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Beta Yadollahi

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Cellular and Molecular Medicine)

GRADE / DEGREE

Department of Cellular and Molecular Medicine

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

The Role if Gas3 in Skin Tumorigenesis

TITRE DE LA THÈSE / TITLE OF THESIS

David Lohnes

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

Christine Pratt

Kursad Turksen

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

The Role of Gas3 in Skin Tumorigenesis

by

Beta Yadollahi

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Abstract

Retinoic acid (RA) plays an integral role during embryonic development, in addition to being a potential chemotherapeutic and chemopreventative agent in various cancer models due to its growth inhibitory and pro-differentiation properties. RA signaling occurs in a ligand-dependent manner through a family of nuclear receptors consisting of the RARs and RXRs. RA elicits growth inhibitory effects in transformed wild type keratinocytes, however the target genes that mediate this outcome remain largely unidentified. In order to isolate genes that could mediate the latter effects, a suppressive subtractive hybridization experiment was conducted by our lab and revealed *growth arrest specific 3 (gas3)* as a candidate gene. *Gas3* is expressed in the skin and is induced by RA as early as two hours post-treatment in tissue culture models. We investigated a possible relationship between *gas3* and epidermal tumorigenesis and found that this gene behaves in a context dependent, strain-specific manner as both an RA-mediated tumor suppressor and an oncogene. To further investigate this, I first demonstrated that RA-induced growth arrest in keratinocytes is mediated in part by *gas3*. Additionally, previous work from our lab demonstrated that *gas3*-null mice from an FVB/NJ background evaded tumor formation following the two-stage carcinogenesis protocol. I demonstrate that this occurs as a result of an increase in apoptotic response following chemical mutagenesis in *gas3* mutants. Conversely, I found that Gas3 mutants on a C57Bl/6 background behave as wild type animals with respect to carcinogenesis, suggesting that genetic modifiers between these two strains impact on Gas3 function with respect to tumorigenesis. Finally, I found that p21 activity is affected by the loss of gas3, which may begin to elucidate how gas3 impacts on carcinogenesis.

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List of Abbreviations

Adh - alcohol dehydrogenase

AP-1 - activator protein 1

Apc^{Min} - adenomatous polyposis coli- multiple intestinal neoplasia

APL - acute promyelocytic leukemia

ATBC - Alpha-Tocopherol Beta-Carotene

Atf - activating transcription factor

BCC - basal cell carcinoma

BMP - bone morphogenic protein

Ca^{2+} - calcium

CARET - Carotene and Retinol Efficacy Trial

Cdk - cyclin-dependent kinase

CMT - Charcot-Marie-Tooth

Ctfr - cystic fribrosis transmembrane conductance regulator gene

DMBA - 7,12-dimethyl-benz-[α]-anthracene

DR - direct repeat

DSS - Dejerine-Sottas syndrome

E - embryonic

EMT - epithelial-mesenchymal transition

FGF - fibroblast growth factor

Gas3 - growth arrest specific 3

GFP - green fluorescent protein

H&E - hemotoxylin and eosin

Ha-ras - Harvey-ras

HAT - histone acetyltransferase

HDAC - histone deacetylase

HNPP - hereditary neuropathy with liability to pressure palsies

IKK - inhibitor κB kinase

IκB - inhibitor κB

K - keratin

Ki-ras - Kirsten-ras

Klf4 - kruppel-like factor 4

MEF - mouse embryonic fibroblast

Mom1 - modifier of Min 1

NF-κB - Nuclear factor κB

NMSC - non-melanoma skin cancer

PI - phosphoinositide

PIP3 - phosphoinositide (3,4,5)-triphosphate

PKC - protein kinase C

Pmp22 - peripheral myelin protein 22

PNS - peripheral nervous system

RA - retinoic acid

Raldh - retinal dehydrogenases

RAR - retinoic acid receptor

RARE - retinoic acid response element

Rb - retinoblastoma

RBP4 - retinol-binding protein 4

RXR - retinoid X receptor

SBC - sunburn cell

SCC - squamous cell carcinoma

SDR - short-chain alchohol dehydrogenase/reductase

siRNA - short interfering RNA

Tgase - transglutaminase

TGF-β - transforming growth factor β

TGF-βR - transforming growth factor β receptor

TPA - 12-*O*-tetradecanoylphorbol-13-acetate

Tr - trembler

TRE - TPA response element

UV - ultraviolet

Wnt - wingless

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Firstly, I would like thank my supervisor, Dr David Lohnes, for giving me the opportunity to work on this project. Through many challenges he provided me with encouragement, insight and support, all of which contributed tremendously to the success of my graduate studies. I would also like to express my gratitude to members of my advisory committee, Dr Stephen Lee and Dr Kursad Turksen, for their help and advice throughout the course of my project. I would like to thank Dr Nadine Wiper-Bergeron for being an endless source of knowledge, for always listening, and whose humor and wit truly made my days enjoyable. Much appreciation to Dr Christine Pratt who has shown me exactly what a strong woman in science exemplifies.

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CHAPTER 1

Introduction

1.1 Vitamin A

Vitamin A plays essential roles during embryonic development, as well as in tissue homeostasis in the adult (De Luca, 1991; De Luca et al., 1996; Gudas, 1994). Vitamin A imbalance during embryogenesis inhibits proper developmental processes (Ross et al., 2000). For example, an insufficiency of vitamin A during development leads to defects in embryo growth, an arrest in vascularization, and cardiac defects, among other malformations, with extreme cases leading to resorption (Maden et al., 1996; Thompson et al., 1969; Wellik and DeLuca, 1995). In addition to an integral role during development, vitamin A is also involved in growth, vision, and reproduction in the adult organism. Vitamin A excess, in contrast, leads to membrane lysis and liver toxicity in the adult, and has a broad spectrum of teratogenic effects on the embryo (Sass et al., 1997).

Retinoic acid (RA), the major biologically active form of vitamin A, regulates developmental processes by activating gene transcription in the embryo (Ross et al., 2000). RA is not distributed homogenously throughout the embryo, but instead is restricted in a number of developing tissues and organs (Balkan et al., 1992; De Luca, 1991). This distribution is controlled by RA-synthesizing and degrading enzymes and allows for proper RA availability within the developing embryo (Ross et al., 2000).

A relationship between vitamin A and cancer has been widely investigated. Epidemiological studies revealed that individuals with a lower vitamin A intake are at a higher risk of developing cancer (Hong and Itri 1994), and vitamin A deficient animals show an increased susceptibility to chemical carcinogenesis (Moon, R.C et al., 1994). Furthermore, in animal carcinogenesis models, retinoids have demonstrated an ability to

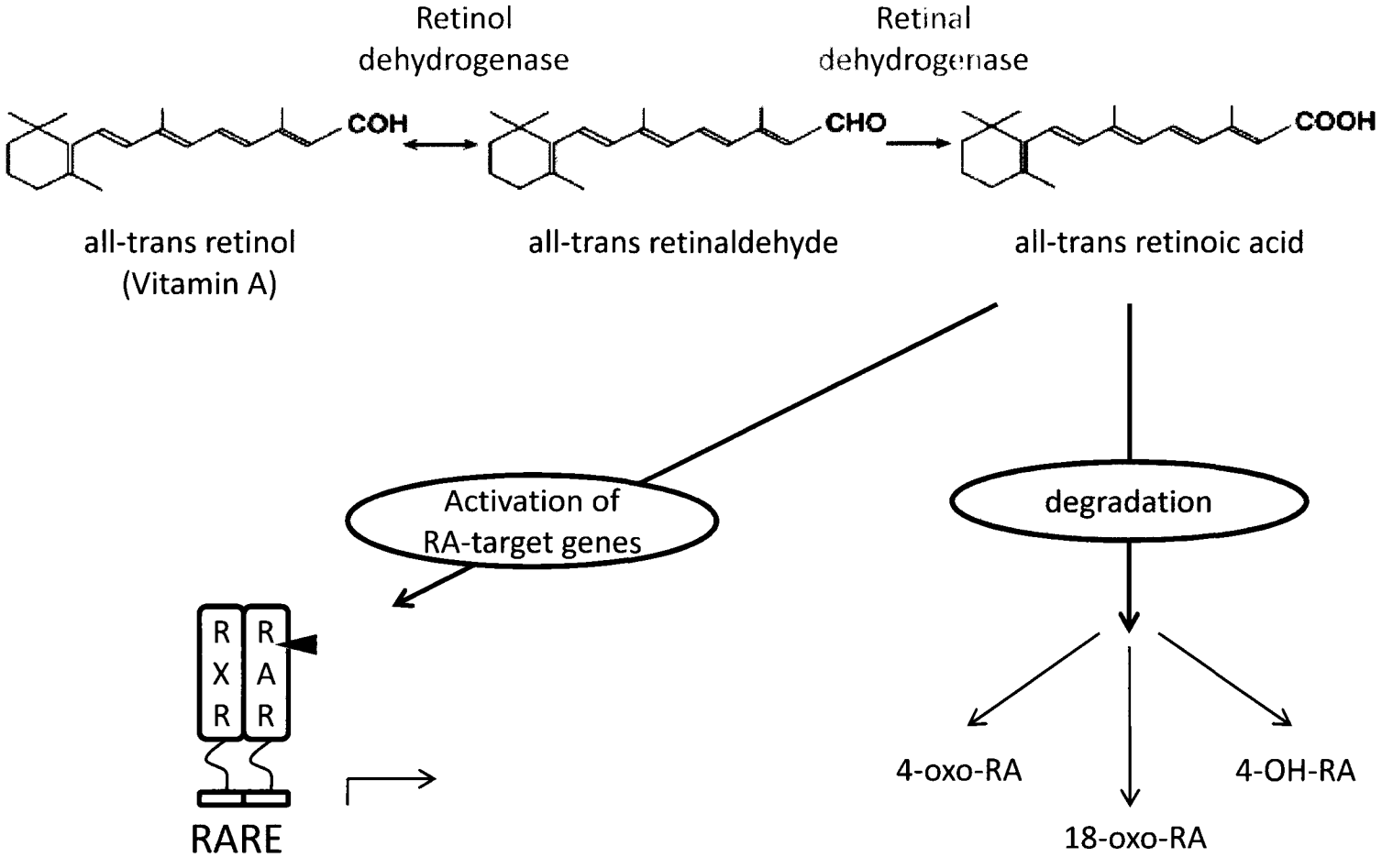
suppress tumor development in a number of tissues including skin, breast, lung, prostate, bladder, liver and pancreas (Sun and Lotan, 2002).

1.2 RA Metabolism

Vitamin A, also referred to as retinol, cannot be synthesized by the body, and must be consumed via dietary intake in the form of carotenoids from plants or retinyl esters from animal products (Maden et al., 2007). Derivatives of vitamin A, referred to as retinoids, are predominantly stored in the liver as retinyl esters, however the lungs, bone marrow, and kidneys can also be sites of storage (Blomhoff and Blomhoff, 2006). Retinol bound to retinol-binding protein 4 (RBP4) is released from such stores, circulates in the bloodstream and enters cells via the aid of STRA6, a transport protein (Maden et al., 2007) or simply diffuses passively across membranes. Upon entering a cell, retinol binds to CRBPI/II, a retinol binding protein that is thought to promote RA synthesis by presenting retinol to retinol dehydrogenase enzymes (Ross et al., 2000). RA synthesis from retinol is a two-step oxidation process, of which retinol dehydrogenases catalyze a reversible oxidation reaction, converting all-trans-retinol to all-trans-retinaldehyde (retinal) (Chen et al., 1995) (Figure 1.1). Retinol dehydrogenases are members of the alcohol dehydrogenase (Adh) family, of which there exist two types; medium-chain cytoplasmic alcohol dehydrogenase (Adh) and short-chain membrane-bound alchohol dehydrogenase/reductase (SDR) (Ross et al., 2000).

Figure 1.1 Retinoid metabolism and catabolism. Two distinct oxidation reactions are involved in the conversion of dietary vitamin A into all-trans retinoic acid (RA). Once RA is synthesized it can be degraded by CYP26 enzymes into polar metabolites destined for elimination, or go on to activate RA-responsive genes by binding to a set of nuclear retinoid receptors (see text for details).

Adapted from Ross S., McCaffrey P., Drager U., and De Luca L., *Physiological Reviews*, 2000, 80: 1021-1054.


