice compared to CDK4$^{R24C/R24C}$ mice after 25 weeks of DMBA/TPA treatment. Relative mRNA transcript levels of endothelin 1 (*Edn1*), hepatocyte growth factor (*Hgf*), Kit ligand (*Kitlg*), proopiomelanocortin (*Pomc*), fibroblast growth factor 2 (*Fgf2*) and microphtalmia-associated transcription factor (*Mitf*) were measured. (B) Enhanced expression of autocrine factors in skin of RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ mice compared to CDK4$^{R24C/R24C}$ 25 weeks after DMBA/TPA treatment. Relative mRNA transcript levels of heparin-binding EGF-like growth factor (*HbEgf*), interleukin 1-alpha (*Il1a*) and cyclooxygenase 2 (*Cox2*) were measured. (C) Western blot analysis of different proteins in the mitogenic MAPK pathway on skin extracts from RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ [MT] and CDK4$^{R24C/R24C}$ [CT] mice 25 weeks after DMBA/TPA treatment. Antibodies used were against p38, phospho-p38, JNK, phospho-JNK. Data (A and B) represents mean ± SEM. All reactions were performed in triplicates using skin samples collected from a minimum of three different mice in each group. Statistical analyses were done using GraphPad Prism software and significance level (*) were set at $p < 0.05$. # = Not significant.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(-1.4 kb)
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+1
Hgf
(-9.1 kb)
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(-1.3 kb)
GGTTCAAGGACA
+1
p53
(-6.9 kb)
AGGACAgccAGGGCT
(-1.3 kb)
AGGTCAggAGGGAG
+1
Pomc
(-5.5 kb)
AGGACAtgctAGGTCA
(-3.5 kb)
AGTTCAAGGTCA
(-1.4 kb)
AGGGCAgAGGTGG
+1
Kitlg
(-6.4 kb)
AAGTCActcacTCCAGT
(-5.1kb)
AGGACAGGTTCA
+1
B
Relative %Input
0
2
4
6
8
RXRα WT +Ab
RXRα WT -Ab
RXRα ep-/- +Ab
Edn1
Hgf
p53
Pomc
Kitlg

Figure 2.6

Figure 2.6. Nuclear receptor RXRα associates with target gene promoter encoding soluble growth factors in primary keratinocytes. (A) Putative RXREs identified upstream of the transcriptional start site of genes for specific soluble growth factors such as *Edn1*, *Hgf*, *p53*, *Pomc* and *Kitlg*. Arrows indicate position of primers designed to capture all potential binding sequences, asterisks (*) indicate location of results shown in figure. The position of transcriptional start site is indicated as +1, promoter diagrams not to scale. (B) Chromatin immunoprecipitation (ChIP) assay was performed on primary keratinocytes using antibody against RXRα protein and results were analyzed by qPCR using specific primers (indicated in A). RXRα association on consensus sequences for RXRα/NR heterodimers located on the promoters of *Edn1*, *Hgf*, *p53*, *Pomc* and *Kitlg* genes in wild type keratinocytes was compared to cells lacking RXRα protein or wild type cells immunoprecipitated with an irrelevant antibody. Endothelin 1, EDN1; proopiomelanocortin, POMC; hepatocyte growth factor, HGF; Kit ligand, KITLG. Statistical analyses was done by Student's unpaired T-test using GraphPad Prism software and significance level (*) were set at $p < 0.05$.

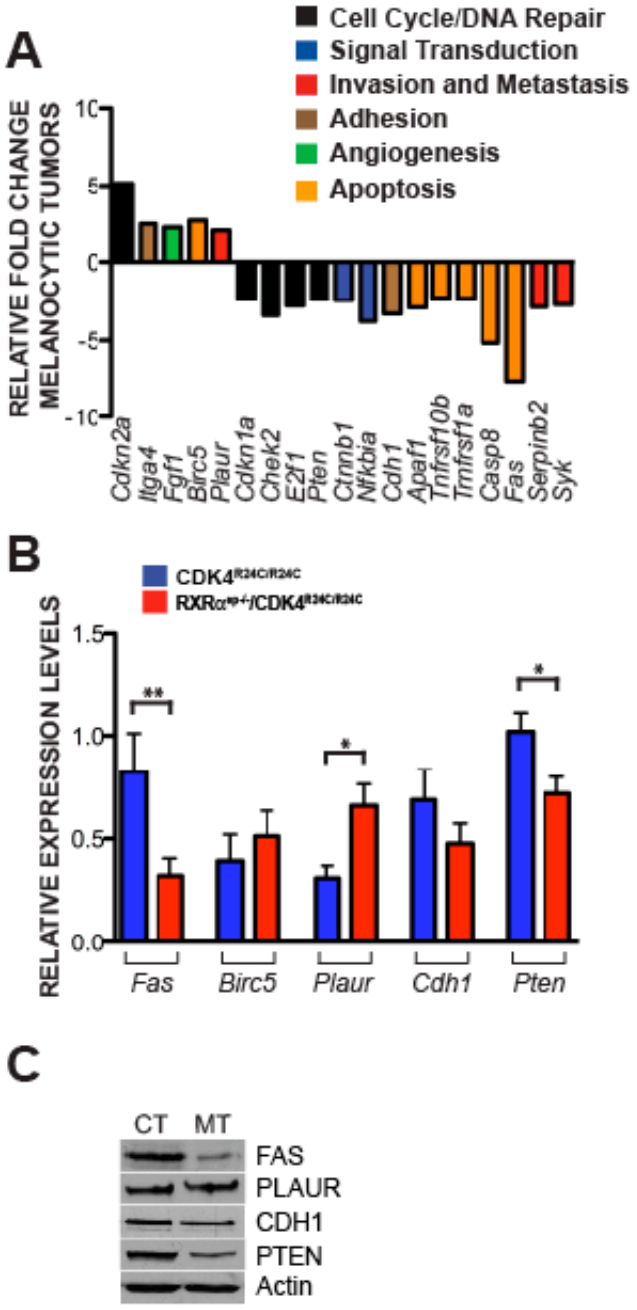
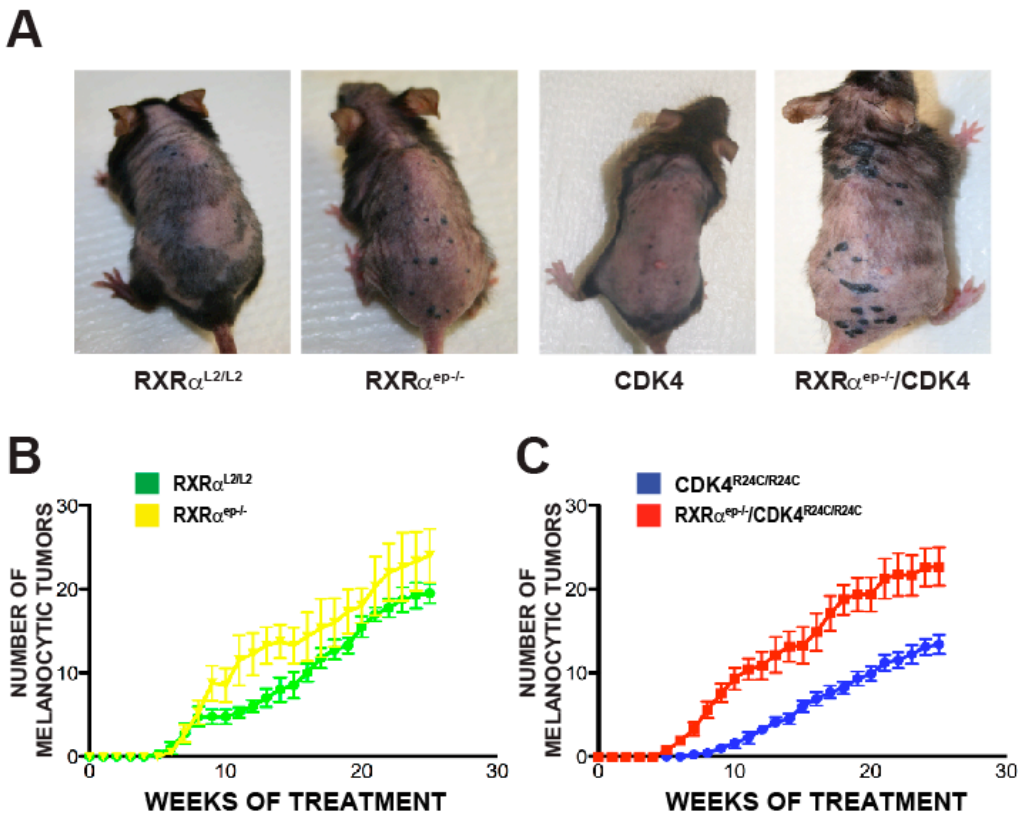
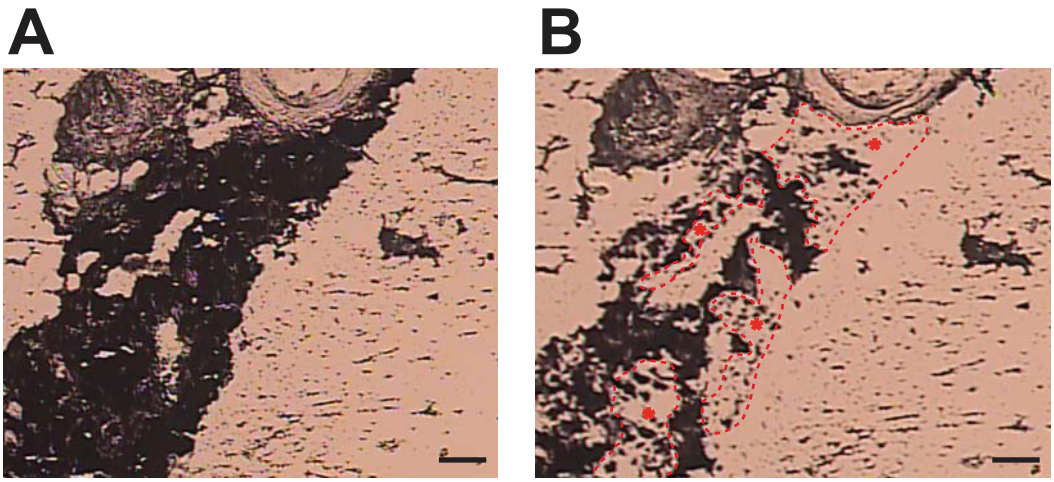

A
Cell Cycle/DNA Repair
Signal Transduction
Invasion and Metastasis
Adhesion
Angiogenesis
Apoptosis
RELATIVE FOLD CHANGE MELANOCYTIC TUMORS
Cdkn2a
Itga4
Fgf1
Birc5
Plaur
Cdkn1a
Chek2
E2f1
Pten
Ctnnb1
Nfkbia
Cdh1
Apaf1
Tnfrsf10b
Tmfrsf1a
Casp8
Fas
Serpinb2
Syk
B
CDK4R24C/R24C
RXRα+/+/CDK4R24C/R24C
RELATIVE EXPRESSION LEVELS
1.5
1.0
0.5
0.0
**
*
*
Fas
Birc5
Plaur
Cdh1
Pten
C
CT
MT
FAS
PLAUR
CDH1
PTEN
Actin

Figure 2.7

Figure 2.7. Altered expressions of genes in laser capture microdissected (LCM) melanocytic tumors. (A) Relative fold change differences ≥ 2 of RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ mRNA transcripts taken from LCM isolated melanocytes compared to CDK4$^{R24C/R24C}$ mice. Amplified cDNA was analyzed using a RT^2 Nano PreAMP cDNA Synthesis Kit and evaluated using a Mouse Cancer PathwayFinder PCR Array System. Gene expression was normalized to a panel of housekeeping genes (*B2m*, *Hprt1*, *Rpl13a*, *Gapdh*, *Actb*). (B) Validation of PCR array results using relative mRNA transcript levels of *Fas*, *Birc5*, *Plaur*, *Cdh1* and *Pten* from LCM captured melanocytes of CDK4$^{R24C/R24C}$ and RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ mice treated with DMBA/TPA for 25 weeks. (C) Validation of PCR array results using western blot detection for protein levels in both RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ [MT] and CDK4$^{R24C/R24C}$ [CT] melanocytic tumors using antibodies against FAS, PLAUR, CDH1 and PTEN. All experiments were done using a minimum of three biological replicates from each group of mice. Data represents three individual experiments and in all cases are expressed as mean +/- SEM. Statistical significance was calculated using unpaired Student's t-test with GraphPad Prism software. Statistical relevance indicated as follows; * = $p < 0.05$, ** = $p < 0.01$.



Supplemental Figure 2.1. Formation of aggressive melanoma in RXRα$^{ep-/-}$/CDK4$^{R24C/R24C}$ bigenic mice. (A) Representative gross morphology of RXRα$^{L2/L2}$, RXRα$^{ep-/-}$, CDK4$^{R24C/R24C}$ and RXRα$^{ep-/-}$/CDK4$^{R24C/R24C}$ dorsal skin after DMBA/TPA treatment. (B) Rate of melanocytic tumor formation from RXRα$^{L2/L2}$ and RXRα$^{ep-/-}$ skin after 25 weeks of DMBA/TPA treatment. (C) Rate of melanocytic tumor formation from CDK4$^{R24C/R24C}$ and RXRα$^{ep-/-}$/CDK4$^{R24C/R24C}$ dorsal skin after DMBA/TPA treatment.



Supplemental Figure 2.2. Laser captured microdissection on sections from melanocytic tumors. (A, B) Representative pre- and post-laser captured microdissection images taken from frozen sections of RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ melanocytic tumors. Red dashed line indicates melanocytic cells that have been removed for RNA isolation. Scale bar = 62μm.

Table 2.1 Primer sequences used in RT-qPCR assays

Gene	Sense	Antisense
Edn1	5'-ACTTCTGCCACCTGGACATC-3'	5'-GTCTTTCAAGGAACGCTTGG-3'
Hgf	5'-AGGAACAGGGGCTTTACGTT-3'	5'-GCTGCCTCCTTTACCAATGA-3'
Kitlg	5'-TCCGAAGAGGCCAGAAACTA-3'	5'-TCAGATGCCACCATAAAGTCC-3'
Pomc	5'-GTACCCCAACGTTGCTGAGA-3'	5'-GACCTGCTCCAAGCCTAATG-3'
Fgf2	5'-CGGTCACGGAAATACTCCAG-3'	5'-TATGGCCTTCTGTCCAGGTC-3'
Mitf	5'-TGAAGCAAGAGCATTGGCTA-3'	5'-TAGCTCCTTAATGCGGTCGT-3'
HbEgf	5'-GGACAGATCTGAACCTTTTCA-3'	5'-GCAGTAGTCCTTGTATTTCCT-3'
Il1α	5'-TCACCTTCAAGGAGAGCCG-3'	5'-ATCTGGGTTGGATGGTCTCTT-3'
Cox2	5'-TCAAAACCGTGGGGAATGTAT-3'	5'-AGGATGTAGTGCACTGTGTTT-3'
Fas	5'- TATCAAGGAGGCCCATTTTGC-3'	5'- TGTTTCCACTTCTAAACCATGCT-3'
Birc5	5'- GAGGCTGGCTTCATCCACTG-3'	5'- CTTTTTGCTTGTTGTTGGTCTCC-3'
Plaur	5'- TGCCGGGGACCAATGAATC-3'	5'- GAGGGTCAGGAGCAGAGAG-3'
Cdh1	5'- CAGGTCTCCTCATGGCTTTGC-3'	5'- CTTCCGAAAAGAAGGCTGTCC-3'
Pten	5'- TGGATTCGACTTAGACTTGACCT-3'	5'- GCGGTGTCATAATGTCTCTCAG-3'
Hprt	5'-TGACACTGGCAAAACAATGCA-3'	5'-GGTCCTTTTCACCAGCAAGCT-3'

Table 2.2 Primer sequences used in ChIP assays

Gene	Sense	Antisense
Edn1	5'-GTTCTTAGTGACCTAAAGCTGCTG-3'	5'-GGAGTAGAGGCCAAAATAGACTCA-3'
Hgf	5'-GGAGAGGGAGAAGGAGAGAGAG-3'	5'-ATAACTGGTCATGAGGGAATGG-3'
p53	5'-GCACCGGTTCAAAGTCTGTATT-3'	5'-CTGGGAACGGTAATGCACTCTA-3'
Pomc	5'-TTGGAGAGTCACAGAATATTCCAC-3'	5'-GGATGGATGGGTTTTCTTTATG-3'
Kitlg	5'-AACTCCAGCCTCTGTGTGTGTA-3'	5'-TTTCCTGGGTTACAGAGAATGG-3'

SUPPLEMENTARY TABLE 2.1

PROPORTION OF SAMPLES LACKING KERATINOCYTIC EXPRESSION OF RXRα

TYPE OF SAMPLE	TOTAL NUMBER OF SAMPLES ANALYZED	LOSS OF EPIDERMAL EXPRESSION OF RXRa
Normal skin	30	0/30
TAN[1] skin	18	0/18
Benign nevi	27	0/13[2]
In situ melanoma	3	2/3
Malignant melanoma	128	4/5[3]

[1]Tumor adjacent normal

[2]For benign nevi only 13 of the 27 samples analyze had epidermal tissue present.