Retinol
dehydrogenase
Retinal
dehydrogenase
COH
CHO
COOH
all-trans retinol
(Vitamin A)
all-trans retinaldehyde
all-trans retinoic acid
Activation of
RA-target genes
degradation
R
X
R
R
A
R
RARE
4-oxo-RA
18-oxo-RA
4-OH-RA

Medium-chain alcohol dehydogenases consist of five classes of zinc-dependent cytosolic enzymes, Adh1, 2, 4, 7 and 8, of which only Adh1 and Adh4 are able to oxidize retinol to retinaldehyde (Duester, 2000; Ross et al., 2000). Adhs are not only responsible for the metabolism of retinoids, but also ethanol and several other alcohols and aldehydes (Duester et al., 1999). There exists eights classes of SDRs (RoDH1-4, CRAD1,2, RDH5, and retSDR1), however unlike Adhs their predominant metabolic substrates are androgens, although alcohols and aldehydes are not excluded. As such SDRs have been implicated in both androgen and retinoid metabolism (Duester, 2000).

The second step of RA synthesis involves an irreversible oxidation reaction converting retinal to all-trans-retinoic acid (RA) (Ross et al., 2000) (Figure 1.1). This is mediated by a family of retinal dehydrogenases (Raldhs), and include Raldh1, Raldh2, and Raldh3 (Gagnon et al., 2003). These enzymes are evolutionarily conserved across vertebrate species, including human, mouse and *Xenopus*, however their expression patterns differ slightly (Duester, 2000). In the mouse, Raldh1 is present in the embryonic dorsal retina, in addition to several adult tissues (Ang and Duester, 1999; Haselbeck et al., 1999). Raldh2 is detected in chick and mouse embryos as well as reproductive organs of the adult mouse (Berggren et al., 1999; Swindell and Eichele, 1999). Raldh2 plays a critical role in regulating RA synthesis during embryonic development. Embryos lacking Raldh2 expression suffer from many developmental malformations such as hindbrain patterning defects, smaller somites, a shortened anteroposterior axis, and cardiac defects and die early in gestation (Mic et al., 2002; Niederreither et al., 1997). Raldh3 is primarily expressed in the ventral retina of mouse and chick embryos, as well as in the olfactory pit, and has been implicated playing a role in normal nasal development

(Begemann et al., 2001; McCaffery et al., 1999; Mic et al., 2002). Nonetheless, despite their differential expression patterns, it is evident that Raldhs1-3 play fundamental roles in RA synthesis.

1.3 RA Catabolism

RA distribution within specific tissues is essential for normal development. An imbalance of RA levels, whether it is insufficiency or excess, can lead to severe malformations, therefore is it imperative that RA distribution is controlled by complementing synthesis and degradation. RA catabolism is mediated by members of the cytochrome P-450 oxidase superfamily, *CYP26A1*, *B1* and *C1* (Fujii et al., 1997; Hollemann et al., 1998; Ray et al., 1997; White et al., 1996). These enzymes oxidize RA into 4-hydroxy-RA, 18-oxo-RA, or 4-oxo-RA, which can be further modified to form more polar metabolites (Napoli, 1996) or conjugated before destined for elimination (Samokyszyn et al., 2000). None of the latter forms have been shown to have any essential physiological roles, at least in embryonic development (Niederreither et al., 2002).

Of the three CYP26 enzymes, CYP26A1 and CYP26B1 have been best characterized. *CYP26A1* expression during early stages of embyrogenesis is detected in the tail bud, and at later stages in the hindbrain and retina (MacLean et al., 2001). Genetic studies reveal the importance of CYP26A1 function during embryonic development, as loss of this enzymes leads to excess RA exposure causing terotogenic effects, including spina bifida and caudal truncation and lethality (Abu-Abed et al.,

2001). *CYP26A1* is also a direct RA-target gene and following RA exposure, its expression is induced in several adult tissues, suggestive of an autoregulatory feedback system that controls RA levels within tissues (Taimi et al., 2004). *CYP26B1* expression is observed in a number of adult tissues such as the dermal layer of the skin, hair follicles and several regions the brain, including the pons, cerebellum and hippocampus. Similar to *CYP26A1*, *CYP26B1* is also induced by RA, but its expression in adult tissues like the brain and skin is mainly constitutive (Abu-Abed et al., 2002).

1.4 Retinoid Receptors and RA Signaling

Once RA has been synthesized, it has two fates to which it can commit; (1) catabolism leading to elimination or (2) activation of RA-responsive genes (Petkovich, 2001). RA regulated gene expression through binding to a subset of nuclear retinoid receptors, members of the steroid hormone receptor family (Mangelsdorf and Evans, 1995). The retinoid receptors are comprised of the retinoic acid receptors (RARα, RARβ, and RARγ) and the retinoid X receptors (RXRα, RXRβ, and RXRγ), each subtype expressed as several isoforms. Retinoid signaling is dependent on the heterodimeric interaction between RAR and RXR members (Altucci and Gronemeyer, 2001; Chambon, 1996) and subsequent activation by ligand binding (Heyman et al., 1992). RAR is activated by the all-trans conformation of RA as well as isomers such as 13-cis-RA, with the former being the most abundant biologically active retinoid and having the highest affinity for RAR (Repa et al., 1993). 9-cis-RA has not been isolated from the mouse, although it is present in other vertebrates, and can also bind to both RARs and RXRs

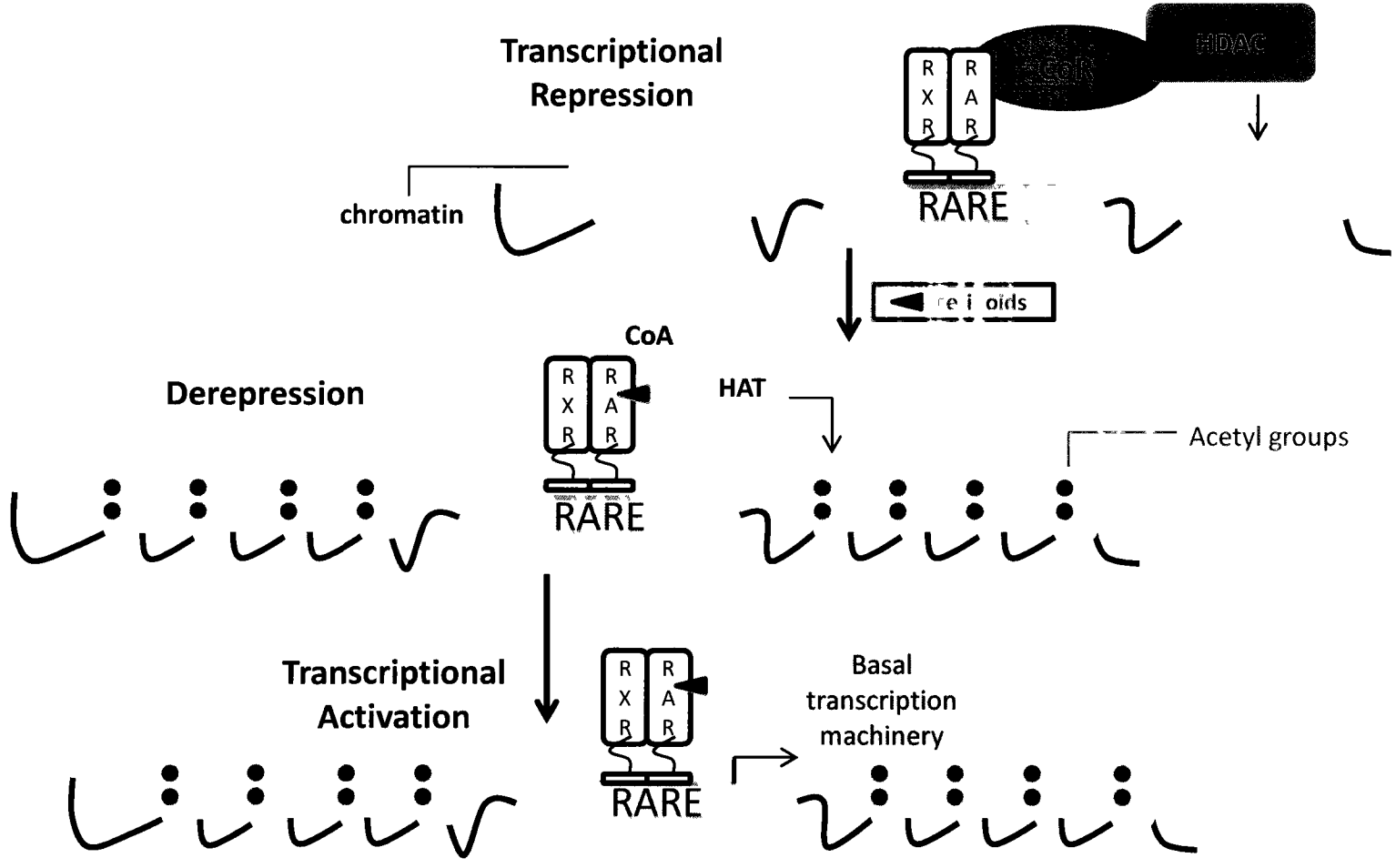
(Mic et al., 2003; Repa et al., 1993). RXRs have also been implicated as heterodimeric partners for several other nuclear receptors, including those for thyroid hormone, vitamin D, and various orphan receptors (Kliewer et al., 1992; Mangelsdorf and Evans, 1995), but do not appear to require a ligand for their function (Lemon and Freedman, 1996; Nagy et al., 1995; Shiohara et al., 1999; Willy et al., 1995).

The RAR/RXR heterodimer binds to specific DNA elements, termed RA response elements (RAREs) situated within the promoter or enhancer region of target genes (Soprano et al., 2004). RAREs are characterized by direct repeats (DRs), composed of two hexamers of a consensus sequence 5'-PuGGTCA, separated by one, two or five nucleotides, denoted as DR1, DR2 and DR5 respectively (Chambon, 1996). The RXR/RAR heterodimer binds to DR2 and DR5 elements (Rastinejad et al., 2000), and is positioned such that the RXR occupies the 5' upstream half-site, and the RAR occupies the 3' half-site (Soprano et al., 2004). However, there exists a number of variant RAREs (Giguere, 1994; Kato et al., 1995; Mangelsdorf and Evans, 1995). DR1 elements are also bound efficiently by other nuclear receptors such as COUP-TFII homodimers and PPAR/RXR heterodimers (Nakshatri and Chambon, 1994).

The transcriptional activity of RXR/RAR heterodimers is regulated through associations with co-repressor and co-activator complexes (Figure 1.2). In the absence of RA, the RAR/RXR heterodimer recruits co-repressors such as N-CoR or SMRT and mSin3 (Alland et al., 1997; Heinzel et al., 1997; Nagy et al., 1997). In turn, these

Figure 1.2 RA signaling. The transcriptional regulation of RA target genes is ligand dependent. In the absence of RA, the RAR/RXR heterodimer recruits a co-repressor (CoR) complex and histone deacetylases (HDACs) leading to chromatin condensation and repression of transcription. RA binding to the receptor causes the dissociation of co-repressors and subsequent recruitment of histone acetyltransferase(HAT)-containing co-activators (CoA), leading to the transcriptional activation of RA responsive genes (see text for details).

Adapted from Altucci L., and Gronemeyer H., *Nature Reviews Cancer*, 2001, 1: 181-193.


Transcriptional Repression
HDAC
R X R
R A R
RARE
chromatin
CoA
Derepression
HAT
Acetyl groups
Transcriptional Activation
Basal transcription machinery

co-repressors recruit histone deacetylases (HDACs) which function to remove acetyl groups from core histone tails which is believed to cause a local change in chromatin structure, leading to a reduction in transcription. Upon ligand binding, there is a dissociation of the co-repressor proteins and association of a histone acetyltransferase (HAT)-containing co-activator complex encompassing, among other members, CBP/p300 (Sun and Lotan, 2002). As a result, histone proteins are acetylated, chromatin decondenses and transcription of the target gene increases.

1.5 Structure and Components of the Skin

The skin and its appendages comprise the body's largest organ, playing a number of physiological roles including providing a barrier against microorganisms, as well as preventing dehydration (Fuchs, 2007). The skin is divided into three distinct components, the epidermis, the dermis and the hypodermis (subcutaneous fat) (Kanitakis, 2002).

The epidermis is extremely resilient, able to withstand daily physical and chemical insults. It is composed of a thin layer of stratified squamous epithelium separated from the underlying dermis, hair follicles and sweat glands by a basement membrane (Fuchs and Raghavan, 2002). There exists several different epidermal cell types, including melanocytes, Langerhans cell, Merkel cells and keratinocytes, with the latter making up 90-95% of the total cell population of the epidermis. Each cell type has a specific function in the skin. Melanocytes produce melanin which gives skin its pigmentation. Melanin is produced through a series of enzymatic reactions mediated by

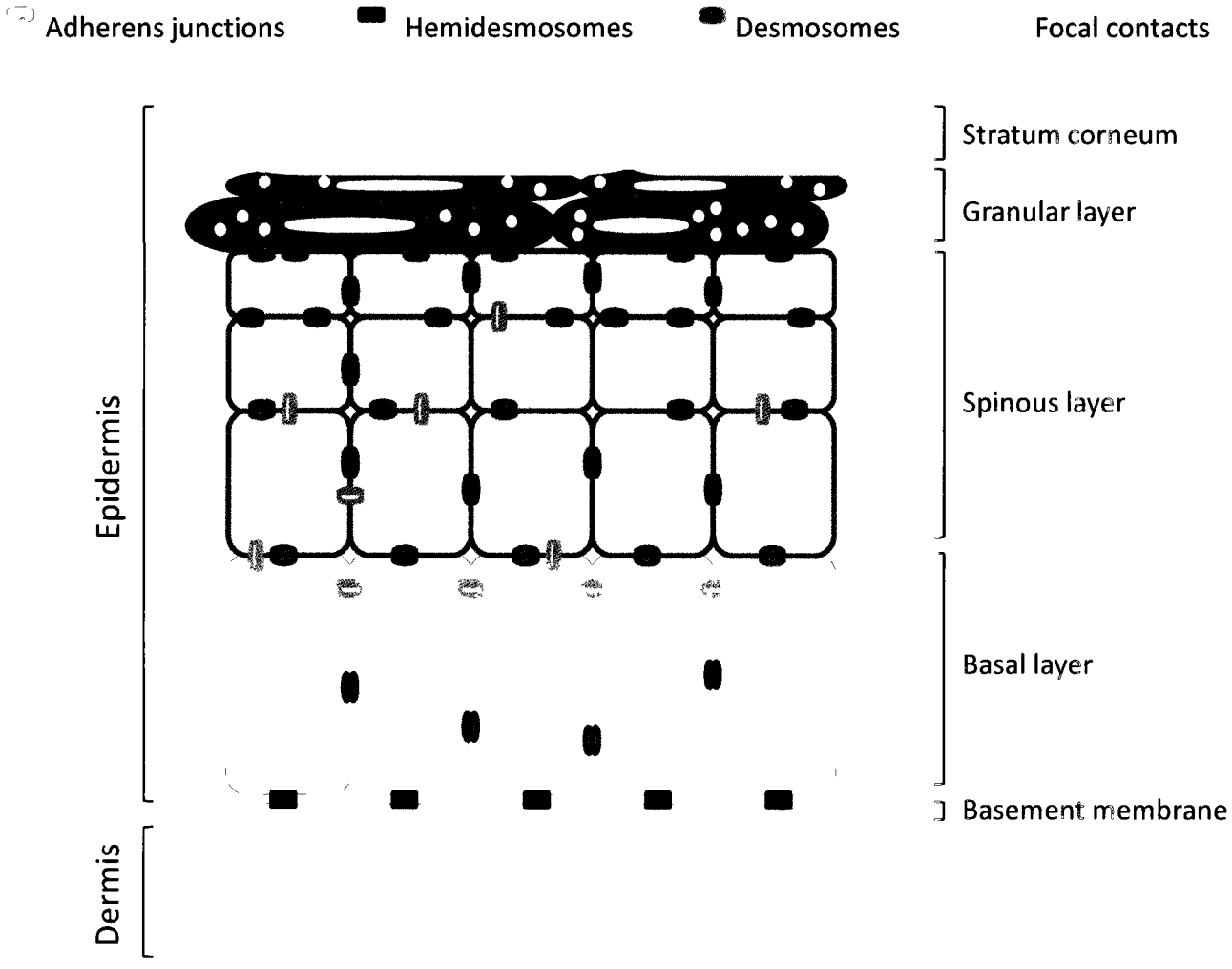
tyrosinase from the amino acid substrate tyrosine, and is stored in melanosomes. The diverse skin and hair pigmentations observed among individuals are due to differences in activity and distribution of melanosomes within the epidermal layer and hair bulb. Langerhans cells are present in all stratified epithelia and constitute 3-6% of the cells in the epidermis. They are dendritic antigen presenting cells and act by uptaking exogenous deposits on the skin and presenting them to naïve T-cells for further processing by the immune system. Merkel cells are located in the basal layer of the epidermis as well as in the hair follicle. They function as sensory receptors, specifically mechanoreceptors, and form synapses with dermal sensory axons (Kanitakis, 2002). Keratinocytes play a more essential role as they are responsible for the synthesis of keratin, a structural protein that provides strength and support to the epidermis (Fuchs, 1995; Hearing, 2005; Romani et al., 1989; Tachibana, 1995).

1.6 Organization and Function of the Epidermis

The epidermis is organized into four distinct layers (Figure 1.3). Directly adjacent to the basement membrane lays the basal layer, followed by the spinous layer, granular layer and stratum corneum, respectively, in ascending order (Fuchs and Raghavan, 2002). The basal layer (stratum basale) is composed of columnar keratinocytes, and contains a population of self-renewing epidermal stem cells. Most of the cells in the basal layer are the immediate progeny of these stem cells, transit amplifying cells, which undergo several rounds of division before

Figure 1.3 Structure and components of the skin. The skin is divided into 3 distinct components: the epidermis, dermis and hypodermis. The epidermis itself is divided into four layers consisting of the basal layer, spinous layer, granular layer and stratum corneum. Each layer uniquely and equally contributes to the self-renewal property and barrier function of the epidermis (see text for details).

Adapted from Fuchs E., and Raghavan S., *Nature Genetics Reviews*, 2002, 3:199-209.


Adherens junctions
Hemidesmosomes
Desmosomes
Focal contacts
Stratum corneum
Granular layer
Spinous layer
Basal layer
Basement membrane
Epidermis
Dermis

they commit to a differentiated state and begin to ascend to the surface of the skin, forming the various layers of the epidermis. Once these cells have reached the surface, they are dead, flat and enucleated, and are eventually shed and replaced (Fuchs and Raghavan, 2002). This property of self-renewal takes approximately 40-56 days in humans (Gelfant, 1976; Halprin, 1972) and 8-10 days in mice (Potten et al., 1987), and is continuous throughout life (Watt, 2002).

The spinous layer (stratum spinosum) is the first suprabasal layer and is the thickest layer of the epidermis (Fuchs and Raghavan, 2002). The cells from the spinous layer arise from the mitotic cells from the basal layer. Cells in the basal layer express keratin 5 and 14 (K5 and K14) and as they begin to migrate up towards the spinous layer, they switch expression to keratin 1 and 10 (K1 and K10) (Fuchs and Green, 1980). This change of keratin expression remains the most identifiable marker of an epidermal cell committing to a terminally differentiated state.

As cells of the spinous layer begin to ascend to the granular layer (stratum granulosum), they produce small basophilic electron dense particles called keratohyalin granules (Fuchs, 2007). These granules form a network of filagrin and keratin proteins which function as a protective barrier. Furthermore, the granular layer contains lamellar granules that contain lipid precursors that eventually migrate to the stratum corneum to coat the surface of the skin, functioning as a barrier against foreign matter (Kanitakis, 2002). The stratum corneum is the most superficial layer of the epidermis and creates an impenetrable seal to the external environment. It is composed of dead, enucleated squamous keratinocytes, called corneocytes, that shed continuously (Fuchs, 2007; Kanitakis, 2002).

The epidermis and dermis are separated by a basement membrane and functions to provide mechanical support, as well regulating the passage of lymphocytes during wound healing and immunological responses (Kanitakis, 2002). The dermis ultimately serves to protect the epidermis and its appendages, and is divided into two layers; papillary (superficial) layer and reticular (deep) layer (Odland, 1991). The papillary layer contains elastin, collagen, fibroblasts, dendrocytes, mast cells, and vascular and nerve plexuses. The reticular layer is composed of thicker bundles of collagen, and also harbors vascular and nerve plexuses (Kanitakis, 2002). Fibroblasts are the principal cell type of the dermis and are responsible for synthesizing collagen and elastin; fibers that provide strength and elasticity to the skin and are considered the two major constituents of the dermis (Odland, 1991).

1.7 Skin Development

The development of the epidermis involves the temporal expression of specific genes, as well as signaling cascades that act synergistically to form the layers of the epidermis. This process is completed in utero, and in mice takes approximately 10 days, commencing at embryonic day 8.5 (E8.5) (Koster and Roop, 2007). During development, gastrulation forms three primary germ layers; the endoderm, mesoderm and ectoderm, with the latter contributing to the nervous system and the skin (Wang and Steinbeisser, 2009). There exists a series of signaling cascades involved in determining whether an ectodermal cell commits to an epidermal cell or neural cell fate. This decision involves wingless (Wnt) and fibroblast growth factor (FGF) signaling, as well as

bone morphogenic proteins (BMPs) (Fuchs, 2007). In the presence of Wnt, FGF signaling to the ectoderm is inhibited, permitting BMP signaling and leading to an epidermal fate.

Once the epidermal fate has been specified, cells are prompted to differentiate into the different layers of the epidermis; p63, a homologue of p53, is the first transcription factor identified to play a critical role in epidermal stratification (Green et al., 2003). Mice lacking p63 exhibit an absence of epidermal stratification and barrier formation, and suffer perinatal death due to dehydration (Koster et al., 2004). ΔNp63α, an isoform of p63, is involved in both the development and maintenance of the basal layer of the embryonic epidermis. It is imperative that the rate of proliferation and terminal differentiation of embryonic basal keratinocytes be highly regulated, as it is this layer populates the entire epidermis (Koster and Roop, 2007). ΔNp63α maintains the proliferation of embryonic basal keratinocytes by directly inhibiting the cyclin-dependent kinase (cdk) inhibitors p21 and 14-3-3σ (Dellambra et al., 1995; Dellambra et al., 2000; Missero et al., 1995; Nguyen et al., 2006; Pellegrini et al., 2001; Westfall et al., 2003). ΔNp63α also promotes terminal differentiation of basal keratinocytes by inhibiting genes required for cell cycle progression, such as *cyclin B2* and *cdc2* (Testoni and Mantovani, 2006). Although the functions of ΔNp63α seem contradictory, it has been suggested that its role in the maintenance of epidermal proliferation occurs at early stages, whereas its impact on differentiation occurs during later stages of basal layer specification (Koster and Roop, 2007).

Terminal differentiation in the spinous and granular layer is prompted by an increase in extracellular calcium (Ca^{2+}) levels (Koster and Roop, 2007). This increase in

Ca^{2+} leads to the activation of the protein kinase C (PKC) pathway, and PKC proteins contribute to the keratin switch that occurs when cells from the spinous layer (expressing K5 and K14) terminally differentiate to cells of the granular layer expressing K1 and K10 (Dlugosz and Yuspa, 1993).

The formation of the epidermal barrier, the stratum corneum, is the final and most crucial component of epidermal stratification. Kruppel-like factor 4 (Klf4) is a transcription factor expressed in the spinous and granular layers and mediates the formation of the epidermal barrier (Garrett-Sinha et al., 1996; Segre et al., 1999). *Klf4*$^{-/-}$ mice exhibit severe barrier formation defects due to the immature development of cornified envelopes (Segre et al., 1999). It is clear that a fully developed and functional epidermis needs to be established in utero as defects in this process often lead to early post-natal complications and lethality.

1.8 Skin Cancer

Skin cancer incidence continues to escalate and it is estimated that rates will further increase. There exists two types of skin cancers, melanoma and non-melanoma skin cancer (NMSC). Melanomas are less frequent but are responsible for the majority of deaths associated with skin cancer (de Vries et al., 2005). NMSC is the most common human malignancy (Neville et al., 2007), accounting for approximately 40% of all diagnosed cancers (Nguyen and Ho, 2002). The mortality associated with NMSC is low, however there are more than one million newly diagnosed cases each year and

approximately 20% of Americans will eventually develop this form of cancer during their lifetime (Chen et al., 2001).