[3]For malignant melanoma only 5 of the 128 samples analyzed had epidermal tissue present.

SUPPLEMENTARY TABLE 2.2
RELATIVE FOLD CHANGE OF GENE EXPRESSION IN RXRα$^{ep-/-}$/CDK4$^{R24C/R24C}$ MTs

Gene Name	Description	Fold Change
Apaf1	Apoptotic peptidase activating factor 1	-2.90
Bcl2l1	Bcl2-like 1	-3.72
Birc5	Baculoviral IAP repeat-containing 5	+2.76
Casp8	Caspase 8	-5.16
Cdh1	Cadherin 1	-3.31
Cdk2	Cyclin-dependent kinase 2	-2.51
Cdk4	Cyclin-dependent kinase 4	-2.75
Cdkn1a	Cyclin-dependent kinase inhibitor 1A (P21)	-2.30
Cdkn2a	Cyclin-dependent kinase inhibitor 2A	+5.14
Cflar	CASP8 and FADD-like apoptosis regulator	-2.05
Chek2	CHK2 checkpoint homolog (S. pombe)	-3.41
Col18a1	Collagen, type XVIII, alpha 1	-2.47
Ctnnb1	Catenin (cadherin associated protein), beta 1	-2.45
E2f1	E2F transcription factor 1	-2.79
Fas	Fas (TNF receptor superfamily member 6)	-7.71
Fgf1	Fibroblast growth factor 1	+2.33
Itga2	Integrin alpha 2	-4.29
Itga4	Integrin alpha 4	+2.54
Jun	Jun oncogene	-3.02
Mta1	Metastasis associated 1	-2.88
Nfkbia	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	-3.79
Nme4	Non-metastatic cells 4, protein expressed in	+2.53
Pdgfb	Platelet derived growth factor, B polypeptide	-3.43
Pik3r1	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	-2.56
Plau	Plasminogen activator, urokinase	-2.08
Plaur	Plasminogen activator, urokinase receptor	+2.13
Pten	Phosphatase and tensin homolog	-2.38
Serpinb2	Serine (or cysteine) peptidase inhibitor, clade B, member 2	-2.88
Syk	Spleen tyrosine kinase	-2.72
Tgfb1	Transforming growth factor, beta 1	-3.14
Tnfrsf10b	Tumor necrosis factor receptor superfamily, member 10b	-2.27
Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	-2.30
Vegfa	Vascular endothelial growth factor A	-2.69
Vegfb	Vascular endothelial growth factor B	-3.98

Supplemental Table 2.2. Relative fold changes of gene expression within melanocytic tumors from RXRα$^{ep-/-}$/CDK4$^{R24C/R24C}$ bigenic mice. List of genes on Cancer PathwayFinder PCR array exhibiting a fold change greater than 2.

Endothelin-1 is a Transcriptional Target of p53 in Epidermal Keratinocytes and Regulates UV Induced Melanocyte Homeostasis

Chapter 3

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3.1 Abstract

Keratinocytes contribute to melanocyte activity by influencing their microenvironment, in part, through secretion of paracrine factors. Here we discovered that p53 directly regulates *Edn1* expression in epidermal keratinocytes and controls UV-induced melanocyte homeostasis. Selective ablation of EDN1 in murine epidermis ($EDN1^{ep-/-}$) does not alter melanocyte homeostasis in newborn skin but decreases dermal melanocytes in adult skin. Results showed that keratinocytic EDN1 in a non-cell autonomous manner controls melanocyte proliferation, migration, DNA damage and apoptosis after UVB irradiation. Expression of other keratinocyte derived paracrine factors did not compensate for the loss of EDN1. Topical treatment with EDN1 receptor (EDNRB) antagonist BQ788 abrogated UV induced melanocyte activation and recapitulated the phenotype seen in $EDN1^{ep-/-}$ mice. Altogether, present studies establish an essential role of EDN1 in epidermal keratinocytes to mediate UV induced melanocyte homeostasis *in vivo*.

3.2 Introduction

Endothelin 1 (EDN1) belongs to a group of related peptides (EDN1, EDN2 and EDN3) encoded by separate genes. The mature 21-amino acid peptides are expressed in a variety of tissue types and activate two distinct G protein-coupled receptors (EDNRA and EDNRB) with varying affinities (Yanagisawa et al., 1988; Arai et al., 1990; Sakurai et al., 1990). The EDNRB receptor is required for melanocyte development from a neural crest lineage (Reid et al., 1996; Opdecamp et al., 1998). Mice deficient in EDN1 have respiratory complications as well as craniofacial abnormalities (Kurihara et al., 1994), while those lacking EDNRB have a spotted color resulting from loss of epidermal melanocytes (Hosoda et al., 1994). Although EDN1 was initially characterized as a potent vasoconstrictive peptide, it also functions as a keratinocyte-derived paracrine factor for melanocytes. Previous *in vitro* studies have shown that ultraviolet B (UVB) exposure increases secretion of keratinocyte-derived EDN1 which regulates proliferation, melanogenesis, migration and dendricity of human melanocytes via a receptor-mediated pathway (Imokawa et al., 1992; Imokawa et al., 1995; Hara et al., 1995; Horikawa et al., 1995), and this stimulatory effect is dampened by the EDNRB antagonist BQ788 (Wu et al., 2004). There was also evidence that EDNRB serves as a tumor progression marker of human melanoma, as increased

expression of EDNRB was found in human melanoma biopsies and usage of EDNRB antagonists inhibited melanoma growth (Demunter et al., 2001; Lahav et al., 1999). Additionally, EDN1 was shown to be upregulated in the epidermis of mice after UVB irradiation (Ahn et al., 1998), potentially contributing to the photoprotective effects of EDN1 on melanocytes (Kadekaro et al., 2005). The melanocytic response to UV exposure in mouse skin has been characterized, where follicular melanocytes migrate upward along the outer root sheath and populate the basal layer of the interfollicular epidermis. This epidermal melanocyte population peaks around 72hrs post-UV and begins to decrease 1-2 days later (Rosdahl and Szabo, 1978; Walker et al., 2009). Melanin pigmentation is a result of reciprocal interaction between epidermal keratinocytes and follicular melanocytes through production of various signaling molecules and activation of corresponding receptors on the target cells (Slominski et al., 2004; Slominski et al., 2005b). However, the full spectrum of keratinocytic influence on melanocyte activity after UV exposure remains to be determined.

Our previous work in an RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ cutaneous melanoma model suggested a paracrine contribution by several keratinocyte-derived soluble factors including EDN1 in disease progression (Hyter et al., 2010). Here we discovered a direct *in vivo* transcriptional regulation of keratinocytic *Edn1* by the tumor-suppressor p53 in epidermal keratinocytes in response to

UV irradiation. We also demonstrate that *in vivo* disruption of keratinocyte-derived EDN1 signaling *in vivo* alters melanocyte proliferation and decreases epidermal and dermal melanocyte populations in both normal and UV exposed mouse skin. EDN1 also has a protective role against UVR induced DNA damage and apoptosis. Similar effects on UV-induced melanocyte proliferation and DNA damage are observed in p53-null mice. Inhibition of EDN1 signaling by topical application of an EDNRB antagonist BQ788 on mouse skin has similar effects as epidermal EDN1 ablation. Furthermore, treatment of primary murine melanocytes with BQ788 abrogates signaling downstream of this receptor. These findings further support the role of a micro-environmental influence driven by p53 in keratinocytes on the behavior and activation of melanocytes.

3.3 Results

Transcriptional regulator p53 directly and positively regulates *Edn1* expression in epidermal keratinocytes after UV irradiation

We wanted to elucidate mechanism(s) of *Edn1* regulation in keratinocytes in response to UV radiation. Induction of p53 after UV exposure protects epidermal keratinocytes against DNA damage skin carcinogenesis (Jiang et al., 1999), and directly regulates *p21* expression to limit cell proliferation. Similar regulation of *Pomc* by p53 in epidermal keratinocytes has been postulated to mediate UV-induced hyperpigmentation in murine skin (Cui et al., 2007). However, this linear mode of action does not fully explain the eumelanogenesis demonstrated by the melanogenic phenotype of POMC-null mice (Slominski et al., 2005a). To determine if p53 is an upstream regulator of *Edn1* gene in epidermal murine keratinocytes, we performed *in silico* promoter analysis using the fuzznuc and ConTra programs. Three potential p53 consensus binding motifs were identified in proximal, mid and distal promoter region of *Edn1* within 5kb upstream of the transcriptional start site (Figure 3.1A). Additional *in silico* studies were performed on the human *EDN1* promoter and potential p53 binding sequences were also discovered (Figure 3.1A). To determine if p53 is recruited onto the promoter of murine *Edn1*, we performed *ex vivo* chromatin immunoprecipitation (ChIP) assay on extracts

from primary keratinocytes exposed to UV irradiation using primers designed to capture the three regions, as well as the 3' UTR region. *p21*, a p53 target in keratinocytes, was used as a positive control (Liu and Pelling, 1995). As reported earlier, amplification of isolated chromatin DNA revealed increased promoter occupancy by p53 on the *p21* promoter following UV irradiation (UVR) (Figure S3.1C). The distal promoter region (relative to the transcriptional start site) of *Edn1* displayed a preferential increase in p53 binding post-UVR compared to other regions and to mock irradiated cells (Figure 3.1B). These results suggest a possible direct regulation of *Edn1* expression by p53 in epidermal keratinocytes. In order to validate these findings, we performed RT-qPCR analyses for *Edn1* expression in the epidermis of both wildtype (WT) and p53-null ($p53^{-/-}$) adult mice after UVR exposure. A significant decrease in the expression of *Edn1* was observed in the epidermis of $p53^{-/-}$ mice 8hrs post-UVR compared to wildtype skin (Figure 3.1C). Altogether, these results establish *Edn1* as a direct transcriptional target of p53 in murine keratinocytes.

Keratinocyte specific ablation of EDN1 alters melanocyte homeostasis in the skin of adult mice

The *in vivo* role of keratinocyte-derived EDN1 in regulating epidermal homeostasis and melanocyte activity is unknown. In the present study, we

investigated the role of this signaling molecule on keratinocyte and melanocyte homeostasis in murine skin. To this end, floxed EDN1$^{L2/L2}$ mice (Huang et al., 2002; Shohet et al., 2004) were bred with a K14-Cre deleter mouse strain, which drives expression of Cre-recombinase in the basal cells of developing epidermis, thereby generating EDN1$^{ep-/-}$ mice (selectively lacking EDN1 in murine epidermis). Deletion of *Edn1* in presence of Cre was confirmed by PCR using specific primers on DNA isolated from neonatal EDN1$^{L2/L2}$ (control, CT) and EDN1$^{ep-/-}$ mice skin (Figure 3.2A). RT-qPCR analyses on RNA isolated from the epidermis of CT and EDN1$^{ep-/-}$ mice showed a significant downregulation of *Edn1* transcripts in the mutant skin, confirming ablation of *Edn1* in epidermal keratinocytes (Figure 3.2B). Basal levels of EDN1 mRNA seen in the EDN1$^{ep-/-}$ skin are most likely due to less frequent *Edn1* expressing cells within the dermal compartment. No compensatory up-regulation of *Edn3* (another endothelin family member expressed in skin) transcripts was observed by RT-qPCR in EDN1$^{ep-/-}$ skin compared to CT skin (Figure 3.2B). Gross morphological examination did not reveal any difference between the skin of EDN1$^{L2/L2}$ and EDN1$^{ep-/-}$ mice.

To determine whether loss of keratinocytic EDN1 altered keratinocyte proliferation and differentiation *in vivo*, we performed histological and immunohistochemical (IHC) analysis on dorsal skin sections from CT and mutant neonatal (P2) and adult (P42) mice. Histological analyses on H&E

stained skin sections from CT and mutant mice did not reveal any differences in epidermal thickness between the two groups in the neonatal or adult skin (Figure S3.2A, B and S3.6A, B, C). Furthermore, expression pattern for markers of proliferation [Ki67, PCNA, keratin-14 (K14)] and differentiation [K10 and loricrin] were similar between CT and EDN1$^{ep-/-}$ P2 and P42 skin (Figure S3.2C, D and S3.6C and data not shown). These results indicate that loss of keratinocytic EDN1 is dispensable for normal epidermal homeostasis of the developing and adult murine epidermis. Fontana-Masson (FM) and TRP1 (melanocyte specific marker) IHC staining on adult skin sections showed a significant decrease in the number of dermal melanocytes in the mutant skin compared to CT skin. However, no differences in dermal melanocyte number were observed between the neonatal CT and mutant skin (Figure 3.2C, D and E). Altogether, these findings suggest that EDN1 mediated paracrine signaling from keratinocytes is necessary for maintenance of dermal melanocyte populations in unirradiated adult murine skin.

Role of keratinocytic EDN1 in regulating UV induced proliferation, DNA damage repair and apoptosis of melanocytes *in vivo*

UV irradiation induces secretion of EDN1 by epidermal keratinocytes (Imokawa et al., 1992). In order to investigate the effects of UV irradiation in absence of EDN1, we subjected neonatal EDN1$^{L2/L2}$ and EDN1$^{ep-/-}$ mice to UVR exposure and analyzed its responses on skin keratinocytes and

melanocytes. Briefly, P2 pups were exposed to a single dose of 600mJ/cm^2 UVR and dorsal skin samples were collected after 24, 48, 72 and 96hrs post-treatment. As reported earlier, *Edn1* transcript level was significantly increased in the epidermis of CT skin at 24hrs post-UVR (Ahn et al., 1998) (Figure 3.3A). No *Edn1* induction was observed in the mutant skin, further confirming Cre-mediated excision of *Edn1* gene in their epidermis (Figure 3.3A). Although *Edn1* expression decreased in the CT skin after 24hrs post-UVR, its transcript level remained significantly higher at later timepoints compared to the EDN1$^{ep-/-}$ skin. RT-qPCR analyses did not reveal any compensatory upregulation of *Edn3* transcript levels or alteration in the expression level of other keratinocyte derived soluble factors, such as *Fgf2, Hgf, Pomc* or *Kitlg*, in the EDN1$^{ep-/-}$ neonatal mice skin at all timepoints post-UV irradiation (Figure 3.3A and Figure S3.3A). These results suggest that any effects on melanocyte activity after UV exposure observed in the present study are solely due to the loss of EDN1. H&E staining on paraffin skin sections from CT and mutant mice did not reveal any differences in epidermal thickness between the CT and mutant skin post-UVR, implying that the loss of keratinocytic EDN1 did not influence UV-induced epidermal hyperplasia (Figure S3.6A and S3.6B). To further evaluate any altered responses to UVR, we performed FM staining for pigmented melanocytes on skin sections from CT and mutant mice at different timepoints after UV exposure. We did not observe an increase in epidermal

melanocyte population until 72hrs post-UVR in both the groups, and the number of epidermal and dermal melanocytes was significantly reduced in the EDN1$^{ep-/-}$ skin compared to CT skin at 72 and 96hrs post-UVR (Figure 3.3B, C and D).

In an attempt to elucidate the mechanistic basis of reduced melanocyte population from the hair follicles in the EDN1$^{ep-/-}$ skin, IHC analyses was performed on CT and mutant skin to investigate the proliferative potential, DNA damage and apoptotic responses of melanocytes post-UVR. Co-immunostaining of paraffin skin sections with proliferation markers PCNA or Ki67 with TRP1 allowed us to quantify the proliferative responses to UV exposure in epidermis, dermis and melanocytes. A significant decrease in the number of proliferating melanocytes was seen in the mutant skin after 72hrs UVR using both the proliferation markers, which correlated with the decreased numbers of total melanocytes at that timepoint (Figure 3.4A and B and S3.5A and B, left panels). Similarly, IHC analyses for UVR induced DNA damage, using co-labeled anti-CPD and anti-TRP1 antibodies, showed a significant decrease in clearance of thymine dimers in melanocytes from EDN1$^{ep-/-}$ skin at the same timepoint (Figure 3.4A and B, middle panel). Analyses of melanocyte apoptosis by a modified TUNEL-IHC assay revealed a modest increase in apoptotic melanocytes at 24hrs post-UV irradiation in the EDN1$^{ep-/-}$ skin compared to CT (Figure 3.4A and B, right panel). We did not observe any

difference in the rate of proliferation, DNA damage or apoptosis in epidermal keratinocytes of the CT and mutant skin post-UVR (Figure S3.6C). Similarly, the dermal proliferation was comparable between CT and MT skin post-UVR at all timepoints. However, we observed a decrease in DNA damage and apoptosis in the dermis after 72 and 24hrs post-UVR, respectively (Figure S3.6D). Altogether, our *in vivo* results suggest that keratinocytic EDN1 alone is an important determinant of melanocyte homeostasis and plays a significant role in the proliferation and photoprotection of melanocytes after UV irradiation in neonatal mice.