Ultraviolet (UV) radiation, in particular UVA and UVB from sun exposure, is the primary cause of most NMSC, and as such there is a heightened awareness to adopt protective measures in order to reduce this risk (Nguyen and Ho, 2002). The two primary types of NMSC are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), which contributes to 75% and 20% of all NMSC cases, respectively (American Cancer Society, 2006). Although SCC is less common than BCC, it is more aggressive and causes the most morbidities and mortalities from NMSC (Nguyen and Ho, 2002). The cellular mechanisms underlying the onset and development of SCC still remain undefined. One model in particular, the two-stage carcinogenesis protocol, has been adapted in order to study the cellular, biochemical and molecular changes associated with multistage skin carcinogenesis.

1.9 The Mouse Skin Model of Multistage Carcinogenesis

Malignant transformation of epidermal cells is multifactorial, encompassing stepwise accumulation of genetic and epigenetic alterations (Hummerich et al., 2006). The two-stage carcinogenesis model has been employed by many researchers as a tool for studying the mechanisms that impact on epidermal SCC. In this system, benign tumors (papillomas) are chemically induced on the dorsal skin of mice by a single topical application of an initiating carcinogen, such as 7,12-dimethyl-benz-[α]-anthracene (DMBA), followed by repeated topical treatments with a hyperproliferative tumor

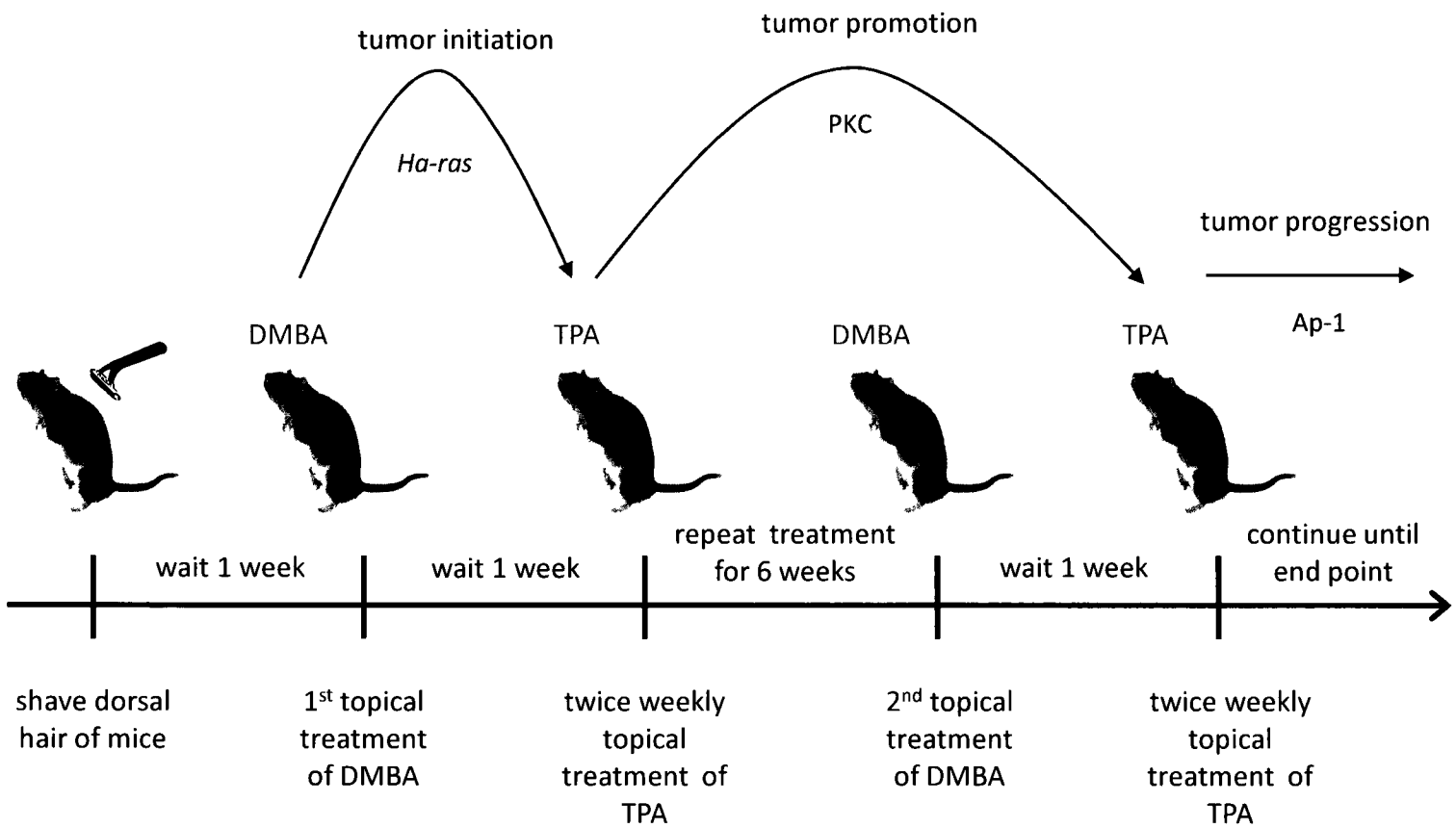
promoting agent such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Slaga, 1984; Yuspa, 1994). TPA-mediated tumor promotion typically commences one week after DMBA exposure, and continues until the desired end point (Figure 1.4). Papillomas may arise as early as 6 weeks following initiation of TPA treatment. While benign, papillomas are predisposed to conversion to squamous cell carcinoma commencing at approximately 20 weeks (Abel et al., 2009).

DMBA is a polycyclic aromatic hydrocarbon that creates the first genetic insult in the target cell, hypothesized to be epidermal stem cells residing in the basal layer (Parkinson, 1985; Yuspa and Poirier, 1988). Once DMBA enters the epidermal cells, it needs to be metabolized to elicit its procarcinogenic properties. Cytochrome P-450 oxidase enzymes metabolize DMBA into a variety of intermediates, such as epoxides, dihydrodiols, quinines, and phenols (Dipple et al., 1984b; Gelboin, 1980; Hall et al., 1990; Pelkonen and Nebert, 1982). The latter metabolites bind to DNA, forming adducts (DiGiovanni et al., 1984; Dipple et al., 1984a). This DNA-carcinogen interaction, where DMBA intercalates into the major groove of DNA, leads to many irreversible frameshift, transversion, and other point mutations, often leading to activating mutations affecting members of the *ras* family, which can confer a growth advantage to the cell (Yuspa et al., 1980; Yuspa et al., 1982).

Members of the *ras* family, including Harvey (Ha), Kirsten (Ki) and N-*ras*, are

Figure 1.4 Two-stage murine carcinogenesis protocol. In this model, papillomas are chemically induced with topical administration of an initiating carcinogen DMBA, followed by several repeated applications of TPA, a tumor promoter. Tumor initiation causes activation of the *Ha-ras* oncogene, whereas tumor promotion stimulates the PKC pathway. The AP-1 transcription factor is involved in the transformation of a benign papilloma to invasive SCC (see text for details).

Adapted from Foster-Hunt C., A thesis submitted to the Faculty of Graduate Studies and Research, University of Ottawa, 2007.


tumor initiation
tumor promotion
Ha-ras
PKC
tumor progression
Ap-1
DMBA
TPA
DMBA
TPA
wait 1 week
wait 1 week
repeat treatment for 6 weeks
wait 1 week
continue until end point
shave dorsal hair of mice
1st topical treatment of DMBA
twice weekly topical treatment of TPA
2nd topical treatment of DMBA
twice weekly topical treatment of TPA

frequently mutated in a number of human and animal tumors (Barbacid, 1987; Weinberg, 1985). The *ras* genes are members of the small GTP-binding proteins and are involved in a multitude of signal transduction pathways (Malumbres and Barbacid, 2003). Common point mutations in mammalian tumors are found in codons 12, 13 or 61, which renders Ras in a constitutively active GTP-bound form resulting in uncontrolled output signals (Balmain and Brown, 1988; Barbacid, 1987; Lowy and Willumsen, 1993; Sekiya et al., 1984). The *Ha-ras* oncogene has been identified as a primary DMBA target for the initiation stage of papilloma development (Ise et al., 2000; Pierceall et al., 1992; Rehman et al., 2000). Indeed, essentially all epithelial tumors initiated with DMBA harbor an A to T transversion in codon 61, causing Ha-ras to be in a permanently active state (Brown et al., 1990).

The DMBA-mediated activation of Ha-ras in epidermal cells is the initial stage in a multistep process of carcinogenesis induction. TPA is the most common tumor promoting agent used in the two-stage carcinogenesis protocol. It is a potent phorbol ester and, when applied topically, causes extensive cellular and morphological damage to the epidermis (DiGiovanni, 1992). Following TPA treatment, cells in the basal layer of the epidermis are stimulated to increase their mitotic activity causing an expansion of nucleated cell layers (Raick, 1973). This chemically-induced hyperplasia also causes an increase in keratinization of the upper layers of the epidermis (Bach and Goerttler, 1971; Balmain, 1976; Raick, 1973). Thus, subsequent to TPA treatment certain DMBA-induced mutated cells are prompted to undergo continuous clonal expansion leading to the formation of a papilloma (Kemp, 2005). TPA directly activates protein kinase C (PKC), a serine/threonine kinase that mediates multiple cellular responses, including

proliferation, and also induces RNA, DNA and protein synthesis, as well as increasing the production of growth and inflammatory factors, causing oxidative stress, oedema and hyperplasia of the epidermis (DiGiovanni, 1992). Unlike initiation, tumor promotion is a reversible step, and the epidermis is able to revert back to a normal phenotype within 2-3 weeks following TPA withdrawal (Raick, 1973).

Papillomas are typically generated as early as six weeks into treatment, however if TPA-mediated promotion continues past twenty weeks, papillomas can progress to SCC (DiGiovanni, 1992). There are several cellular changes that occur during this conversion, such as the acquisition of additional chromosomal abnormalities including trisomies of chromosomes 6 and 7, mutations in p53 (Aldaz et al., 1989; Ruggeri et al., 1991) and promotion of epithelial-mesenchymal transition (EMT) (Caulin et al., 1993; Chan et al., 2008; DiGiovanni, 1992; Navarro et al., 1991).

The transcription factors comprising activator protein 1 (AP-1) have been implicated in contributing to the conversion of a benign skin tumor to malignancy (Yuspa et al., 1991). AP-1 is a dimeric complex consisting of members of the Jun, Fos, activating transcription factor (Atf) and Maf protein families (Eferl and Wagner, 2003). Fos and Jun heterodimers are able to recognize and bind to TPA response elements (TREs) (Angel et al., 1987) and regulate the expression of genes that function to control cell differentiation, proliferation and apoptosis (Bowden et al., 1994). In addition, TPA has been shown to upregulate AP-1 target genes thus sustaining its role in tumor progression.

1.10 Retinoid Signaling in Skin

RA regulates several important cellular functions in normal and neoplastic skin, such as growth, differentiation and inflammation (Baron et al., 2005). For instance, when RA is topically applied to adult skin it causes hyperplasia and hyperproliferation of basal keratinocytes, which causes thickening and differentiation of the suprabasal layers (Fisher and Voorhees, 1996). Topical RA treatment also disrupts the cohesiveness of the stratum corneum causing mild dehydration of the adult skin due to water loss (Elias et al., 1981).

The epidermis expresses RARγ and RARα, as well as RXRα and RXRβ, with RARγ and RXRα as the predominant retinoid receptors (Fisher and Voorhees, 1996). Targeted gene disruption of RAR isoforms has elucidated the function of this retinoid receptor in the mouse epidermis. The epidermis of RARα-null mice appeared histologically normal, as did that of RARγ-null mice conditionally lacking RARα in keratinocytes (Chapellier et al., 2002). The skin of RARγ-null newborn mice appeared glossy, however was also histologically normal. Further studies demonstrated that both RARα and RARγ are dispensable for the homeostatic renewal of keratinocytes in the adult mouse, and given that these are the predominant retinoid receptors expressed in the epidermis, it was proposed that RA was not required for the self-renewal process of keratinocytes in the adult mouse epidermis (Calleja et al., 2006).