In order to determine if the non-cell autonomous role of keratinocytic EDN1 is mediated through EDNRB receptor on melanocytes, selective EDNRB antagonist BQ788 was applied to the dorsal skin of wildtype C57BL/6 neonatal mice concurrent with UVR exposure (Ishikawa et al., 1994; Lahav et al., 1999). A significant decrease in the number of epidermal melanocytes was observed by FM staining, and by IHC for TRP1+ cells, 72hrs post-UVR in the BQ788 treated group compared to the vehicle treatment (Figure 3.3E). We did not notice effects on the dermal melanocyte population, possibly due to an inadequate penetration of BQ788 through the skin barrier (Figure S3.4C). No significant difference in the percentage of $TRP1^{+}/PCNA^{+}$ proliferating melanocytes was observed by IHC analyses between the two groups (BQ788 vs vehicle) of mice (Figure S3.4C). Altogether, results demonstrated that *in*

vivo blocking of EDNRB activity recapitulates the impaired paracrine effects on melanocytes observed in EDN1$^{ep-/-}$ mice. Present results further support the *in vivo* contribution of EDN1/EDNRB signaling pathway to UVR induced melanocyte activation, migration and proliferation, and demonstrates the feasibility of topical treatments for manipulating melanocyte activation in skin.

Phenotypic similarities of the melanocytic response post-UV exposure between EDN1$^{ep-/-}$ and p53$^{-/-}$ mice

The ability of p53 to bind and regulate melanogenic factors in mice has been previously reported. In order to corroborate our findings for transcriptional regulation of murine *Edn1* by p53, we utilized p53-null (p53$^{-/-}$) mice to evaluate the melanocytic response after UV-exposure. FM staining and IHC utilizing antibodies against TRP1 showed a dramatic decrease in the number of melanocytes that populate the murine epidermis 72hrs after UV exposure (Figure 3.5A). That was further verified by quantifying both the FM-positive and TRP1-positive cells to ensure that only those from the melanocytic lineage are counted (Figure 3.5B). Furthermore, IHC analyses for UV-induced DNA damage and melanocyte proliferation using anti-CPD and anti-PCNA or anti-Ki67 antibodies confirmed a significant increase in DNA damage-retaining melanocytes and a decrease in proliferating melanocytes in the p53-null skin (Figure 3.5C and S3.5A and B). Similar results were obtained after UVR in the

EDN1$^{ep-/-}$ mice skin at that time point. Altogether, our results support an important role of p53, upregulated following UV exposure, to bind multiple factors and assist in the regulation of paracrine melanogenic networks.

EDN1 mediates activation of MAPK and PKC signaling through its receptor EDNRB in murine melanocytes

Previous studies in human melanocytes have highlighted the contributions of both MAPK and PKC activation by EDN1 signaling (Imokawa et al., 1996; Sato-Jin et al., 2008; Imokawa et al., 2000). To verify expression of EDNRB receptor in keratinocytes and mature murine melanocytes, we performed immunoblots for EDNRB on lysates prepared from primary keratinocytes and melanocytes (Figure 3.6A). We hypothesized that lack of activation of MAPK and PKC pathways downstream of EDNRB in melanocytes contribute to their impaired activation and proliferation in EDN1$^{ep-/-}$ mice. To test that, we investigated the effects of exogenous EDN1 on activation of those pathways in murine melanocytes. Briefly, PKC and MAPK activation was analyzed in primary murine melanocytes cultured in complete or minimal medium supplemented with EDN1 and in presence of EDNRB antagonist BQ788. Western blot analyses on cell extracts from melanocytes conditioned with complete or minimal medium supplemented with EDN1 indicated strong phosphorylation of p42/p44 (pERK) proteins compared to those exposed to

minimal media (Figure 3.6B). Parallel treatment with BQ788 completely abrogated ERK1/2 phosphorylation, indicating EDNRB receptor mediated activation of MAPK in presence of EDN1 (Figure 3.6B). Furthermore, PKC activity was measured to confirm activation of this pathway downstream of EDNRB. Treatment of minimal medium with EDN1 induced PKC activation in a time-dependent manner, and the kinase activity was completely abolished upon treatment with BQ788, confirming the role of EDNRB receptor for this signaling (Figure 3.6C). Altogether, above results confirm EDNRB receptor mediated activation of downstream MAPK and PKC pathways in murine melanocytes in presence of exogenous EDN1, and establishes a parallel between mouse and human melanocytes.

It is possible that the loss of keratinocytic EDN1 in our EDN1$^{ep-/-}$ model leads to decreased follicular melanocytes migrating along the upper root sheath, resulting in a lower population of epidermal melanocytes post-UV exposure. We therefore investigated the effects of exogenous EDN1 on the migration of wild type melanocytes using an *in vitro* real-time transwell migration assay. Results indicated that EDN1 is sufficient to increase the rate of transwell migration of cultured melanocytes compared to minimal media alone, supporting the role of keratinocytic EDN1 as a melanocyte chemoattractant (Figure 3.6D).

In order to determine the *in vivo* physiological effects of EDN1 signaling on melanocytes we further performed IHC for pERK on $EDN1^{L2/L2}$ and $EDN1^{ep-/-}$ neonatal mouse skin after UV exposure. Strikingly, there was a strong induction of pERK 24hrs after UV exposure in the control and mutant epidermis (Figure 3.6E). That upregulation decreases at 48hrs and reverted to basal levels by 72hrs post-UVR (Figure 3.6E and F). Importantly, a similar induction of pERK was seen in follicular melanocytes as well, although that induction was modestly reduced in the skin of $EDN1^{ep-/-}$ mice 24hrs post-UVR (Figure 3.6G). These results verify the *in vivo* activation of MAPK pathway in murine melanocytes post-UV exposure.

3.4 Discussion

EDN1 contributes to human melanogenesis and is upregulated in keratinocytes exposed to UVR. In the present study we discovered *Edn1* is a transcriptional target of p53 in epidermal keratinocytes and established an essential role of EDN1 in regulating melanocyte homeostasis and UVR induced photoprotection. Our previous work demonstrated a positive regulation of *Edn1* by RXRα in murine keratinocytes (Hyter et al., 2010). That led us to investigate the mechanisms of regulation of this paracrine factor after exposure to the solar carcinogen UVR. Our RT-qPCR data on p53 null epidermis, together with the ChIP assay for p53 recruitment on the distal region of *Edn1* gene promoter in murine keratinocytes post UVR, have established p53 as a positive transcriptional regulator of *Edn1* gene in UVR exposed mouse skin. Recently, multiple studies have investigated the role of p53 transcriptional regulation on pigmentation and *in silico* analyses have confirmed presence of p53 binding motifs on promoters of melanogenic factors *KITLG* and *FGF2* (Wei et al., 2006). Concerning p53 regulated UVR induction of *Pomc* (precursor to α–MSH) (Cui et al., 2007), we were able to show a recruitment of p53 on the *Pomc* promoter in murine keratinocytes (Figure S3.1A). However, under our experimental conditions *Pomc* transcript levels were unaltered in the epidermis of $p53^{-/-}$ mice before and after UV irradiation

(Figure S3.1B). This is consistent with unaltered melanin pigmentation observed after *Pomc* deletion on the same genetic background (Slominski et al., 2005a), suggesting EDN1 signaling could provide alternate mechanisms of eumelanogenesis within the skin. Of note, melanocytes are known to upregulate expression of *MC1R* in response to EDN1 (Tada et al., 1998). Elevated expression of *p53* together with *KITLG, EDN1* and *POMC* has been previously reported in the epidermis of hyperpigmented human skin, further suggesting a possible role of p53 in activating pigmentation networks (Murase et al., 2009). Also, ChIP combined with a yeast-based assay led to the identification of p53 binding sites on the promoter of *EDN2*, an EDN1 family member, in human mammary epithelial cells (Hearnes et al., 2005). The phenotypic similarities of the melanocytic response observed between $EDN1^{ep-/-}$ and $p53^{-/-}$ mice post-UV exposure underscores the role of keratinocytic p53 to regulate expression of multiple paracrine factors (e.g. EDN1) and mediate melanocyte homeostasis.

The loss of dermal melanocyte populations observed in unirradiated $EDN1^{ep-/-}$ adult mice further emphasizes the existence of paracrine relationship between keratinocytes and melanocytes and underscores the existence of distinct signaling pathways in modulating epidermal vs. dermal melanocytes (Aoki et al., 2009). As this discrepancy in dermal melanocytes is only present in adult and not neonatal mice, it suggests a temporal aspect of EDN1-EDNRB

signaling during post-developmental homeostasis and emphasizes EDN1 function in maintenance of adult melanocyte homeostasis. Although epidermal melanocytes are absent in adult mouse skin compared to humans, resident dermal melanocytes are present (Fitzpatrick and Shiseidō, 1981). It is believed that epidermal melanocytes and dermal melanocytes represent distinct populations of cells exhibiting different sensitivities to signaling factors (Aoki et al., 2009; Slominski et al., 2005b). Reliance of dermal melanocytes on EDN1/EDNRB signaling could partly account for the decrease in the number of viable dermal melanocytes in our $EDN1^{ep-/-}$ mice model and establishes a non-cell autonomous role of keratinocytic EDN1 in melanocyte homeostasis. However, no alterations of epidermal proliferation and/or differentiation were seen in the skin of $EDN1^{ep-/-}$ mice due to the loss of keratinocytic EDN1, suggesting that EDN1 is dispensable for controlling epidermal homeostasis. That was in agreement with an earlier report showing no increase in proliferation of cultured human keratinocytes in response to exogenous EDN1 (Yohn et al., 1994).

Our results demonstrate that the overall decrease in the melanocyte population observed 72 and 96hrs post UVR could be, at least in part, due to decreased melanocyte proliferation around that timepoint. The significant and paradoxical decrease of melanocyte proliferation 72hrs after UVR indicates a possible temporal role of EDN1-EDNRB signaling in regulating melanocyte

homeostasis at the point when melanocytes are activated and migrating out of the hair follicles to repopulate the interfollicular epidermis (Walker et al., 2009). By 96hrs post UVR the percentage of proliferating melanocytes is again similar between the two lines, though total numbers of melanocytes are decreased in EDN1$^{ep-/-}$ skin, suggesting a response to alternative keratinocyte-derived paracrine factors. In addition, the decrease in CPD clearance along with increased apoptosis and altered melanocyte migration could be other potential mechanisms contributing to the overall decrease in melanocyte population in the mutant skin post UVR. Previous studies utilizing cultured human melanocytes have demonstrated the contribution of EDN1 supplementation in enhancing melanocytic proliferation (Yada et al., 1991). Our present *in vivo* observation of decreased melanocyte proliferation in absence of keratinocytic EDN1, but in the presence of other diffusible paracrine factors (KITLG, HGF, FGF2 and α–MSH), further corroborates those *in vitro* studies. The synergistic induction of multiple UV-induced paracrine factors such as EDN1, FGF2 and POMC-derived peptides makes it difficult to elucidate individual contributions in an *in vivo* model (Swope et al., 1995; Tada et al., 1998). Detecting EDNRB expression on primary murine melanocytes, along with a reduced number of proliferating epidermal melanocytes noted in our EDN1$^{ep-/-}$ skin post-UVR, suggests a role for EDN1/EDNRB signaling in melanocyte activation. It has been previously shown that exogenous EDN1

can protect cultured human melanocytes from UVR-induced apoptosis, while at the same time enhancing repair of CPD formations in those cells (Kadekaro et al., 2005). The reduced clearance of UVR induced thymine dimers and the modest increase in melanocyte apoptosis observed post-UVR in our EDN1$^{ep-/-}$ mice further validates the previous *in vitro* findings. Our results suggest that cells of the melanocytic lineage have impaired CPD repair capacity in absence of EDN1 signaling compared to other cell types of the skin, thereby predisposing them towards neoplastic transformation. Similar phenotypes in p53$^{-/-}$ mice further support the importance of p53 signaling to the UVR melanocytic response. Additional studies are required to determine which paracrine factors, besides *Edn1* and *Pomc*, are upregulated by p53 in keratinocytes after UV exposure.

Our present results demonstrate that selective inhibition of EDN1 receptor (EDNRB) in cultured murine melanocytes abrogated downstream signaling by inhibiting PKC activation and MAPK pathway, corroborating previous results in human melanocytes (Imokawa et al., 1996; Imokawa et al., 2000; Sato-Jin et al., 2008). The activation of MAPK and PKC by EDN1 in cultured human melanocytes is synergistically enhanced with the addition of KITLG (Imokawa et al., 2000), supporting the role of multiple keratinocyte-derived signaling factors working in tandem to initiate melanocytic activity. Our *in vivo* IHC data for ERK activation suggest that loss of keratinocytic EDN1 did not significantly

abolish pERK induction in melanocytes, possibly due to the presence of additional keratinocytic-derived paracrine mitogenic factors in the cellular millieu. Due to the increasing reliance on mouse melanoma models for translational therapeutic approaches in humans, it is important to verify that comparable transduction mechanisms exist downstream of the EDNRB receptor in murine melanocytes. Our data of reduced epidermal melanocyte population after topical treatment with EDNRB receptor antagonist supports our *in vivo* observation where expression of several UVR induced paracrine factors such as KITLG, α–MSH, HGF and FGF2 could not compensate for the loss of EDN1 in the murine epidermis. The modulation of MITF-M has been linked to EDN1 stimulation (Sato-Jin et al., 2008), thereby connecting growth factor receptor signaling to the master regulator of melanocytes. It has also been previously shown that contribution of EDN1 to migration of human melanocytes is superior to FGF2, KITLG and a α–MSH analog (Horikawa et al., 1995; Scott et al., 1997). Our studies of melanocyte migration in presence or absence of EDN1 suggests a possible *in vivo* role of keratinocytic EDN1 in stimulating follicular melanocyte migration to the murine epidermis post-UVR.

Our present findings shed light on the complex interplay between keratinocytes and melanocytes in skin in response to UV irradiation and provide evidence for the possibility of therapeutic manipulation of those pathways. We discovered that p53 regulated secretion of diffusible factors

such as EDN1 from epidermal keratinocytes can initiate signaling cascades within melanocytes and control melanocyte homeostasis in response to solar UV-irradiation. What is not currently understood is how these factors act in combination to establish the full spectrum of receptor-mediated cellular responses. Additional studies are necessary to further elucidate the temporal events that occur post-UV exposure within the skin. Understanding how paracrine factors act as photoprotectors against DNA damage and prevent malignant transformation of melanocytes in the cellular microenvironment is crucial for improved treatment of UV-induced skin cancers.

3.5 Methods

Mice

Generation of EDN1$^{L2/L2}$ mice has been previously described (Huang et al, 2002). To selectively ablate EDN1 in epidermal keratinocytes, mice carrying LoxP-site-containing (floxed) *Edn1* alleles were bred with hemizygous K14-Cre transgenic mice (Li et al., 2001) backcrossed to a C57BL/6 background in order to generate EDN1$^{ep-/-}$ mice in mendelian ratios. A semiquantitative PCR was performed in an Eppendorf thermal cycler using primers to amplify the *Cre*, L2 and L- *Edn1* alleles. Mice were housed in our approved University Animal Facility with 12h light cycles, food and water were provided ad libitum, and institutional approval was granted for all animal experiments.

UVR treatment

P2 EDN1$^{L2/L2}$ and EDN1$^{ep-/-}$ mice were exposed to a single dose of 600mJ/cm^2 of UVB light from a bank of four Philips FS-40 UV sunlamps. The irradiance of the sunlamps was measured with an IL-1400A radiometer with an SEE240 UVB detector (International Light). Mice were euthanized 24, 48, 72 and 96hrs after UVR and skin samples retrieved. 0hr samples were taken from P2 mice not exposed to UVR. Cohorts of 5 to 8 age-matched mice from multiple litters were utilized per time point. C57BL/6 p53$^{-/-}$ adult mice were exposed to 600mJ/cm^2 of UVB followed by biopsies taken at 8, 24 and 72hrs post-UVR,

while C57BL/6 $p53^{-/-}$ neonatal mice were treated with the same dosage at P3.

BQ788 topical application

BQ788 (A.G. Scientific) was dissolved in ethanol to a 200μM working solution and 20μl was applied to the dorsal skin of P1 C57BL/6 mice once a day for seven treatments. 600mJ/cm^2 UVB exposure was administered on day P3 and skin samples were collected 72 and 96hr post-UVR. Ethanol application alone was used for vehicle control samples.