1.11 RA and RARs in Skin Cancer

RA and its synthetic derivatives have long been investigated as potential pharmacological agents in cancer therapy and prevention. Early small scale clinical trials had suggested that retinoids demonstrated therapeutic effects in treating cancers of the skin (Kraemer et al., 1988; Lippman et al., 1988; Peck et al., 1988), lungs (Pastorino et al., 1988), head and neck (Hong et al., 1990), and bladder (Studer et al., 1984). They had also shown promise in the treatment of preneoplastic disorders such as leukoplakia (Hong et al., 1986) and myelodysplastic syndromes (Besa et al., 1990). Acute promyelocytic leukemia (APL) however, is the only successful example where RA (in combination with chemotherapy) has been demonstrated to induce complete remission of a disease (Tallman et al., 1997). APL is the most common subtype of acute myeloid leukemia, and in almost all cases is characterized by the reciprocal translocation between the RARα gene on chromosome 17 and the PML gene on chromosome 15 (He et al., 1999). In the presence of physiological levels of RA, the RARα-PML fusion protein interacts strongly with co-repressor complexes such as SMART or N-CoR and recruits HDACs, causing transcriptional repression blocking the normal maturation and differentiation of promyelocytes (Grignani et al., 1998; He et al., 1998; Lin et al., 1998). Pharmacological levels of RA causes the dissociation of co-repressors from the fusion protein, permitting co-activator association and proper differentiation of the malignant cells resulting in remission of the disease (He et al., 1998; Lin et al., 1999). In spite of this, while treatment with RA and chemotherapy induces remission in the majority of APL cases, approximately 30% of patients relapse or become resistant to retreatment with RA (Soprano et al., 2004).

In regards to skin cancer, the exact mechanism of retinoid action is not known, however it is speculated to act by inhibiting the promotion step of tumor growth (Grubbs et al., 1990; Verma et al., 1983). One of the first studies suggesting a link between RA and cancer inhibition involved the two-stage murine carcinogenesis model, where topical administration of RA in conjunction with TPA inhibited the formation of both papillomas and carcinomas. However, when applied only after DMBA treatment, RA was unable to reduce tumor formation (Verma et al., 1980). Thus RA hinders the development of papillomas by inhibiting the TPA-mediated tumor promotion step (Verma et al., 1979).

In the therapeutic treatment of skin tumors, it is believed that retinoids are most effective when administered topically, intraperitoneally or orally (Bollag, 1971; Pawson et al., 1977; Verma and Boutwell, 1977). For instance, topical administration of RA has been correlated with the regression of cutaneous lesions of malignant melanoma (Levine and Meyskens, 1980) and of dysplastic nevi (Edwards and Jaffe, 1990). It is suggested that individuals suffering from xeroderma pigmentosum develop skin cancer at a reduced rate following oral administration of 13-cis-RA (Kraemer et al., 1988). However, in the early 1990s, two large-scale studies, the Alpha-Tocopherol Beta-Carotene Cancer Prevention (ATBC) Trial, and the Carotene and Retinol Efficacy Trial (CARET), investigated whether oral administration of α-tocopherol (a form of vitamin E) or β-carotene to individuals with a history of smoking or asbestos exposure could reduce the risk of lung cancer (Duffield-Lillico and Begg, 2004). Both the ATBC and CARET trials had to be terminated early due a significant increase in lung cancer and mortality incidences in experimental subjects compared to placebo (Duffield-Lillico and Begg,

2004; Omenn et al., 1996). Clinical trials have therefore been largely halted until we understand the basis of these negative outcomes.

Investigation of the expression levels of retinoid receptors during cancer progression have been used to determine if there was a correlation between malignancy and retinoid signaling (Xu et al., 2001). There have been several reports of altered or aberrant retinoid receptor expression in a number of cancers. For instance, in head and neck (Lotan et al., 1995), lung (Sun and Lotan, 2002) and esophageal cancers (Qiu et al., 1999) RARβ expression is decreased. In breast cancer, RARα and β are downregulated (Xu et al., 1997), and in prostate cancer RARβ and RXRβ are affected (Lotan et al., 2000). Most notably, a progressive decrease in RARα, RARγ, RXRα, and RXRβ expression in the epidermis is observed in the malignant progression from normal skin to SCC (Xu et al., 2001). In the two-stage murine carcinogenesis protocol, treatment with TPA causes marked decreases in RARα and RARγ expression in the epidermis (Marks and Furstenberger, 1990). In vitro studies indicate that keratinocytes transduced with *Ha-ras* exhibit a similar decrease in RARα and RARγ proteins (Darwiche et al., 1996) and RARαγ-null and RARγ-null keratinocytes are predisposed to Ras-induced tumorigenesis consistent with RARγ acting as a tumor suppressor in murine keratinocytes (Chen et al., 2004). Exogenous RA also inhibits the growth of transformed keratinocytes in culture primarily through RARγ, with a minor contribution by RARα (Goyette et al., 2000).

1.12 Screening for RA Target Genes in Keratinocytes

It is understood that RARs are involved in retinoid-mediated growth arrest in keratinocytes, and that RARγ is largely responsible for eliciting this effect, at least in the mouse (Goyette et al., 2000). Our group was interested in identifying target genes that could mediate the anti-tumorigenic effect of RA in transformed keratinocytes. In order to investigate the latter, a suppressive subtractive hybridization technique was conducted comparing genes induced by RA in wild type relative to RARαγ-null keratinocytes. Several candidate genes were revealed, such as *TransglutaminaseI (TgaseI)* and *TransglutaminaseII (TgaseII)*, which were already characterized as direct RA-target genes; *Gas3* was also recovered as a novel candidate RA-target gene. Northern blot analyses revealed that in wild type keratinocytes *gas3* expression was induced as early as 2 hours post-treatment with RA, whereas its expression was absent in RARαγ-null keratinocytes (Laforest, 2004). The identification of two partially redundant DR2 RARE motifs in the *gas3* locus, as well as ChIP studies indicating occupancy of this region of the promoter by RXR/RAR, further substantiated *gas3* as a direct RA target gene (Foster-Hunt 2007).

1.13 Growth Arrest Specific 3 (Gas3)

Growth arrest specific (gas3), also known as *peripheral myelin protein 22 (pmp22)* is a 22 kDa membrane bound tetraspan glycoprotein that was initially described as one of six genes induced by growth arrest in NIH 3T3 fibroblasts (Schneider et al., 1988). Gas3 is a 160 amino acid protein (Suter and Snipes, 1995) and is a member of the

Pmp22/epithelial membrane protein/eye lens-specific membrane protein 20/claudin (Pmp22/Emp/MP20/claudin) mammalian superfamily (Van Itallie and Anderson, 2006). Gas3 is present in both neural and non-neural tissues, and its expression is regulated by two tissue-specific promoters (Suter et al., 1994). *Gas3* expression in non-neural tissues is a result of use of the proximal promoter, while the distal promoter controls *gas3* expression in neural tissues (Bosse et al., 1994; Suter et al., 1994). Although the exact mechanism of action of *gas3* currently remains unclear, this complex transcriptional regulation suggests that it has distinct functions in neural and non-neural tissues (Suter et al., 1994).

1.14 Expression and Function of Gas3 in Neural Tissues

The highest level of *gas3* expression is found in the peripheral nervous system (PNS), in myelin-forming Schwann cells (Kuhn et al., 1993; Snipes et al., 1992). *Gas3* comprises approximately 2-5% of the total myelin in the PNS, confined mostly to compact myelin (Snipes et al., 1992; Spreyer et al., 1991; Welcher et al., 1991). There exist several autosomal-dominant hereditary peripheral neuropathies associated with *gas3* mutations, including Charcot-Marie-Tooth (CMT) disease, Dejerine-Sottas syndrome (DSS) and hereditary neuropathy with liability to pressure palsies (HNPP) (Suter and Snipes, 1995). Although differing in their level of severity, these diseases impact on myelin formation by Schwann cells, which affects both motor and sensory peripheral nerves (Bai et al., 2010). Individuals suffering from DSS and a small portion of CMT1A patients (a subtype of CMT and the most common of the inherited peripheral

neuropathies) carry a missense mutation in the second transmembrane domain of *gas3* (Suter et al., 1992) (Figure 1.5). The majority of CMT1A patients however, have acquired the disease as a result of a 1.5 megabase duplication on chromosome 17p11.2-p12, which includes the entire *gas3* locus (Lupski et al., 1991; Matsunami et al., 1992; Patel et al., 1992; Timmerman et al., 1992; Valentijn et al., 1992).

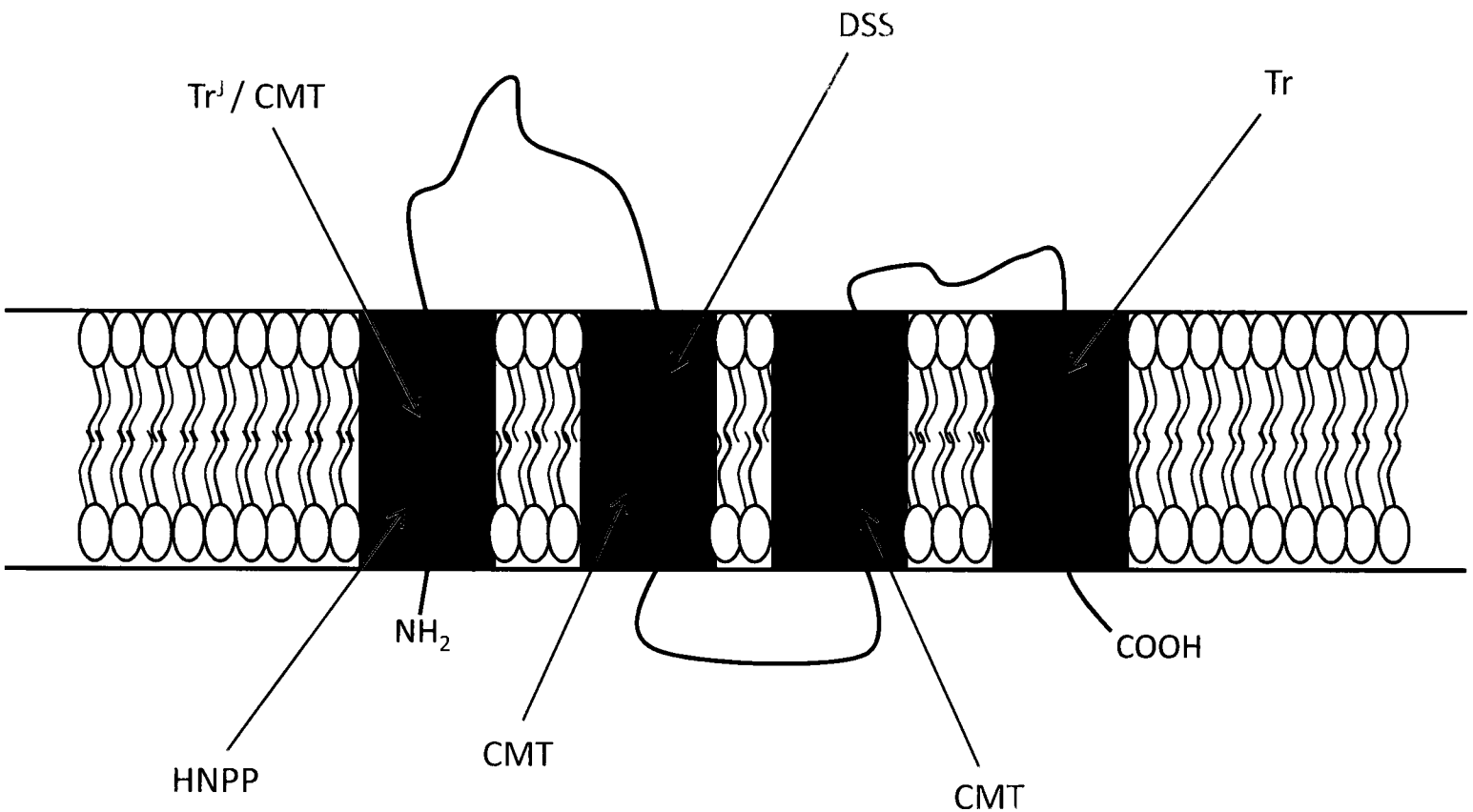
CMT1A is characterized by demyelination and remyelination of axons, as well as onion bulb formation (thin concentric swirls of myelin) (Dyck and Lambert, 1968; Robertson et al., 2002). The onset of this disease typically begins around 20 years of age, and patients often suffer from progressive muscle atrophy and weakness of the distal limbs (Brancolini et al., 1999). Mouse models have been extremely useful in understanding the pathogenesis of these inherited peripheral neuropathies. For example, the *Trembler (Tr)* and *Trembler-J (Tr-J)* mice carry missense mutations in the fourth and first transmembrane domains of *gas3*, respectively, and exhibit similar myelination defects as observed in human cases (Suter et al., 1992); in some cases of DSS and CMT1A patients present the same missense mutations associated with *Tr* and *Tr-J* mice (Roa et al., 1993a; Roa et al., 1993b; Roa et al., 1993c) (Figure 1.5).