Histological analyses

Skin biopsies were fixed and stained with hematoxylin and eosin (H&E) as previously described (Indra et al., 2007). Fontana-Masson staining was performed according to manufactures instructions (American MasterTech). All microscopic studies were performed using a Leica DME light microscope and analyzed using the Leica Application Suite software, version 3.3.1.

Immunohistochemistry

Immunofluorescence studies were performed as previously described (Hyter et al, 2010). The following antibodies were used for immunohistochemistry: anti-PEP1 (kindly provided by V. Hearing, NIH, 1:1000), anti-PCNA (Abcam, ab29, 1:6000), anti-CPD (Kamiya Biomedical Company, MC-062, 1:200), anti-pERK

(Abcam, ab50011, 1:200), anti-Ki67 (Novocastra, NCL-Ki67-MM1, 1:200) and anti-EDN1 (Peninsula Laboratories, T-4050, 1:1000). The secondary antibodies used were goat anti-rabbit CY2 (1:400) and goat anti-mouse CY3 (1:1000) (Jackson ImmunoResearch). For dual TUNEL-IHC staining, the DeadEnd™ TUNEL System (Promega) was combined with the above protocol. Sections stained without primary antibody was used as a negative control, and all experiments were performed in triplicates. All images were captured using a Zeiss AXIO Imager.Z1 with a digital AxioCam HRm and processed using AxioVision 4.7 and Photoshop. Data were analyzed using ImageJ software (NIH), multiple IHC fields on each slide from all groups were randomly chosen and 10-15 fields per slide were counted. The slides were analyzed independently in a double-blinded manner by two investigators and significance was determined using a Student's t-test.

Reverse transcription–quantitative PCR (RT-qPCR) analyses

Total RNA was extracted from whole dorsal skin or epidermal tail skin using Trizol (Invitrogen) and cDNA was created using SuperScript III RT (Invitrogen). Amplification was performed on an ABI Real Time PCR machine using a QuantiTect SYBR Green PCR kit (Invitrogen), and all targets were normalized to the internal control *Hprt*. All reactions were performed in triplicates using a minimum of three biological replicates from each group of mice. Melting curve

analyses were performed to ensure specificity of amplification. Statistical analysis was done with GraphPad Prism software.

Immunoblotting analyses

Primary C57BL/6 murine melanocytes were obtained from the Yale University Cell Culture Core. Cells were maintained in a complete melanocyte growth medium consisting of F-12 nutrient mixture (Ham), 8% FBS, bovine pituitary extract (25μg/mL), TPA (10ng/mL), 3-isobutyl-1-methylxanthine (22μg/mL) and 1X antibiotic/antimycotic. Melanocytes were starved into a quiescent state using a minimal culture medium containing F-12 nutrient mixture (Ham), 8% FBS and 1X antibiotic/antimycotic for 72hrs prior to experiment. Cells were pre-incubated with the EDNRB antagonist BQ788 (A.G. Scientific) or vehicle in minimal culture medium for 60 minutes prior to treatment, followed by addition of EDN1 (Sigma). All treatments were prepared in minimal culture medium and cells exposed to minimal medium alone or complete melanocyte growth medium were used as negative and positive controls, respectively. At the appropriate time points, protein lysates were obtained by collecting cells in a lysis buffer (20mM HEPES, 250mM NaCl, 2mM EDTA, 1% SDS, 10% glycerol, 50mM NaF, .1mM hemin chloride, 5mM NEM, 1mM PMSF and 10μg/mL leupeptin and aproptinin) followed by sonication. Protein concentration was performed using the BCA assay (Thermo Scientific). Equal amounts of protein

extract (15μg) from each lysate were resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The blots were blocked overnight with 5% nonfat dry milk and incubated with specific antibodies. The antibodies used were rabbit anti-endothelin receptor B (#AER-002, Alomone labs), total ERK (#4695, Cell Signaling) and phospho-ERK (#9101, Cell Signaling). After incubation with the appropriate secondary antibody, signals were detected using immunochemiluminescent reagents (GE Healthcare, Piscataway, NJ**)**. Equal protein loading in each lane was confirmed with a b-actin antibody (#A300-491, Bethyl).

PKC kinase activity assay

Murine melanocytes were grown and treated as described above. Cells were lysated at the designated timepoints using cold lysis buffer (20mM MOPS, 50mM β-glycerolphosphate, 50mM sodium fluoride, 1mM sodium vanadate, 2mM EGTA, 2mM EDTA, 1%NP40, 1mM DTT, 1mM benzamidine, 1mM PMSF and 10μg/mL leupeptin and aproptinin). Cold lysates were collected and sonicated to ensure disruption and cytosolic fraction was collected using centrifugation. Protein concentration was determined as described above. Relative kinase activity was measured using the PKC Kinase Activity assay kit (Enzo) according to the manufacturers instructions.

Real-time migration assay

Murine melanocytes were starved into a quiescent state using a minimal culture medium for 48hrs prior to experiment as described above. Cells were seeded into a CIM-plate 16 using an xCELLigence system (Roche) at a density of $2x10^4$ according to manufacturer's instructions. All assays were performed in triplicate and data analysis was performed using xCELLigence software.

Chromatin immunoprecipitation

Primary mouse keratinocytes from wild-type B6 pups were exposed without a lid to $10mJ/cm^2$ UV irradiation. Fresh media was applied and the cells were allowed to incubate for 2hrs. Chromatin precipitation was performed as previously described (Hyter et al, 2010) using either 2μg of a p53 antibody (Santa Cruz Biotechnology) or non-specific IgG (Santa Cruz Biotechnology). Experiments were performed a minimum of three times.

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Conflict of interest

The authors declare that no conflicting interests exist

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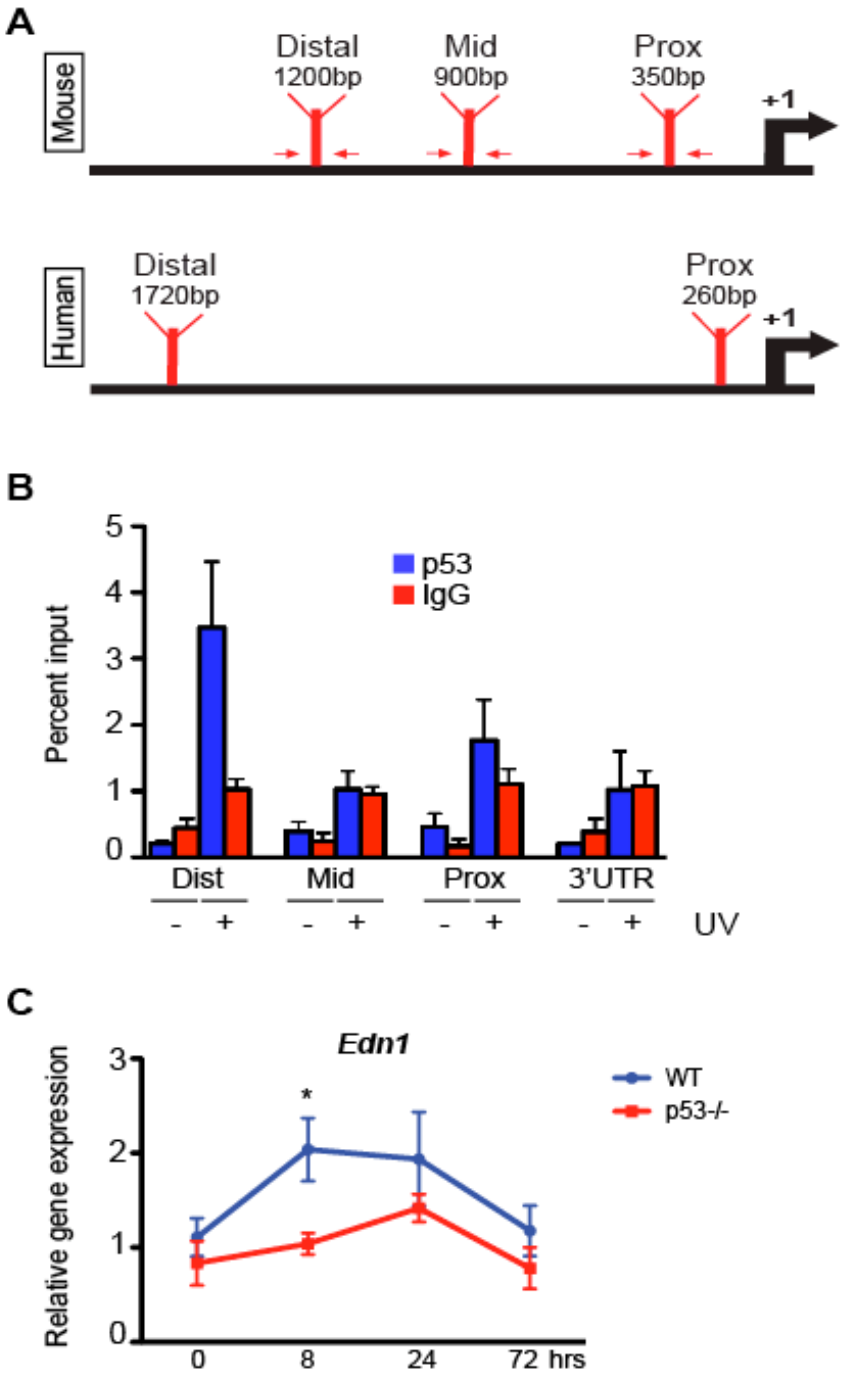
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Dist
Mid
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UV
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Edn1
Relative gene expression
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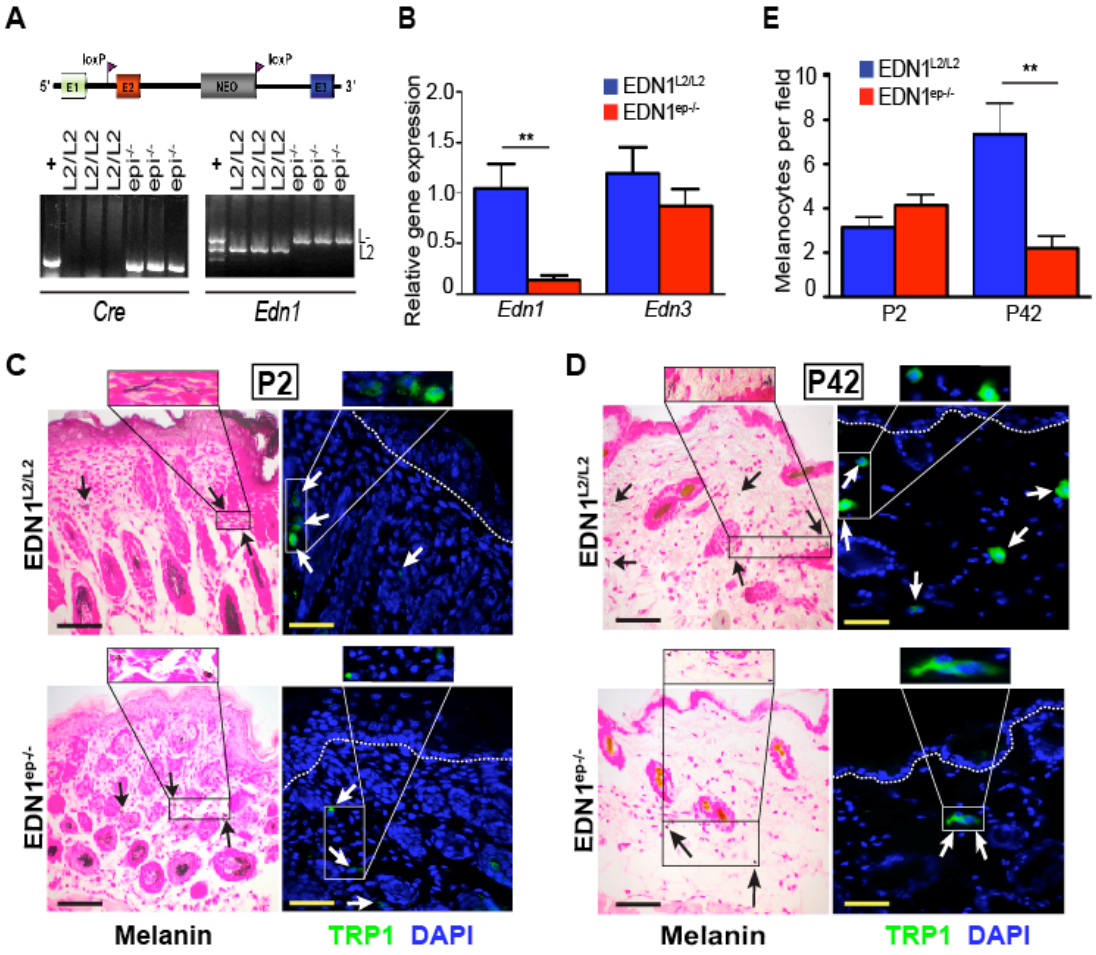
0
8
24
72 hrs

Figure 3.1

Figure 3.1. Positive regulation of *Edn1* expression after UV exposure by p53 in murine keratinocytes. (A) Schematic of predicted p53 binding locations on murine and human *Edn1/EDN1* promoters. Arrows indicate primers designed for chromatin immunoprecipitation (ChIP). (B) ChIP assay on primary murine keratinocytes using anti-p53 antibody following presence or absence of UV exposure. Results were analyzed by qPCR using primers specific to proximal, mid and distal regions (indicated in A). Primers directed against the 3' UTR region of EDN1 and non-specific IgG antibody were used as negative controls. (C) Relative gene expression of *Edn1* in epidermis from adult wildtype C57BL/6 and $p53^{-/-}$ mice at designated time points post-UV exposure. All experiments were done using a minimum of three biological replicates from each group and in all cases are expressed as mean +/- SEM. Statistical analysis was performed using Graphpad Prism, * = $p<0.05$.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loxP
loxP
5'
E1
E2
NEO
E1
3'
+
L2/L2
L2/L2
L2/L2
epi-/-
epi-/-
epi-/-
+
L2/L2
L2/L2
L2/L2
epi-/-
epi-/-
epi-/-
L-L2
Cre
Edn1
B
Relative gene expression
2.0
1.5
1.0
0.5
0
**
EDN1L2/L2
EDN1ep-/-
Edn1
Edn3
E
Melanocytes per field
10
8
6
4
2
0
EDN1L2/L2
EDN1ep-/-
**
P2
P42
C
P2
EDN1L2/L2
EDN1ep-/-
Melanin
TRP1 DAPI
D
P42
EDN1L2/L2
EDN1ep-/-
Melanin
TRP1 DAPI

Figure 3.2

Figure 3.2. Characterization of EDN1$^{ep-/-}$ mice showed reduced epidermal and dermal melanocytes in adult skin. (A) Schematic diagram of Cre-targeted loxP sites flanking exon 2 of *Edn1*. Genotyping results using primers directed against both *Cre* and *Edn1* from epidermal genomic DNA. (B) qPCR analysis of mRNA expression levels for *Edn1* and *Edn3* in untreated P2 mouse whole skin are shown. (C,D) Fontana-Masson (black) and TRP1 (green) staining of untreated P2 and P42 mouse dorsal skin sections, arrows indicate dermal melanocytes; scale bar=20μm. IHC sections counterstained with DAPI (blue) and white dashed lines indicate epidermal-dermal junction. (E) Bar graph comparing melanocytes per field between untreated P2 and P42 EDN1$^{L2/L2}$ and EDN1$^{ep-/-}$ skin. All experiments were done using a minimum of three biological replicates from each group and in all cases are expressed as mean +/- SEM. Statistical analysis was performed by Student's t-test using Graphpad Prism, ** = $p<0.01$.

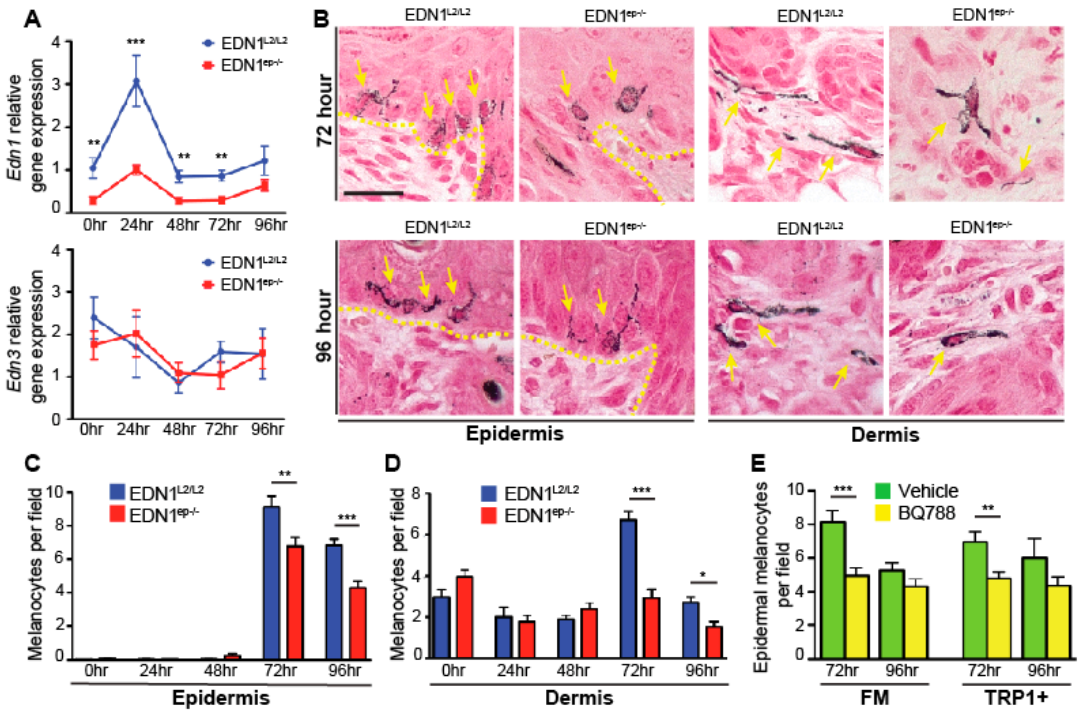

A
Edn1 relative gene expression
Edn3 relative gene expression
EDN1L2/L2
EDN1ep-/-
0hr 24hr 48hr 72hr 96hr
B
72 hour
96 hour
Epidermis
Dermis
C
Melanocytes per field
Epidermis
D
Melanocytes per field
Dermis
E
Epidermal melanocytes per field
Vehicle
BQ788
FM
TRP1+

Figure 3.3

Figure 3.3. Decreased epidermal and dermal melanocytes in EDN1$^{ep-/-}$ and BQ788-treated wildtype neonatal skin post-UVR. (A) Relative gene expression of *Edn1* and *Edn3* over time following UVR of EDN1$^{L2/L2}$ and EDN1$^{ep-/-}$ P2 skin. (B) Fontana Masson (FM) stained images at 72 and 96hr post-UVR of P2 skin, arrows indicate melanocytes. Yellow dashed line represents epidermal-dermal junction, scale bar=20μm. (C) Epidermal melanocyte counts per field in EDN1$^{L2/L2}$ and EDN1$^{ep-/-}$ skin over time post-UVR of P2 skin. (D) Dermal melanocyte counts per field in EDN1$^{L2/L2}$ and EDN1$^{ep-/-}$ skin over time post-UVR of P2 skin. (E) Epidermal melanocyte counts per field in wildtype B6 mice treated with topical BQ788 or vehicle at 72 and 96hrs post-UVR of P3 skin for both FM and IHC (TRP1+). All experiments were done using a minimum of three biological replicates from each group of mice and in all cases are expressed as mean +/- SEM. Statistical analysis was performed using Graphpad Prism, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

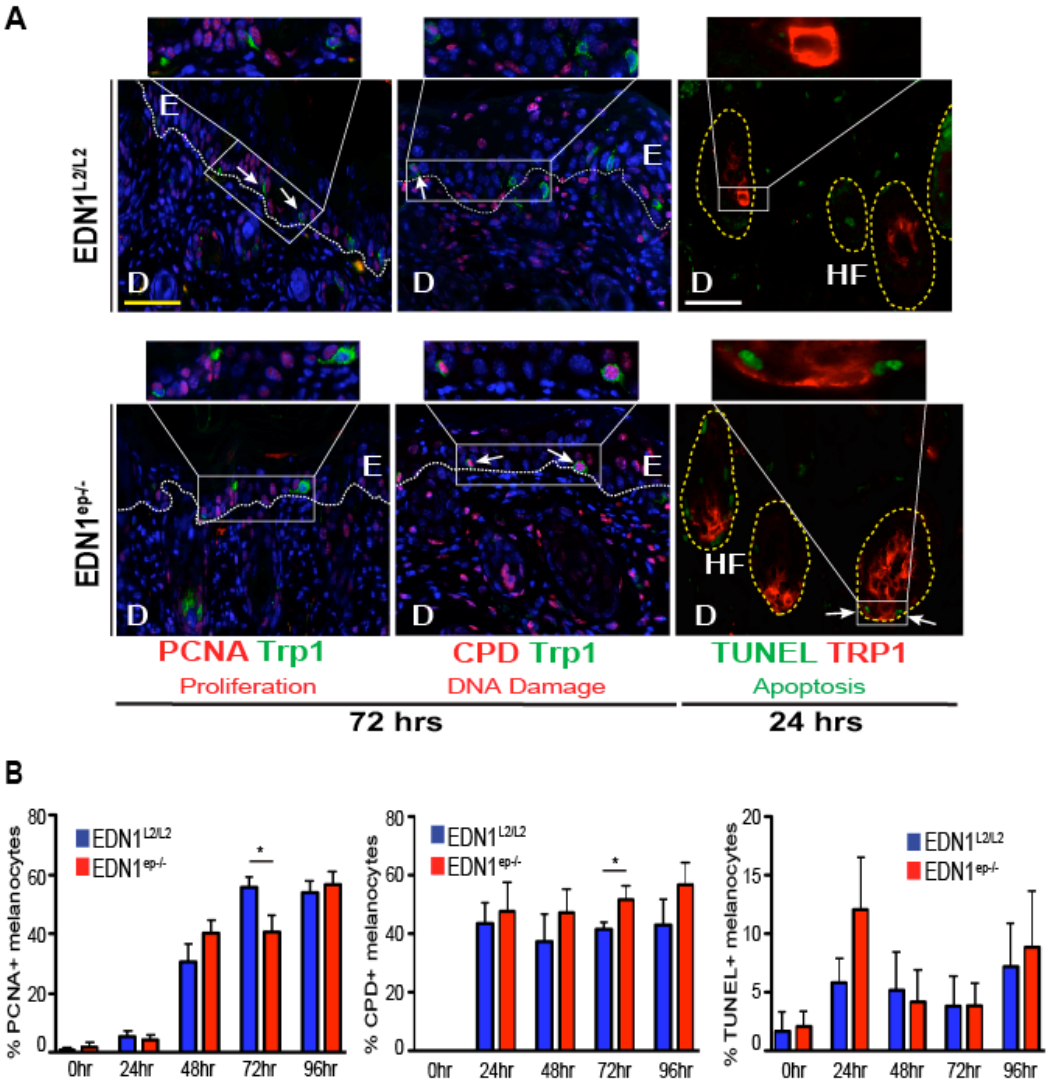

A
EDN1L2/L2
EDN1ep-/-
E
D
HF
PCNA Trp1
Proliferation
CPD Trp1
DNA Damage
TUNEL TRP1
Apoptosis
72 hrs
24 hrs
B
% PCNA+ melanocytes
% CPD+ melanocytes
% TUNEL+ melanocytes
EDN1L2/L2
EDN1ep-/-
0hr
24hr
48hr
72hr
96hr

Figure 3.4

Figure 3.4. Immunohistochemical characterization for proliferation, DNA damage and apoptosis of melanocytes post-UVR. (A) IHC analysis of $EDN1^{L2/L2}$ and $EDN1^{ep-/-}$ skin at 72 or 24hrs post-UVR of P2 mice stained with anti-PCNA (red), anti-CPD (red), anti-TRP1 (green and red) primary antibodies and TUNEL assay (green). Yellow scale bar=62μm, white scale bar=20μm. E=epidermis, D=dermis, HF=hair follicle. PCNA and CPD sections counterstained with DAPI (blue) White dashed line indicates dermal-epidermal junction. (B) Percentage of PCNA, CPD or TUNEL-positive melanocytes out of total DAPI-stained cells between $EDN1^{L2/L2}$ and $EDN1^{ep-/-}$ skin post-UVR of P2 mice. All experiments were done using a minimum of three biological replicates from each group of mice and in all cases are expressed as mean +/- SEM. Statistical analysis was performed using Graphpad Prism, * = $p<0.05$.

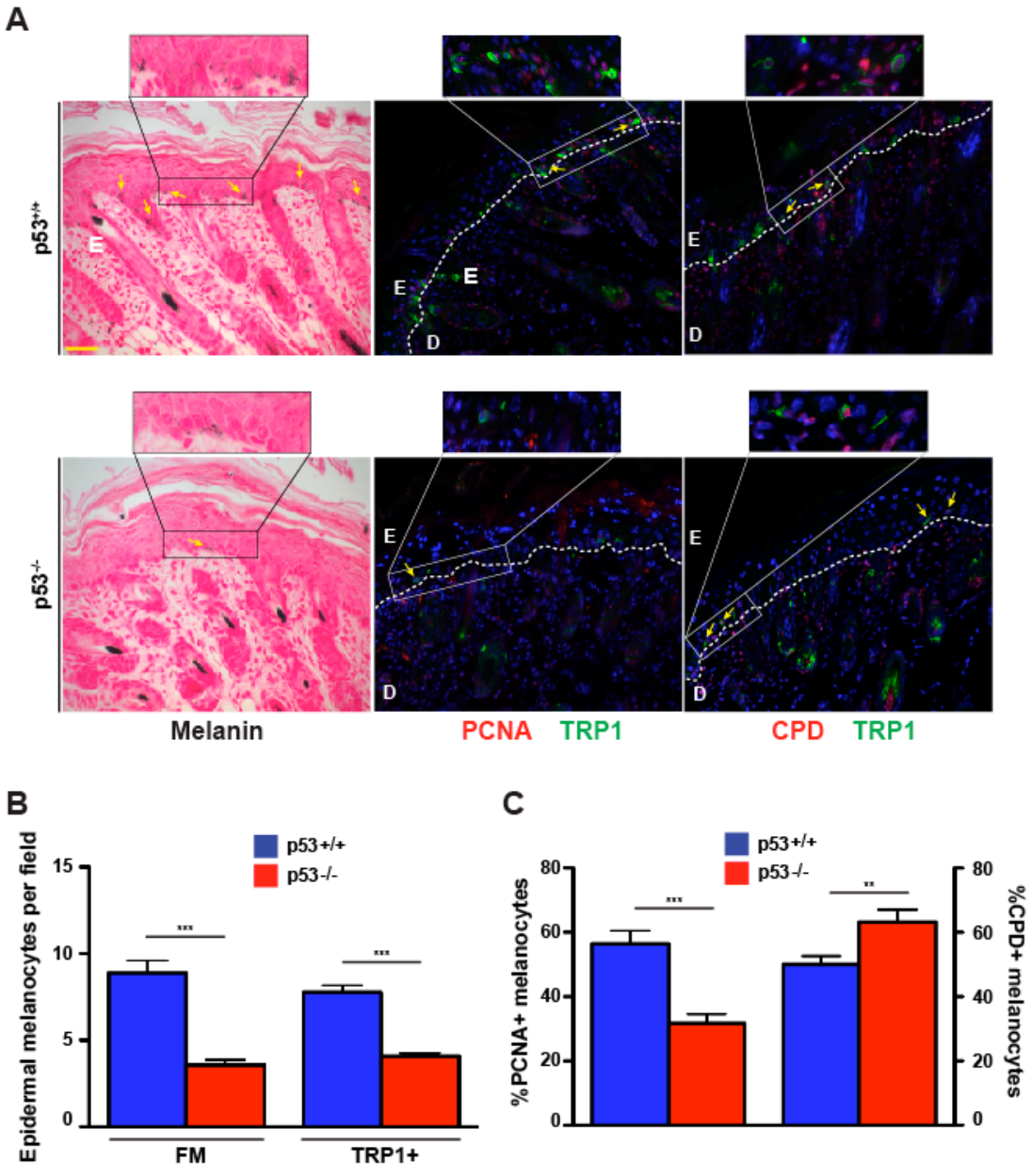

A
p53+/+
p53-/-
E
D
Melanin
PCNA TRP1
CPD TRP1
B
Epidermal melanocytes per field
p53+/+
p53-/-
FM
TRP1+
C
%PCNA+ melanocytes
%CPD+ melanocytes

Figure 3.5

Figure 3.5. Decreased epidermal and dermal melanocytes and altered melanocyte proliferation and DNA damage in $p53^{-/-}$ neonatal skin post-UVR. (A) Fontana Masson (FM) and IHC stained images of $p53^{+/+}$ and $p53^{-/-}$ mouse skin at 72hr post-UV treatment of P3 mice, arrows indicate melanocytes. Anti-PCNA (red), anti-CPD (red), anti-TRP1 (green) primary antibodies were used, PCNA and CPD sections counterstained with DAPI (blue). White dashed line represents epidermal-dermal junction, E=epidermis, D=dermis, scale bar=62μm. (B) Epidermal melanocyte counts per field using both FM+ and TRP1+ cells 72 hours post-UVR of P3 skin in $p53^{+/+}$ and $p53^{-/-}$ mice. (C) Percentage of PCNA or CPD positive melanocytes out of total DAPI-stained cells between $p53^{+/+}$ and $p53^{-/-}$ neonatal mice 72 hours post-UVR of P3 mice. All experiments were done using a minimum of three biological replicates from each group of mice and in all cases are expressed as mean +/- SEM. Statistical analysis was performed using Graphpad Prism, ** = $p<0.01$, *** = $p<0.001$.