1.15 Expression and Function of Gas3 in Non-neural Tissues

Given its abundant expression in Schwann cells, *gas3* has been implicated as a structural protein involved in the maintenance and formation of PNS myelin (Suter and Snipes, 1995). The exact function of *gas3* in non-neural tissues however, remains

Figure 1.5 Structure of *gas3* and associated mutations. *Gas3* is a small 160 amino acid membrane bound tetraspan glycoprotein and mutations of this gene have been associated with several autosomal dominant hereditary peripheral neuropathies. A number of patients suffering from Charcot-Marie-Tooth disease (CMT) harbor mutations in the first, second or third transmembrane domain. Mutations in the first and second domains have also been associated with hereditary neuropathy with liability to pressure palsies (HNPP) and Dejerine-Sottas syndrome (DSS), respectively. Similarly, *Trembler-J (Tr-J)* and *Trembler (Tr)* mouse models also carry mutations in the first and fourth transmembrane domains, respectively (see text for details).

Adapted from Suter U., and Snipes G.L., *Journal of Neuroscience Research*, 1995, 40: 145-151.


DSS
Tr
Tr / CMT
NH2
COOH
HNPP
CMT
CMT

unclear, and it is expressed in numerous tissues outside the PNS. For example, *gas3* is found in epithelial cells of the lungs and intestines (Baechner et al., 1995) and the liver, heart, choroid plexus and skin (Manzow et al., 1996; Notterpek et al., 2001; Roux et al., 2004; Taylor et al., 1995). Notably, gas3 mRNA is also detected in keratinocytes of both neonatal and adult mice (Manzow et al., 1996). Since *gas3* was initially identified in growth arrested NIH 3T3 cells, it has been postulated to play a role in cell cycle regulation (Schneider et al., 1988). In support of this, elevated expression of *gas3* retards the transition from G0/G1 to S phase of the cell cycle, implicating it as a potential regulator of cell proliferation (Zoidl et al., 1995). Furthermore, overexpression of *gas3* in cultured fibroblasts or Schwann cells can trigger cell spreading and apoptosis (Baudet et al., 1998; Brancolini et al., 1997; Fabbretti et al., 1995; Zoidl et al., 1997), potentially via impact on the Rho family of GTPases (Brancolini et al., 1999). The mechanism by which *gas3* elicits both cell death and cell spreading is not known, however it has been suggested that its exposure at the cell surface is required for its signaling activities (Brancolini et al., 2000).

1.16 Rationale

We have previously identified *gas3* as a direct retinoid target gene potentially mediating the anti-tumorigenic effects of RA (Laforest, 2004; Foster-Hunt, 2007). Furthermore, an intriguing relationship between *gas3* and skin tumorigenesis was revealed when its expression was shown to be reduced in chemically induced papillomas (Laforest, 2004). In this regard, *gas3*-null mice (on an FVB/NJ background) were

resistant to tumor formation elicited by the two-stage carcinogenesis protocol. In addition, only wild type mice were responsive to the growth inhibitory effects of RA (Foster-Hunt 2007). A pilot study conducted in the same context utilizing mice on a mixed FVB/NJ-C57Bl/6 background suggested that *gas3*-null mice exhibited a higher tumor burden relative to wild type controls (Foster-Hunt, 2007). Taken together, these results suggest that *gas3* may behave in a context dependent, strain-specific manner as both an oncogene and tumor suppressor.

1.17 Hypothesis and Objectives

I hypothesize that *gas3* is a RA-mediated tumor suppressor in the skin, with potential oncogenic properties. This will be addressed by (i) determining if RA-mediated growth inhibition in keratinocytes is dependent on *gas3*, (ii) utilizing the two-stage carcinogenesis protocol to compare the impact of Gas3 loss of function in FVB/NJ and C57Bl/6 backgrounds to determine if *gas3* impacts on tumorigenesis in a strain-specific manner, (iii) comparing the response of the epidermis of FVB/NJ and C57Bl/6 backgrounds to carcinogenic treatments to determine a cellular mechanism by which *gas3* impacts on epidermal tumorigenesis.

CHAPTER 2

Materials and Methods

2.1 Mice.

Wild type and *Pmp22(gas3)*-null mutants were obtained from *gas3*-heterozygous intercrosses (Adlkofer et al., 1995), through six backcrosses to either FVB/NJ or C57BL/6 backgrounds. *Gas3*-heterozygous mice were obtained from Ueli Suter (Institute of Cell Biology, Department of Biology, ETH Zurich, Switzerland).

2.1.1 Short term chemical treatments. Three different sets of treatments were conducted on wild type and *gas3*-null mice. First, the backs of FVB/NJ mice were shaved and treated with a single topical application of 200 nmol 7,12-dimethylbenz [a] anthracene (DMBA) (Sigma-Aldrich Canada Ltd) in 200µl of acetone (n=3 experimental, n=2 vehicle; acetone was used as control). A cohort of mice were sacrificed 24 hours later, and another subset of DMBA-treated mice were topically treated commencing one week later with two separate applications of 5nmol of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Sigma-Aldrich Canada Ltd) in 200µl of acetone per week for a total treatment of 3 weeks (n=3 experimental, n=3 vehicle). Finally, a set of mice were treated with TPA only, twice a week for a total of 2 weeks (n=3 experimental, n=2 vehicle).

Mice from a C57Bl/6 background were subjected to treatment with DMBA and TPA, and TPA only (n=3 experimental, n=3 vehicle in each case). The dorsal skin of all mice was excised 24 hours following the last treatment and either harvested for protein or fixed in 10% phosphate buffered formalin overnight and paraffin embedded for analyses.

2.1.2 Long term chemical mutagenesis. The two-stage murine carcinogenesis protocol as described in Owens et al., 1999, was conducted on mice of a C57Bl/6 background. The backs of wild type, *gas3*-heterozygous and *gas3*-null animals (n=13 for each

respective genotype) were shaved and treated with 200nmol DMBA. One week later, bi-weekly topical applications of 5nmol TPA were administered. At 6 weeks of treatment, a second application of 200nmol DMBA was administered, and TPA treatment continued for a total of 20 weeks. Twenty four hours following the last treatment, the tumors of each mouse were excised, fixed in 10% phosphate buffered formalin overnight and paraffin embedded for analyses, or snap frozen and stored at -80°C. Tumor incidence and size were monitored weekly throughout the course of the study.

2.2 Terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL). TUNEL analyses to identify apoptotic cells were conducted as per manufactures instructions using the ApopTag® Red *In Situ* Apoptosis Detection Kit (Chemicon® International). Double stranded DNA breaks were visualized using a Rhodamine-coupled anti-digoxigenin antibody and 4',6-diamidino-2-phenylindole (DAPI) used to visualize DNA; Apoptotic epidermal cells were identified by the merged images of the two signals.

2.3 Ki67 Immunohistochemistry. Skin sections were deparaffinized, subjected to antigen retrieval and endogenous peroxidase activity was blocked with 3% H_2O_2 in 1X Tris-buffered saline (TBS). Sections were blocked for one hour at room temperature in 4% bovine serum albumin (BSA), 10% sucrose and 1% fetal calf serum (FCS). Sections were then incubated overnight at 4°C with a rabbit polyclonal antibody against Ki67 (Abcam®) at a dilution of 1:100 in 4% BSA and 10% sucrose, followed by incubation

with a secondary antibody dilution of 1:1000 horseradish peroxidase-conjugated goat anti-rabbit IgG (Abcam®) for two hours at room temperature. Following several washes with TBS/0.1% Tween-20 (TBST), immunoreactivity was revealed using 2.4% diaminobenzidine (DAB), and slides were mounted using Permount medium (Fisher Scientific).

2.4 Maintenance of keratinocyte cell line. Keratinocyte cultures used in these studies were described previously (Goyette et al., 2000). Cells were cultured at 37°C with 5% carbon dioxide in air in S-minimal essential medium (S-MEM, Invitrogen™), supplemented with 10% chelex-treated FCS (Invitrogen™), 1% penicillin/streptomycin, 24μl/ml adenine sulfate, 0.5μM hydrocortisone, 1.5mM $MgCl_2$, 5μg/ml insulin, 1.2 x 10^{-11}M cholera toxin, 10ng/ml epidermal growth factor, 2mM L-Glutamine and 1mM sodium pyruvate.

2.5 RNAi transfections. siRNA targeting mouse *Pmp22* (*Gas3*) (M-042725) was purchased from Dharmacon (Thermo Scientific). To determine an optimal working concentration of siRNA that would provide maximal knockdown of *gas3* expression, 1 x 10^6 keratinocytes were plated in 10 cm tissue cultures dishes and transfected via Lipofectamine™ 2000 (Invitrogen™) with 200pmol, 100pmol, 50pmol, and 25pmol siRNA against Gas3, with an untransfected control. Cells were harvested 72 hours post-transfection and assessed for *gas3* expression via Northern Blot. Subsequent to this, to determine how long *gas3* knockdown persists, 5 x 10^4 keratinocytes were plated in 6-well

tissue culture dishes and transfected with 200pmol siGas3, RNA harvested every 24 hours over a 5 day period and *gas3* expression assessed via Northern Blot. A scrambled siRNA (Santa Cruz Biotechnology) was used as a negative control.

2.6 Northern Blot analyses. Total RNA was isolated from keratinocyte cultures using 1ml of Trizol® Reagent (Invitrogen™) per 1 x 10^7 cells as per the manufactures instructions and the RNA pellet resuspended in 100% deionized formamide and stored at -80°C. For Northern Blot analyses, 15μg of total RNA was run on a 1% agarose-formaldehyde gel in 1X 3-(N-morpholino) propanesulfonic acid (MOPS) based buffer solution (1M MOPS, 2M sodium acetate, 0.5M EDTA sodium salt) for 2 hours, and subsequently washed 3 times 20 minutes in water, followed by a 50 minutes wash in 10X saline-sodium citrate (SSC). RNA was transferred to a Hybond™-N membrane (Amersham Biosciences) overnight via capillary transfer using 10X SSC buffer, crosslinked via UV treatment and hybridized for 2 hours at 42°C in hybridization solution (40% formamide, 0.9M NaCl, 50mM sodium phosphate, 2mM EDTA, 4X Denhardts, 0.1% SDS) supplemented with 5mg/ml herring sperm DNA. Probes were generated using *gas3* or *β-actin* cDNA with the Rediprime™ Random Prime Labeling kit (Amersham Biosciences) and [α^{32}P]CTP (Perkin Elmer). Blots were hybridized overnight at 42°C with approximately 10^6 cpm denatured probe per ml of hybridization buffer, subsequently washed 3 times 30 minutes with 2X SSC, 0.1% SDS at 65°C and signal revealed by autoradiography using Kodak Maximum Sensitivity BioMax Film.

2.7 Tritiated Thymidine Incorporation Assay. 5 x 10^4 keratinocytes were plated in 6-well tissue culture dishes and transfected with 200pmol siGas3 or control scrambled siRNA. Seventy two hours post-transfection 10μM retinoic acid (Sigma-Aldrich Canada Ltd) or vehicle was added for 24 hours, and cells subsequently incubated with tritiated thymidine (Perkin Elmer) at a concentration of 2.5μCi/ml for 4 hours. Cells were washed with 1X ice cold phosphate buffered saline (PBS), 5% trichloroacetic acid (TCA), solublized with 400 μl 10M NaOH and thymidine incorporation read using a liquid scintillation counter (Wallac Win Spectral™).

2.8 Generation of Primary Mouse Embryonic Fibroblast (MEF) Cell Lines. Wild type and *gas3*-null mutants were generated from FVB/NJ *gas3*-heterozygous intercrosses. Embryonic day 16.5 fetuses were removed via caesarean section and genotype was determined by PCR using tail tip DNA from each embryo. Embryos were dissected in PBS containing 1% penicillin/streptomycin and limbs and internal organs were removed in order to isolate skin (epidermis and dermis). The skin was rinsed three times with Dulbecco's Modification Eagle's Medium (DMEM, Wisent Inc) without serum, minced and placed in 0.25% Trypsin, 1mM EDTA (Wisent Inc) at 37°C for 90 minutes with gentle rocking. Cell suspension was washed twice with DMEM containing 10% FBS, pelleted by centrifugation and cultured in DMEM, 10% FBS at 37°C with 5% CO_2. Media was changed daily until cells reached confluency. Cells were immortalized via serial passage.

2.9 Western Blot analysis.

2.9.1 Protein extraction from fresh tissue. Dorsal skin was excised, snap frozen in liquid nitrogen and crushed to a powder using a mortar and pestle. 1ml of RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 2mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (1μg/ml aprotinin, 1μg/ml leupeptin, 1mg/ml pepstatin A and 1mM PMSF) was added, and incubated on ice for 45 minutes. Samples were then homogenized using a Polytron PT 1200C (Kinematica Inc) twice for 15 seconds, centrifuged at maximum speed for 10 minutes at 4°C, and the supernatant used for protein analyses.

2.9.2 Protein extraction from cell cultures. 1 X 10^5 wild type and *gas3*-null MEFs were plated in 10cm tissue culture and cultured to approximately 70% confluency. Cells were then serum starved overnight in DMEM, re-stimulated with serum and harvested for protein at 5, 15, 30 and 60 minutes post serum stimulation. Cells were placed on ice, washed twice with ice cold 1X PBS, scraped with 1ml 1X PBS/1mM EDTA and cells recovered by centrifugation. Cells were then resuspended in 300μl of RIPA buffer containing protease inhibitors, placed on ice for 30 minutes, centrifuged at maximum speed for 2 minutes at 4°C and the supernatant used for protein analyses.

Protein lysates (50μg) were subjected to SDS-PAGE, the gels transferred for 1 hour to an Immobilon-P Transfer Membrane (Millipore) and subsequently blocked in 5% (w/v) non-fat milk in 1X TBST for 2 hours. Membranes were then incubated with gentle rocking overnight at 4°C with 1:1000 anti-phospho-IKKα/β (Cell Signaling Technology), 1:1000 anti-phospho-Smad2 (Cell Signaling Technology), 1:1000 anti-phospho-Akt (Cell

Signaling Technology), 1:250 anti-c-myc, 1:200 anti-p27 or 1:200 anti-p21 (Santa Cruz Biotechnology). Blots were then incubated with 1:5000 HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) and signal revealed with Western Lightning® Plus-ECL (Perkin Elmer) and autoradiography using Kodak Maximum Sensitivity BioMax Film.

2.10 Clonogenic Assays. Clonogenic assays were performed as previously described (Franken et al., 2006). Briefly, 50, 100, 500 or 1000 wild type or *gas3*-null MEFs were plated in 10cm tissue culture dishes and cultured as above grown for 3 weeks with medium changed every two days. Cells were then fixed and stained for 30 minutes using 6% glutaraldehyde and 0.5% crystal violet, rinsed thoroughly with water, dried overnight and macroscopic colonies counted.

CHAPTER 3

Results

3.1 RA-induced Growth Arrest in Keratinocytes is Mediated by *Gas3*

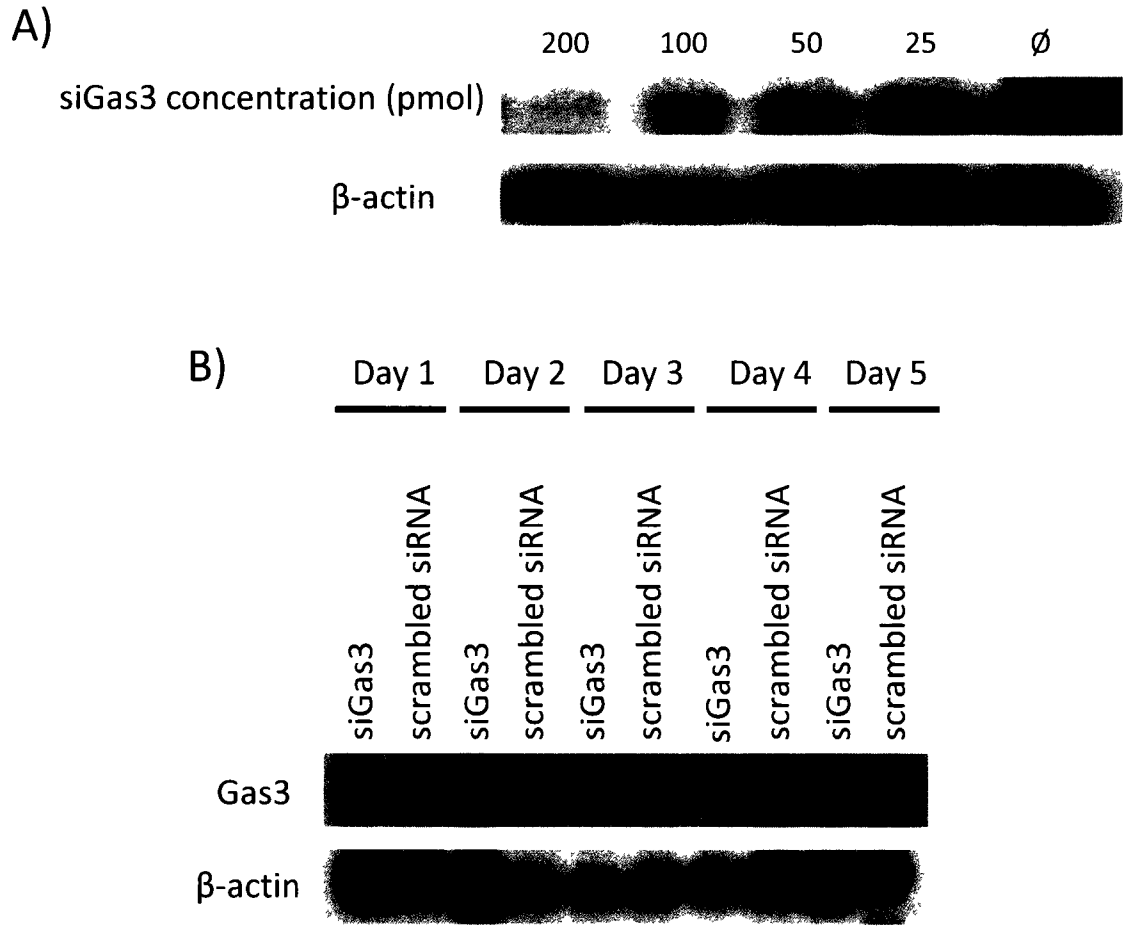
siRNA-mediated gene knockdown to transiently silence gas3 expression was used to determine if RA-induced growth inhibition in wild type keratinocytes required gas3. As such, this entailed transiently silencing *gas3* expression, and subsequently evaluating cell growth in the presence and absence of exogenous RA. RA administration was integral in our experimental design because (i) g*as3* is a direct RA-target gene, and its expression is induced following RA treatment (ii) administration of RA to wild type keratinocytes causes growth inhibition and (iii) Gas3 is associated with growth arrest.

As siRNA-mediated knockdown impacts on mRNA and protein levels, Northern and Western Blot analyses were conducted in order to evaluate *gas3* knockdown efficiency. However, the available antibodies for Gas3 were not suitable for Western Blot analyses, leading to uninterpretable results (data not shown). Alternatively, Northern Blot analyses for *gas3* mRNA were used.

Optimal conditions for knockdown were first established by testing a series of concentrations from 200pmol to 25pmol siRNA, with an untransfected control sample. In subsequent experiments, a non-targeting scrambled siRNA sequence was used as a control in order to ensure a more accurate interpretation of the effects. Seventy two hours post-transfection, RNA was extracted and used to assess *gas3* expression by Northern Blot. Results from this experiment suggested that optimal knockdown of *gas3* was seen at a concentration of 200pmol siRNA (Figure 3.1A).

With an optimal concentration established, a time course analysis was conducted. It was important to evaluate at what time points *gas3* knockdown occurred as well as

Figure 3.1 siRNA mediated knockdown of gas3 in keratinocytes. A) Northern blot analysis using total RNA (15μg) from keratinocytes cultures transfected with 200pmol, 100pmol, 50pmol and 25pmol of siGas3, with an untransfected control. The blot was probed with *gas3* cDNA and revealed 200pmol as the optimal concentration to achieve knockdown. β-actin was used as a loading control. B) A Northern blot analysis probed with *gas3* cDNA using total RNA (15μg) from wild type keratinocytes transfected with 200pmol siGas3 or control non-targetting scrambled siRNA shows significant knockdown of *gas3* expression at day 3 and 4. β-actin was used as a loading control.


A)
200
100
50
25
Ø
siGas3 concentration (pmol)
β-actin
B)
Day 1
Day 2
Day 3
Day 4
Day 5
siGas3
scrambled siRNA
siGas3
scrambled siRNA
siGas3
scrambled siRNA
siGas3
scrambled siRNA
siGas3
scrambled siRNA
Gas3
β-actin

when its expression returned to basal levels, as to provide a time frame in which experiments could be conducted where *gas3* expression is silenced. To this end, cells were transfected with 200pmol siGas3 or scrambled siRNA and harvested every 24 hours over 5 days and *gas3* expression assessed via Northern blot. Significant reduction was observed at day 3 to day 4 (Figure 3.1B). This indicated that experiments had to be conducted 3-4 days post-transfection.

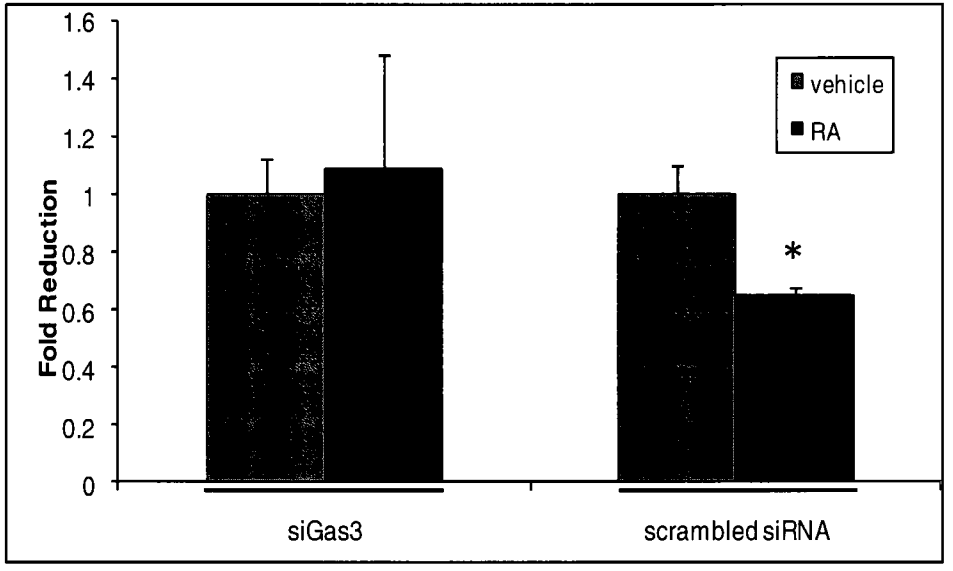
The main goal of this experiment was to determine if Gas3 is required for the growth inhibitory effects of RA in keratinocytes. To this end, experiments were conducted where the growth in the presence of RA in siGas3-treated keratinocytes was assessed using tritiated thymidine incorporation. Seventy-two hours post siRNA transfection, RA was added to cultures for 24 hours, cells were subsequently pulsed with radioactive tritiated thymidine for 4 hours and incorporation assessed as a measure of mitosis. Results indicated that RA was unable to induce growth arrest in siGas3-treated keratinocytes, whereas a 2 fold reduction in mitosis was seen in control samples (Figure 3.2A). In parallel, RNA extracts analyzed *via* Northern blot revealed that, although there is not complete knockdown of *gas3*, RA-mediated induction of *gas3* expression was seen only in cells transfected with scrambled siRNA (Figure 3.2B). Therefore, these findings suggest that RA-mediated growth inhibition of keratinocytes is mediated in part by gas3.