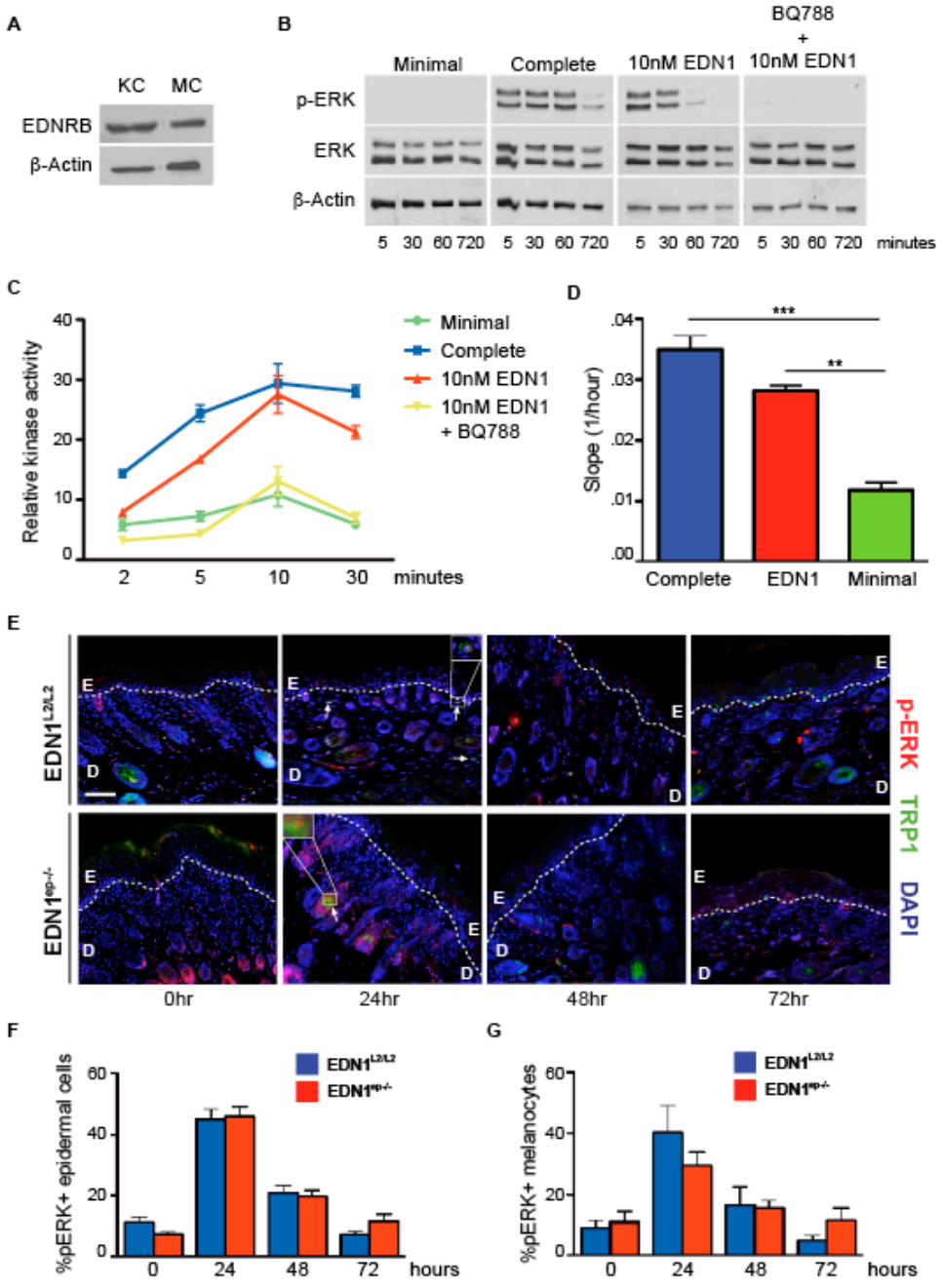
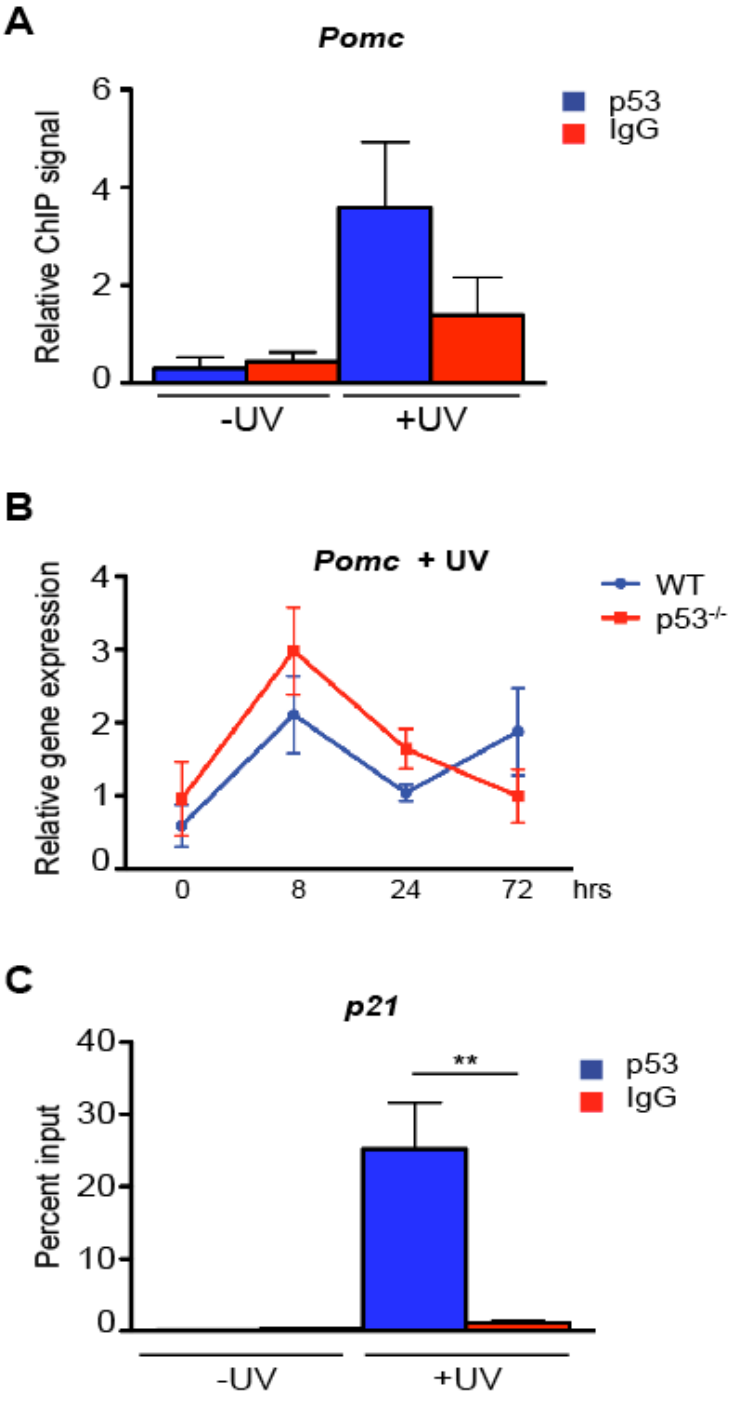

A
KC
MC
EDNRB
β-Actin
B
Minimal
Complete
10nM EDN1
BQ788 + 10nM EDN1
p-ERK
ERK
β-Actin
5 30 60 720 5 30 60 720 5 30 60 720 5 30 60 720 minutes
C
Relative kinase activity
Minimal
Complete
10nM EDN1
10nM EDN1 + BQ788
2 5 10 30 minutes
D
Slope (1/hour)
***
**
Complete
EDN1
Minimal
E
EDN1L2/L2
EDN1ep-/-
p-ERK
TRP1
DAPI
0hr
24hr
48hr
72hr
F
%pERK+ epidermal cells
EDN1L2/L2
EDN1ep-/-
0 24 48 72 hours
G
%pERK+ melanocytes
EDN1L2/L2
EDN1ep-/-
0 24 48 72 hours

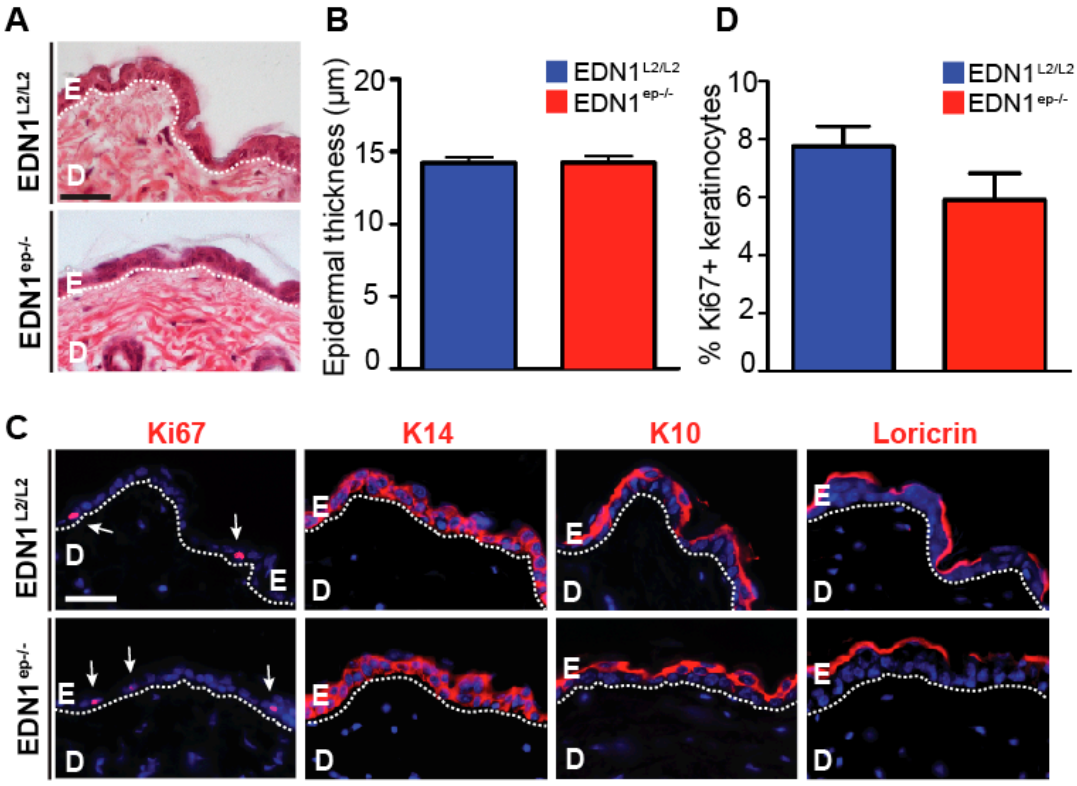
Figure 3.6

Figure 3.6. Activation of MAPK and PKC signaling by EDN1 is specific to EDNRB receptor. (A) Immunoblot analysis of EDNRB expression in lysates from primary murine keratinocytes [KC] and melanocytes [MC], β-actin levels were used as controls. (B) Immunoblot analysis for ERK phosphorylation after addition of exogenous EDN1 with or without the EDNRB antagonist BQ788. Total ERK and β-actin levels were used as controls, as well as minimal and complete mediums. (C) Activation of PKC after addition of exogenous EDN1 with or without the presence of EDNRB antagonist BQ788. All experiments were performed in triplicate and PKC activation results are expressed as mean +/- SEM. (D) Real-time transwell migration assay comparing slopes for exponential migration phase of wildtype melanocytes. (E) IHC analysis of ERK activation in $EDN1^{L2/L2}$ and $EDN1^{ep-/-}$ skin at 0, 24, 48 and 72hrs post-UVR treatment of P2 mice with anti-pERK (red) and anti-TRP1 (green) primary antibodies. All sections are counterstained with DAPI (blue), white dashed line represents epidermal-dermal junction. E=epidermis, D=dermis, white scale bar=62 μm, arrows and white boxes indicate pERK activated melanocytes. (F) Percentage of pERK+ cells out of total DAPI-stained epidermal cells between $EDN1^{L2/L2}$ and $EDN1^{ep-/-}$ mice post-UVR treatment of P2 mice. (G) Percentage of pERK+ melanocytes out of total TRP1+ stained cells in $EDN1^{L2/L2}$ and $EDN1^{ep-/-}$ after UVR treatment of P2 mice. All experiments were done using a minimum of three biological replicates from each group of mice and in all cases are expressed as mean +/- SEM. Statistical analysis was performed using Graphpad Prism, ** = $p<0.01$, *** = $p<0.001$.



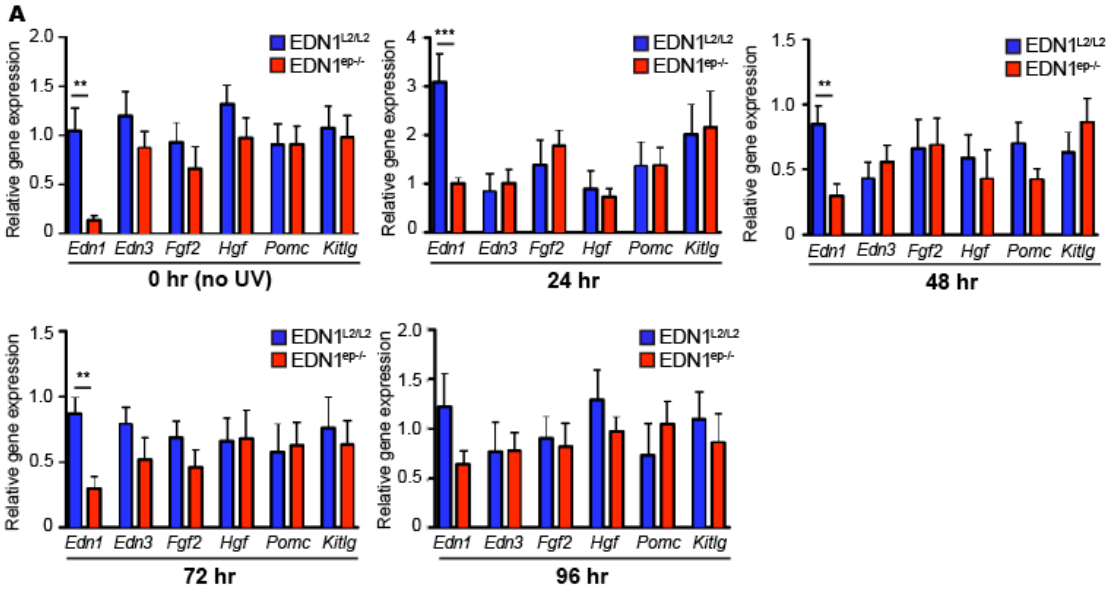
Supplemental Figure 3.1

Supplemental Figure 3.1. Regulation of *Pomc* expression after UV exposure by p53 in murine epidermis. (A) ChIP assay on primary murine keratinocytes using anti-p53 antibody with or without UV exposure. Results were analyzed by qPCR using primers specific to predicted p53 binding location on the *Pomc* promoter. Non-specific IgG antibody was used as a negative control. (B) Relative gene expression of *Pomc* in epidermis from adult wildtype B6 and $p53^{-/-}$ mice at designated time points post-UV exposure. (C) ChIP assay for p53 occupancy on the *p21* promoter of primary murine keratinocytes with or without UV exposure. Results were analyzed by qPCR using primers specific to p53 consensus sequence on *p21* promoter. Non-specific IgG antibody was used as a negative control. All experiments were done using a minimum of three biological replicates from each group and in all cases are expressed as mean +/- SEM. Statistical analysis was performed using Graphpad Prism, ** = $p<0.01$.



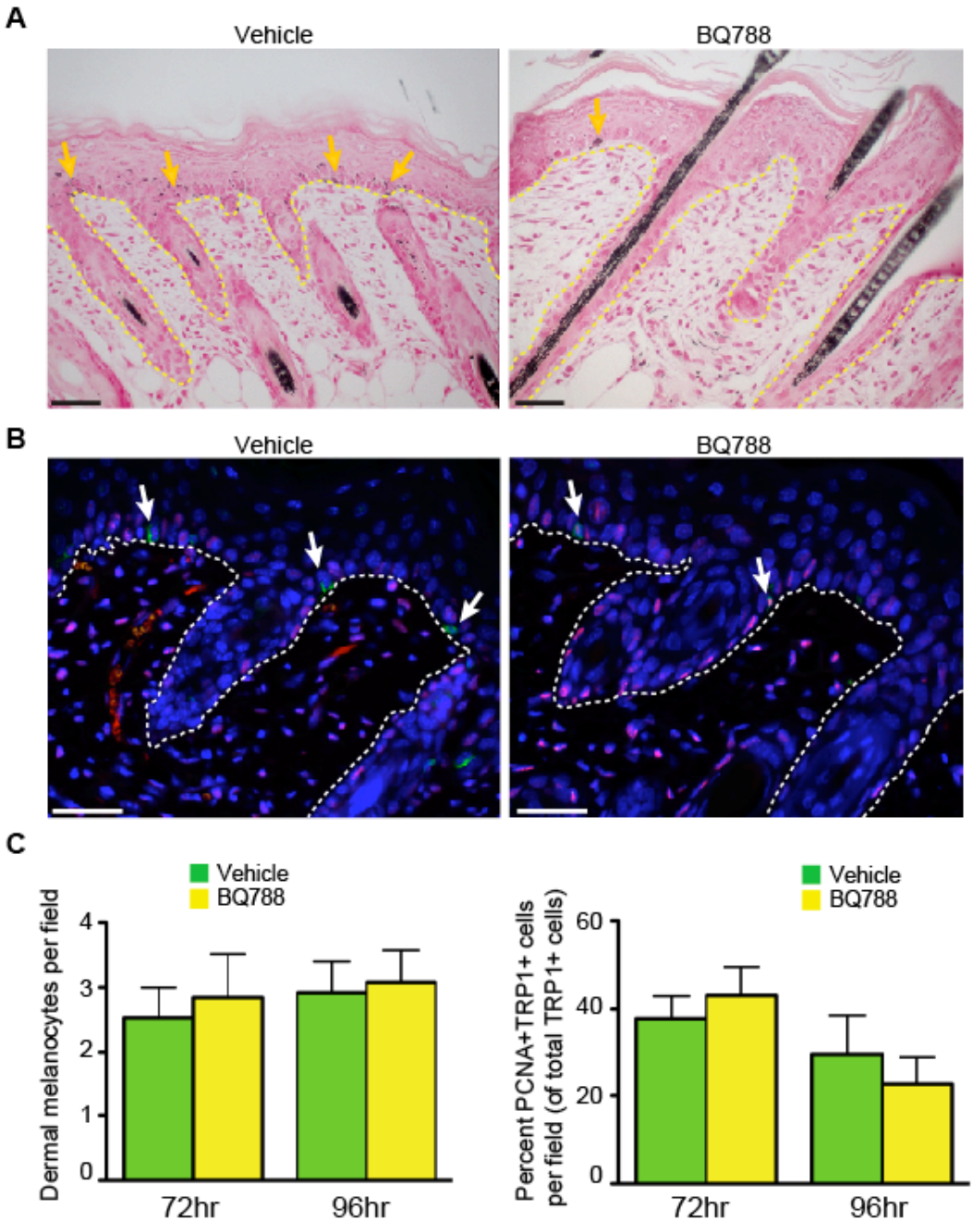
Supplemental Figure 3.2

Supplemental Figure 3.2. Keratinocytic EDN1 ablation does not influence epidermal homeostasis. (A) Hematoxylin & eosin staining of adult $EDN1^{L2/L2}$ and $EDN1^{ep-/-}$ skin. Scale bar=62μm, E=epidermis, D=dermis, white dashed line indicates epidermal-dermal junction. (B) Bar graph showing the epidermal thickness of $EDN1^{L2/L2}$ and $EDN1^{ep-/-}$ adult mice. (C) Representative IHC analysis of adult skin stained with anti-Ki67 (red), anti-K14 (red), anti-K10 (red) and anti-loricrin (red) primary antibodies, blue represents DAPI nuclear staining, arrows indicated Ki67-positive keratinocytes. Scale bar = 62μm, E=epidermis, D=dermis, white dashed line indicates epidermal-dermal junction. (D) Percentage of Ki67-positive stained keratinocytes per microscopic field between $EDN1^{L2/L2}$ and $EDN1^{ep-/-}$ adult mice.



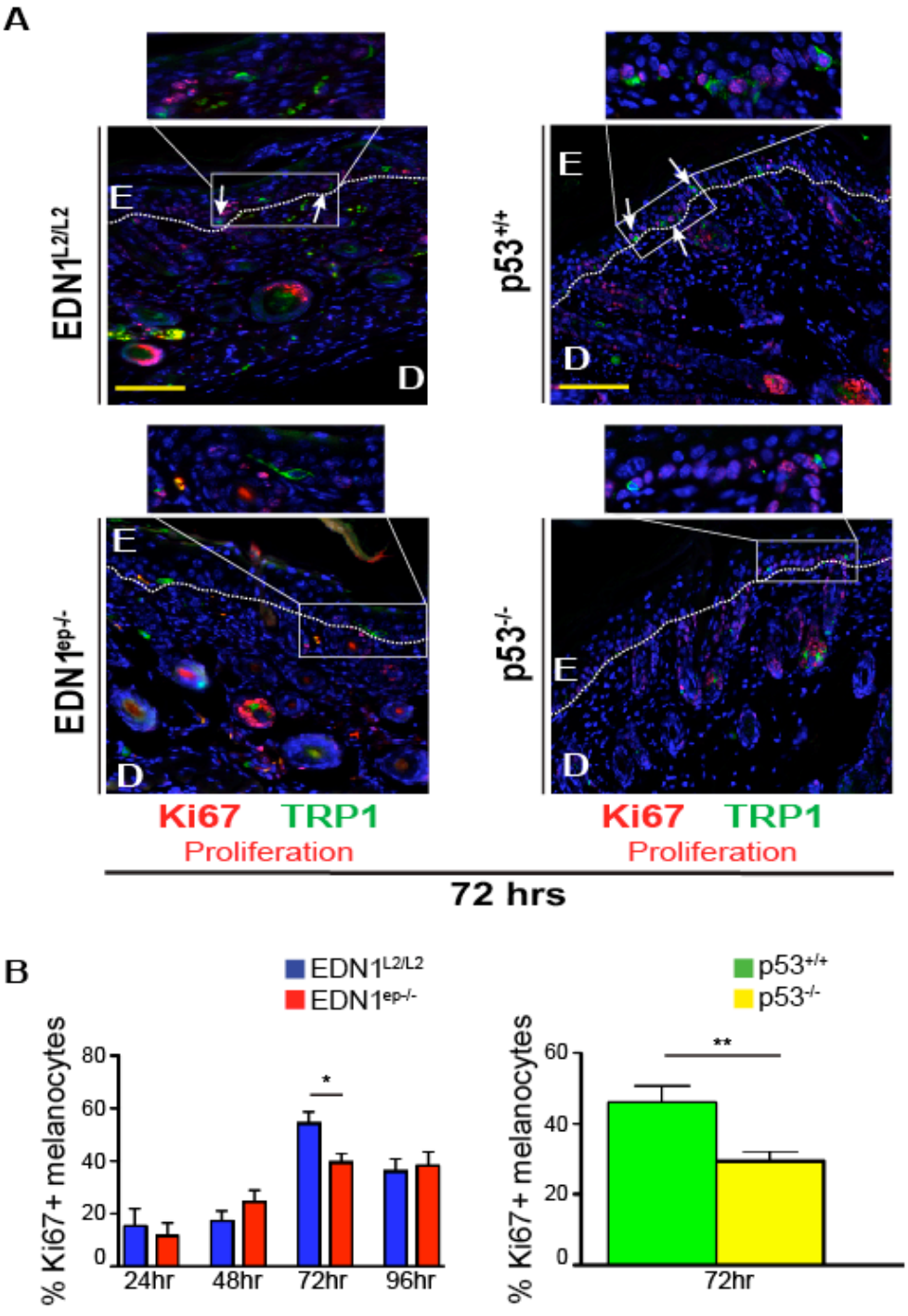
Supplemental Figure 3.3

Supplemental Figure 3.3. Relative *Edn1* mRNA expression was significantly lower in skin of EDN1$^{ep-/-}$ neonatal mice post-UVR compared to EDN1$^{L2/L2}$. (A) qPCR analysis for relative mRNA expression at various time points post-UVB of P2 mice. *Edn3*=endothelin 3, *Fgf2*=fibroblast growth factor 2, *Hgf*=hepatocyte growth factor, *Pomc*=pro-opiomelanotropin, *Kitlg*=Kit ligand. All experiments were done using a minimum of three biological replicates from each group of mice and in all cases are expressed as mean +/- SEM. Statistical analysis was performed using Graphpad Prism, ** = $p<0.01$, *** = $p<0.001$.



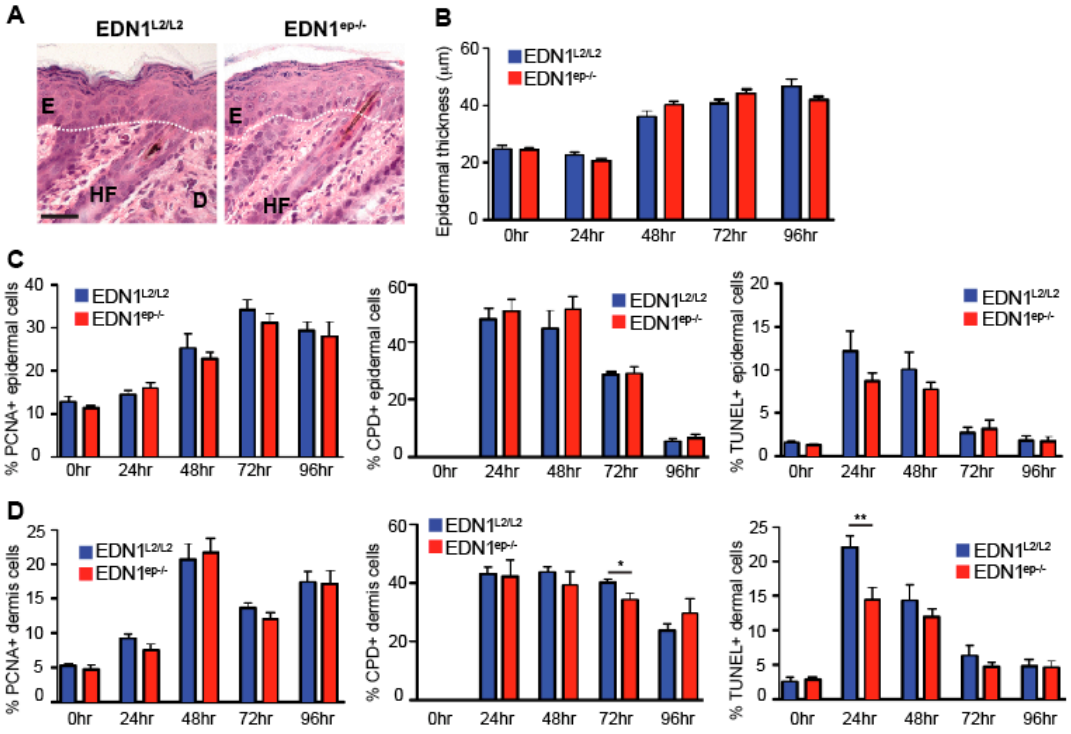
Supplemental Figure 3.4

Supplemental Figure 3.4. Topical application of BQ788 on wildtype neonatal skin does not affect dermal melanocyte populations or overall melanocyte proliferation. (A) Representative FM staining of 72hr post-UVR skin sections from P3 B6 mice exposed to topical application of either vehicle or BQ788 treatments. Arrows indicate melanocytes, yellow dashed line represents epidermal-dermal junction, scale bar=62μm. (B) Representative IHC staining of 72hr post-UVR skin sections from P3 C57BL/6 mice exposed to topical application of either vehicle or BQ788 treatments. Antibodies directed against TRP1 (green) and PCNA (red) were used. Arrows indicate melanocytes and white dashed line represents epidermal-dermal junction, scale bar=62μm. (C) Graphical representation of dermal FM and epidermal IHC melanocyte counts. IHC counts were performed using both PCNA and TRP1 primary antibodies. Total number of TRP1+ cells were counted from the vehicle and BQ788 treated groups from the 72 and 96hr post-UV timepoints. The percentage that were co-labeled PCNA+TRP1+ were graphed, all experiments were done using a minimum of three biological replicates from each group of mice. Data represents three individual experiments and in all cases are expressed as mean +/- SEM. Statistical analysis was performed using Graphpad Prism.



Supplemental Figure 3.5

Supplemental Figure 3.5. Immunohistochemical validation of melanocytic proliferation post-UVR. (A) IHC analysis of $EDN1^{L2/L2}$ and $EDN1^{ep-/-}$ or $p53^{+/+}$ and $p53^{-/-}$ skin at 72hrs post-UVR of P3 mice stained with anti-Ki67 (red) and anti-TRP1 (green) primary antibodies. Yellow scale bar=62μm, E=epidermis, D=dermis, all sections counterstained with DAPI (blue). White dashed line indicates epidermal-dermal junction. (B) Percentage of Ki67-positive melanocytes out of total DAPI-stained cells between $EDN1^{L2/L2}$ and $EDN1^{ep-/-}$ or $p53^{+/+}$ and $p53^{-/-}$ mice post-UVR. All experiments were done using a minimum of three biological replicates from each group of mice and in all cases are expressed as mean +/- SEM. Statistical analysis was performed using Graphpad Prism, * = $p<0.05$, ** = $p<0.01$.



Supplemental Figure 3.6

Supplemental Figure 3.6. Immunohistochemical characterization for proliferation, DNA damage and apoptosis of cutaneous cells post-UVR. (A) Representative H&E stained analysis of $EDN1^{L2/L2}$ and $EDN1^{ep-/-}$ skin 72hrs post-UVR of P2 mice. Scale bar=62μm, E=epidermis, D=dermis, HF=hair follicle. White dashed line indicates epidermal-dermal junction. (B) Epidermal thickness between $EDN1^{L2/L2}$ and $EDN1^{ep-/-}$ skin post-UVR of P2 mice. (C-D) Percentage of PCNA, CPD or TUNEL-positive epidermal and dermal cells out of total DAPI-stained cells between $EDN1^{L2/L2}$ and $EDN1^{ep-/-}$ skin post-UVR of P2 mice. All experiments were done using a minimum of three biological replicates from each group of mice and in all cases are expressed as mean +/- SEM. Statistical analysis was performed using Graphpad Prism, * = $p<0.05$, ** = $p<0.01$.

Table 3.1 Primer sequences used in RT-qPCR assays

Gene	Sense	Antisense
Edn1	5'-ACTTCTGCCACCTGGACATC-3'	5'-GTCTTTCAAGGAACGCTTGG-3'
Edn3	5'-ACACGCTTGCGTTGTACTTG-3'	5'-TTTCTGCTCTCCCGGAATAA-3'
Hgf	5'-AGGAACAGGGGCTTTACGTT-3'	5'-GCTGCCTCCTTTACCAATGA-3'
Kitlg	5'-TCCGAAGAGGCCAGAAACTA-3'	5'-TCAGATGCCACCATAAAGTCC-3'
Pomc	5'-GTACCCCAACGTTGCTGAGA-3'	5'-GACCTGCTCCAAGCCTAATG-3'
Fgf2	5'-CGGTCACGGAAATACTCCAG-3'	5'-TATGGCCTTCTGTCCAGGTC-3'
Hprt	5'-TGACACTGGCAAAACAATGCA-3'	5'-GGTCCTTTTCACCAGCAAGCT-3'

Table 3.2 Primer sequences used in ChIP assays

Gene	Sense	Antisense
Edn1 Dist	5'- GCTGCTGTGTCTCCCCTACT -3'	5'- CCACAAAGGTCCCTTCTTCA -3'
Edn1 Mid	5'- GCCAACGTGTTTTCTGTGTG -3'	5'- TGCGGTCTGTGCTTGATTAG -3'
Edn1 Prox	5'- GGTGGTGGTGGAAAAGTAGG -3'	5'- GGGGCTGATCATTGTTCACT -3'
Edn1 3'UTR	5'- CTTTTGTGGCTGGTTGACCT -3'	5'- CTAATGGCCTGCCAGAGAAG -3'
Pomc	5'-CAGATGCGCCTTGCGCTCAG-3'	5'-ACCTTCCTGGCAGCGCTTC-3'
p21	5'-CCTTTCTATCAGCCCCAGAGGATACC-3'	5'-GACCCCAAAATGACAAAGTGACAA-3'

General Conclusions

Chapter 4

Stephen D. Hyter and Arup K. Indra

4.1 Conclusions

Malignant melanoma is one of the fastest growing cancers in the United States and no effective therapeutic options are available once the primary tumor has disseminated to distal organs. Exposure to solar UV irradiation increases the risk of melanoma progression and many of these effects may be indirectly mediated through keratinocytic upregulation of various cytokines and growth factors that function as paracrine regulators of melanocyte homeostasis. *In vitro* culture studies of melanocytes in keratinocyte-derived conditioned medium have elucidated key factors that contribute to the cellular communication between these two cell types. Histological and biochemical analyses of signal transduction pathways suggest influences of keratinocytes on melanocyte migration, mitogenesis and melanogenesis, particularly after exposure to UV irradiation. The use of genetically engineered animal models allows further exploration of this relationship between two cell types by focusing on the contribution of individual soluble factors *in vivo* within a functional cellular microenvironment. Our own work has utilized $RXR\alpha^{ep-/-}$, $RXR\alpha^{ep-/-}/CDK4^{R24C/R24C}$ and $EDN1^{ep-/-}$ murine models to investigate transcriptional regulation of epidermal keratinocyte-derived soluble factors in response to chemical and/or physical carcinogens. Our studies have also established keratinocytic EDN1 as a critical modulator for maintenance of

melanocyte homeostasis in adulthood and after UV irradiation.

Retinoid X Receptor α (RXRα), a member of the nuclear hormone receptor superfamily, is a central coordinator in transducing cellular signals through heterodimerization with various family members. RXRα ablation in murine epidermal keratinocytes ($RXR\alpha^{ep-/-}$) using Cre/loxP technology has provided evidence that RXRα is intimately involved in modulating expression of various keratinocyte-derived growth factors. An additional point mutation that activates cyclin dependent kinase 4 (CDK4) was used to create $RXR\alpha^{ep-/-}/CDK4^{R24C/R24C}$ bigenic mice that exhibited more aggressive melanocytic growths compared to $RXR\alpha^{ep-/-}$ or $CDK4^{R24C/R24C}$ alone when subjected to DMBA/TPA treatments (Chapter 2). The loss of keratinocytic RXRα induced secretion of paracrine growth factors into the tumor microenvironment. Evidence of recruitment of RXRα on the proximal promoter region of *Edn1* and *Hgf* (relative to TSS) in murine keratinocytes suggests transcriptional regulation of these genes. In addition, altered expression of gene networks related to survival and invasiveness within the melanocytic tumors was linked to loss of keratinocytic RXRα. Finally, evidence of decreased epidermal levels of RXRα protein during human melanoma progression correlated well with the results obtained in the mouse studies. Altogether, results demonstrate the cooperative effects between RXRα and CDK4 in mediating melanocyte proliferation and melanoma formation.

In order to determine the molecular basis of keratinocytic RXRα regulated expression of paracrine factors fully, further studies investigating the mechanism(s) of RXRα binding and transactivation properties need to be performed. Determining the expression patterns of receptors for various paracrine growth factors on carcinogen-induced melanomas generated in RXRα$^{ep-/-}$ mice will corroborate previous studies in human samples (Loftus et al., 1999; Valesky et al., 2002; Sanchez-Mas et al., 2004; Curtin et al., 2006). Receptor inhibition using small-molecule or antibody-based antagonism will reveal the importance of this keratinocyte-derived signaling in formation and malignant progression of the melanocytic tumors from RXRα$^{ep-/-}$ mice. Although we have gained significant insight into the role of EDN1 in melanocyte homeostasis, additional transgenic mice selectively ablated for other mitogenic factors such as KITLG, POMC and/or FGF2 would be useful to elucidate their contributions in regulating melanogenesis and UV-induced melanocyte homeostasis. Analyses of conditioned media from UV-irradiated primary keratinocytes from RXRα$^{ep-/-}$ or RXRα$^{ep-/-}$/CDK4$^{R24C/R24C}$ bigenic mice will help to identify additional keratinocyte-derived soluble growth factors that are released in the microenvironment and can modulate melanocyte homeostasis. Isolation and characterization of such unknown proteins could establish novel molecular signaling between keratinocytes and melanocytes that could influence melanocyte homeostasis.

In the absence of agonistic ligands, RXRα/NR heterodimers act as transcriptional repressors for the various paracrine growth factors as previously described for thymic stromal lymphopoietin (TSLP) in mouse keratinocytes *in vivo* (Li et al., 2006). Ligand binding to its cognate receptor can release the repression exerted by RXRα/NR corepressor complexes and enhance gene expression through modulation of cofactor complexes. In order to reveal the identity of RXRα heterodimeric partner(s), primary murine keratinocytes can be treated with various NR ligands. Selective retinoids, deltanoids, thiazolidinediones, oxysterols and other NR agonists can be utilized to determine activation of specific growth factor expression. Additionally, computational analyses of paracrine gene promoters can be useful to predict RXR/NR binding. Those *in silico* analyses of RXR/NR consensus binding motifs can be partnered with chromatin immunoprecipitation (ChIP) sequencing analyses for histone activation and repression marks to provide a mechanistic basis for RXR regulation of paracrine factor promoter(s). Furthermore, Co-IP and/or ChIP-reChIP experiments investigating the sequential association of RXRα with components of the transcriptional activation or repression complexes will assist in determining the RXR/NR regulation of their target gene promoters.

Endothelins are signaling peptides composed of 21 amino acid residues that play a significant role in early melanocyte development, response to ultraviolet

radiation and pathological conditions including melanoma. We recently demonstrated a direct recruitment of p53 on the distal promoter of murine *Edn1* and a positive regulation in response to UV treatment of primary keratinocytes. A mouse model harboring a selective ablation of keratinocytic EDN1 ($EDN1^{ep-/-}$) demonstrated disruption of melanocytic homeostasis in adult skin, while neonatal $EDN1^{ep-/-}$ mice exhibit impaired melanocytic UV response. The phenotypic similarities observed between $EDN1^{ep-/-}$ and $p53^{-/-}$ mice, related to the altered melanocytic responses post-UV exposure, underscores the role of keratinocytic p53 to regulate expression of multiple paracrine factors (e.g. EDN1) and mediate melanocyte homeostasis. Finally, we demonstrated that EDN1 produces a mitogenic response through the EDNRB receptor via pERK and activated PKC in cultured murine melanocytes and in murine skin *in vivo* (Imokawa et al., 1996; Sato-Jin et al., 2008; Hyter et al., in review). Our data also suggested that EDN1 stimulate melanocyte migration *in vitro* and additional studies are needed to reveal the underlying mechanisms. Details on how melanocytes are able to disconnect from a homeostatic niche and migrate towards an alternate tissue location during melanoma metastasis could provide druggable targets in our attempt to prevent metastatic phenotypes of primary human melanomas.

It is important to establish ultraviolet (UV) irradiation as a causative agent for melanomagenesis. Melanoma resulting from neonatal UV exposure combined

with chronic adult treatment can provide important information regarding driving mutations within the tumors. *Tyr*-NRAS mice containing activated NRAS driven by the tyrosinase promoter demonstrate susceptibility to melanoma formation (Ackermann et al., 2005)**.** To establish the keratinocytic contribution towards solar UV-induced carcinogenesis, we bred our RXR$\alpha^{ep-/-}$ mice to this line and have created a novel RXR$\alpha^{ep-/-}$/*Tyr*RAS+ mouse to investigate the combinatorial effects of both these pathways in UVB-induced melanomagenesis. Briefly, two days old (P2) RXR$\alpha^{L2/L2}$/*Tyr*RAS+ (CT) and RXR$\alpha^{ep-/-}$/*Tyr*RAS+ (MT) mice were subjected to 600mJ/cm^2 UVB irradiation and then chronically exposed to 300mJ/cm^2 UVB three times a week for 30 weeks. At the termination of the treatment period, gross morphological analyses were made, skin samples were collected and processed for histological and immunohistochemical analysis. Whereas both lines experienced chronic UV-induced alopecia, all of the MT mice experienced a total loss of hair from both dorsal and ventral side, most likely due to the additive effects of keratinocytic loss of RXRα (Figure 4.1A). Histologically, CT and MT UV-irradiated adult mice skin displayed dermal pigmentation, due to NRAS activated melanocytes present in the hair follicles populating the dermal compartment (Figure 4.1B). However, after chronic UV treatment, the MT mice displayed denser pigmentation with melanocytes infiltrating into the basal layer keratinocytes and upper epidermis, reminiscent of the pagetoid spread seen in

junctional melanoma (Chudnovsky et al., 2005; Chin et al., 2006). Melanoma cells show cytologic atypia, with large abundant cytoplasm, enlarged and hyperchromatic nuclei, and increased overall size compared with normal melanocytes. Commonly, there was more junctional melanocytic hyperplasia (nests of tumor cells at the basement membrane zone). This is indicative of aggressive melanocytes transitioning through the basement membrane, potentially drawn by increased keratinocyte-derived paracrine signalling (Figure 4.1B). The MT mice also developed significant numbers of dorsal dermal growths compared to the CT mice, demonstrating phenotypic changes attributed to the RXR$\alpha^{ep-/-}$ genotype (Figure 4.1C).

Preliminary IHC analyses on skin sections co-labelled for melanocyte-specific marker TRP1 and the proliferation marker PCNA demonstrated a similar rate of proliferating melanocytes in the dermis of both CT and MT mice skin (Figure 4.2A). In addition, comparable levels of expression of the malignant melanocyte marker HMB45 were detected by IHC in both CT and MT mouse lines (Figure 4.2B). Finally, CD31 labelling for vessels displayed equivalent staining in both RXR$\alpha^{L2/L2}$/*Tyr*RAS+ and RXR$\alpha^{ep-/-}$/ *Tyr*RAS+ dorsal skin, implying equal levels of vascularization within the pigmented dermis (Figure 4.2C). Overall, chronic UV exposure in mice lacking keratinocytic RXRα paired alongside activated N-RAS in melanocytes display a more aggressive phenotype of epidermal invasion compared to those with activated RAS alone.

Additional IHC studies for markers of immunoevasion and investigations into metastatic behaviour are needed to elucidate the cooperativity between the RAS and NR signalling pathways during melanoma progression and metastasis. Similarly, exposure of RXR$\alpha^{ep-/-}$/CDK4$^{R24C/R24C}$ bigenic mice to solar UVB irradiation (physical carcinogen) instead of chemical carcinogen will present a more natural relationship to human melanoma development. Histopathological and immunohistochemical analyses of melanocytic tumors from those mice can then be compared to those from RXR$\alpha^{ep-/-}$/*Tyr*RAS+ mice to determine the cooperative effects between the multiple signalling pathways during melanomagenesis. Our present data and previous studies in mice and human suggest that establishment of a trigenic RXR$\alpha^{ep-/-}$/*Tyr*RAS+/CDK4$^{R24C/R24C}$ mouse line could possibly exhibit enhanced melanomagenesis and increased susceptibility to invasive and metastatic melanoma formation with minimal carcinogenic stress. (Ackermann et al., 2005; Delmas et al., 2007; Contassot et al., 2012).

In conclusion, our work has significantly expanded the body of knowledge regarding keratinocytic influence on the melanoma microenvironment, especially the role of RXRα-mediated regulation of soluble mitogenic factors in chemical/physical carcinogen induced melanomagenesis. This devastating disease is refractory to all current forms of treatment and our studies investigating mechanisms underlying micro-environmental influences on

melanoma promotion and progression are important for the development of novel therapeutic targets to prevent and/or cure melanoma.

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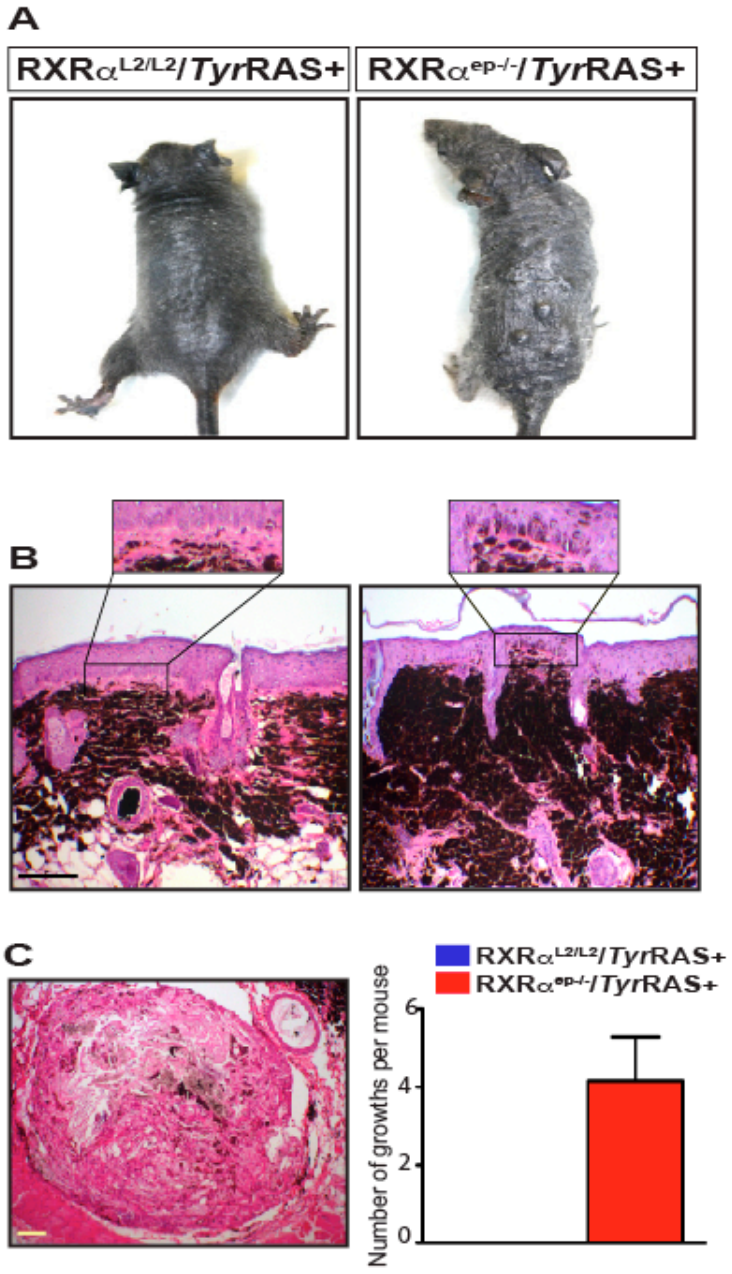
A
RXRα L2/L2/TyrRAS+
RXRα ep-/-/TyrRAS+
B
C
RXRα L2/L2/TyrRAS+
RXRα ep-/-/TyrRAS+
Number of growths per mouse
6
4
2
0

Figure 4.1

Figure 4.1. Phenotypic and histological characterization of RXR$\alpha^{L2/L2}$/*Tyr*RAS+ and RXR$\alpha^{ep-/-}$/*Tyr*RAS+ bigenic mice. (A) Representative gross morphology of dorsal skin from RXR$\alpha^{L2/L2}$/*Tyr*RAS+ and RXR$\alpha^{ep-/-}$/*Tyr*RAS+ after 30 weeks of chronic UV exposure. RXR$\alpha^{ep-/-}$/*Tyr*RAS+ mice develop dermal growths not seen in RXR$\alpha^{L2/L2}$/*Tyr*RAS+ mice. (B) Histological analyses of dermal pigmentation from chronic UV treated RXR$\alpha^{L2/L2}$/*Tyr*RAS+ and RXR$\alpha^{ep-/-}$/*Tyr*RAS+ skin. Hematoxylin and eosin (H&E)-stained 5μm thick paraffin skin sections of biopsies taken 30 weeks after birth, scale bar = 62μm. (C) Representative dermal growth and graphical analyses of endpoint formations present in Rxr$\alpha^{ep-/-}$/TyrRas+ skin after UV treatment.

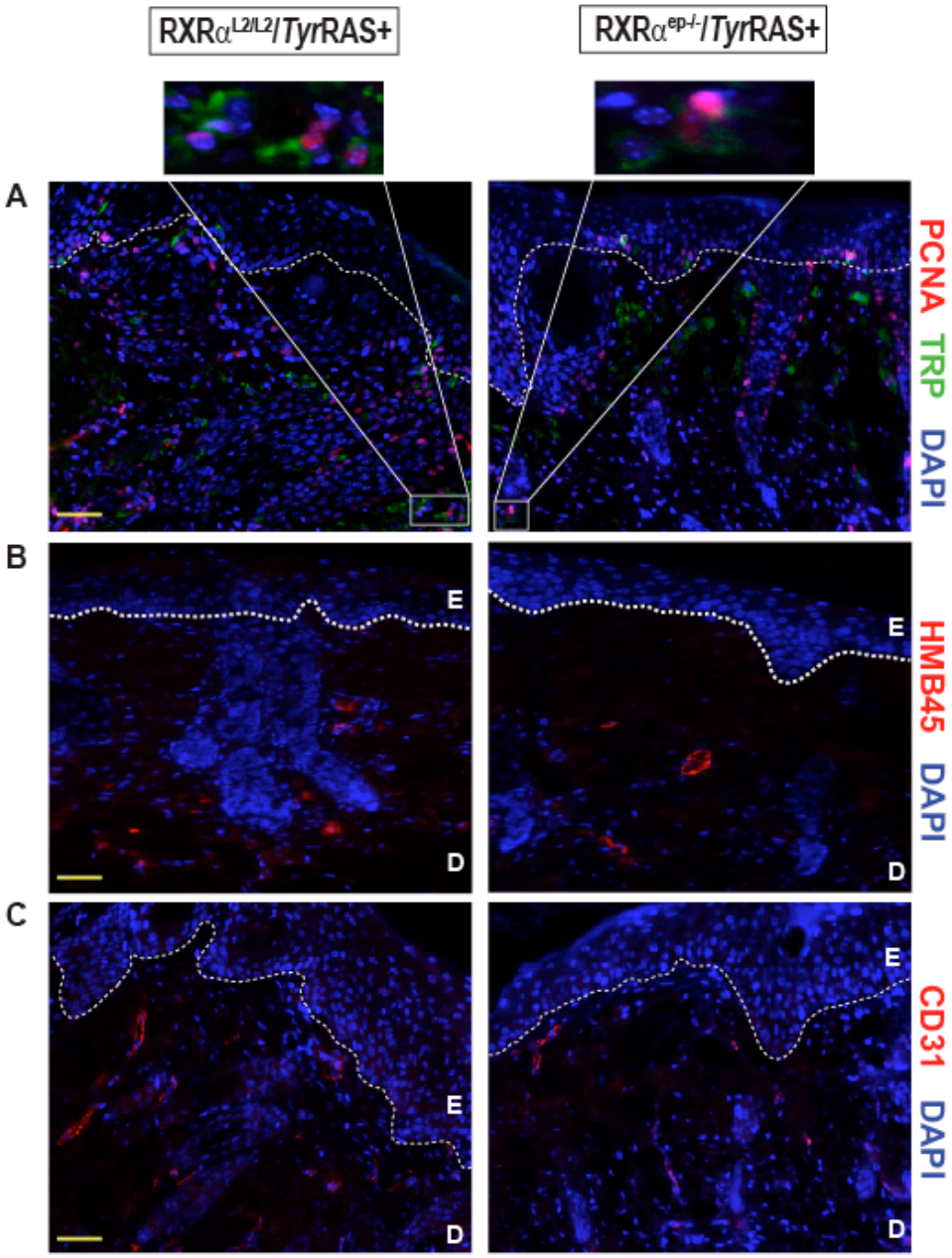
RXRα^L2/L2/TyrRAS+
RXRα^ep-/-/TyrRAS+
A
B
C
PCNA TRP DAPI
HMB45 DAPI
CD31 DAPI
E
D

Figure 4.2

Figure 4.2. Immunohistochemical (IHC) characterization of cutaneous tissue in RXR$\alpha^{L2/L2}$/*Tyr*RAS+ and RXR$\alpha^{ep-/-}$/*Tyr*RAS+ bigenic mice. (A) IHC staining of bleached RXR$\alpha^{L2/L2}$/*Tyr*RAS+ and RXR$\alpha^{ep-/-}$/*Tyr*RAS+ skin sections after 30 weeks of chronic UV treatment using both anti-TRP1 [green] and anti-PCNA [red] antibodies. (B) IHC staining for malignant melanocytes using a cocktail of antibodies directed against melanoma antigens HMB45 and MART-1 [red]. (C) Cutaneous vascularization detected by anti-CD31 antibody [red] within dermal tissure. E, epidermis; D, dermis. Scale bar = 33μm. White dashed line artificially added to indicate epidermal-dermal junction. Blue color corresponds to DAPI staining of the nuclei.