3.2 Evidence for Strain Modifers in Gas3-dependent Tumorigenesis

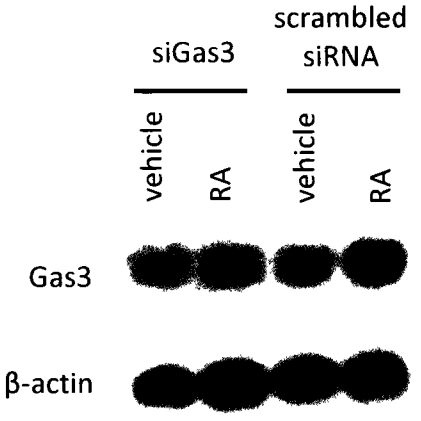
Our initial hypothesis proposing that *gas3* impacts on skin tumorigenesis stemmed from a study conducted on tumors from FVB/NJ mice that were subjected to the

Figure 3.2 siGas3-treated keratinocytes are refractory to RA-induced growth arrest. A) Three days post-transfection with siGas3 or scrambled siRNA, keratinocytes were treated with vehicle or RA (10μM) for 24 hours. Cell growth was evaluated using radioactive tritiated thymidine incorporation (2.5μCi/ml). B) A Northern blot analysis using total RNA (15ug) from lysates collected from B) were probed with *gas3* cDNA to validate knockdown. β-actin was used as a loading control. Error bars represent standard deviation of the mean. Asterisk denotes a statistically significant difference ($p<0.05$) from scrambled siRNA vehicle determined by Student's *t* test.

A)


1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Fold Reduction
vehicle
RA
*
siGas3
scrambled siRNA

B)


siGas3
scrambled siRNA
vehicle
RA
vehicle
RA
Gas3
β-actin

two-stage carcinogenesis protocol. *Gas3* expression in all tumors from these mice was severely diminished (Laforest, 2004). These results, together with the requirement for gas3 in RA-mediated growth inhibition, suggested that *gas3* acts as an RA-dependent tumor suppressor gene and therefore its loss may predispose mice to epidermal carcinogenesis, and/or attenuate the inhibitory effects of RA on tumor promotion. To assess this, the two-stage murine carcinogenesis protocol was used to compare tumor incidence between wild type, *gas3*-heterozygous and *gas3*-null mice treated with vehicle or RA; all animals were on a FVB/NJ background, a strain which has a high susceptibility to tumor formation. Results indicated that all wild type mice exhibited tumor development at the end of the study (Foster-Hunt, 2007). Furthermore, application of RA during promotion caused a delay in tumor onset and decrease in tumor burden in wild type animals (Foster-Hunt, 2007), as expected from past work (Sporn et al., 1976; Verma and Boutwell, 1977; Verma et al., 1979; Verma et al., 1980). *Gas3*-null animals, in contrast, exhibited essentially no tumors (Foster-Hunt, 2007). The latter observations seem counterintuitive to our earlier findings that *gas3* expression was lost in chemically induced papillomas.

A previously conducted pilot study where the two-stage carcinogenesis assay was performed on mice of a C57Bl/6-FVB/NJ mixed background, suggested that *gas3*-null mice exhibited an earlier onset of tumor formation and developed more tumors than wild type and *gas3*-heterozygous mice (Foster-Hunt, 2007). This lead to the hypothesis that *gas3* may impact on epidermal tumorigenesis in a strain-specific manner. The strain-specific differences in the impact of *gas3* status on tumor development may be due to modifier genes that differentially alter the expression and/or activity of gas3.

Based on the pilot study, we set out to further explore the issue of strain-specific modifiers and *gas3*-dependent tumorigenesis. To this end, the *gas3* null allele was placed on a C57Bl/6 background through six backcrosses and the two-stage carcinogenesis protocol repeated on wild type, *gas3*-heterozygous and *gas3*-null mice, with treatment conducted for a total of 20 weeks. Results from this study indicated that wild type mice began forming tumors at 11 weeks of treatment, with 69% of mice displaying an average of 2.6 tumors/mouse, 1.9 mm in size (Figures 3.3, 3.4A and 3.4B). *Gas3*-heterozygous mice exhibited a tumor onset at 10 weeks, with 85% developing tumors an average of 1 tumor/mouse and 2.7 mm in size (Figures 3.3, 3.4A and 3.4B). Similarly, *gas3*-null mice developed tumors at 10 weeks, with 85% of mice presenting an average burden of 1.6 tumors/mouse measuring 2.1 mm (Figures 3.3, 3.4A and 3.4B). We therefore did not observe any significant differences between tumor onset, burden or size between the three genotypes on a C57Bl/6 background (Figure 3.5).

Hematoxylin and eosin (H&E) staining of representative tumors collected from wild type, *gas3*-heterozygous and *gas3*-null C57Bl/6 mice revealed epidermal hypertrophy and hyperkeratosis (Figure 3.6) typical of papillomas induced by this protocol (DiGiovanni, 1992). All tumors appeared as pre-malignant lesions with no evidence of invasion through the basement membrane into the dermis.

Figure 3.3 Behavior of *gas3* in epidermal tumorigenesis (C57Bl/6 mice). Wild type, *gas3*-heterozygous and *gas3*-null mice of C57Bl/6 background were subjected to the two-stage carcinogenesis protocol and tumor onset and burden were monitored weekly.

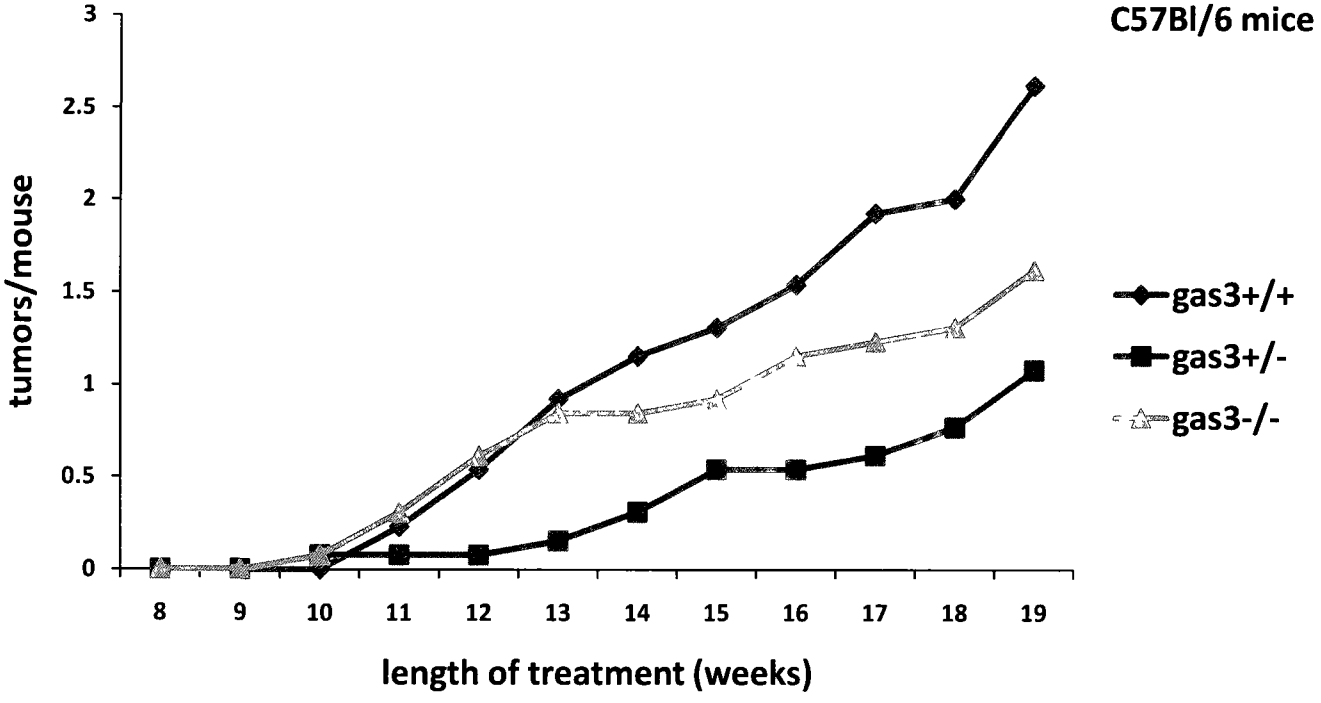

C57Bl/6 mice
tumors/mouse
3
2.5
2
1.5
1
0.5
0
8
9
10
11
12
13
14
15
16
17
18
19
length of treatment (weeks)
gas3+/+
gas3+/-
gas3-/-

Figure 3.4 Behavior of *gas3* in epidermal tumorigenesis (C57Bl/6 mice). The two-stage carcinogenesis protocol was conducted for a total of 20 weeks on mice of C57Bl/6 background. A) At the end point, 69% of wild type and 85% of *gas3*-heterozygous and *gas*-null mice, respectively, developed papillomas. B) Average tumor size at end point of study.

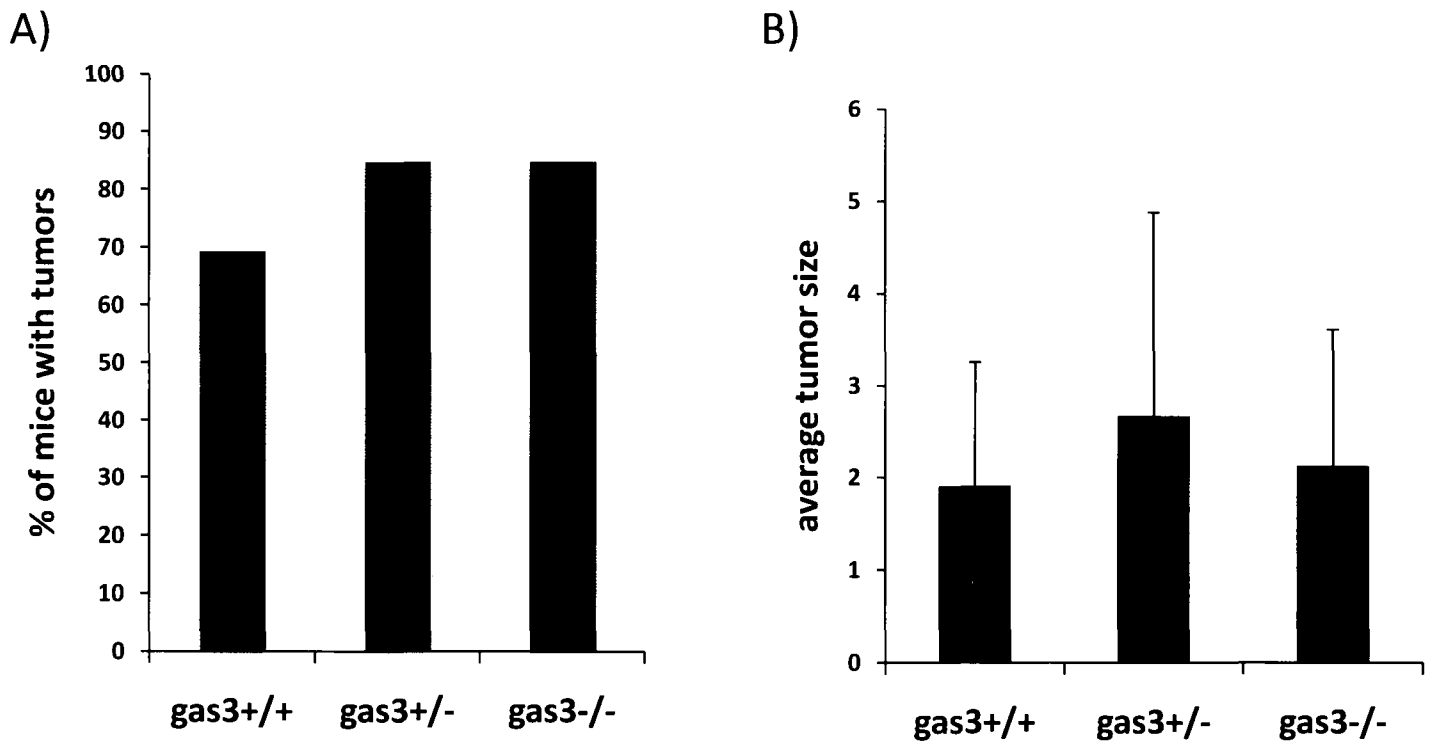
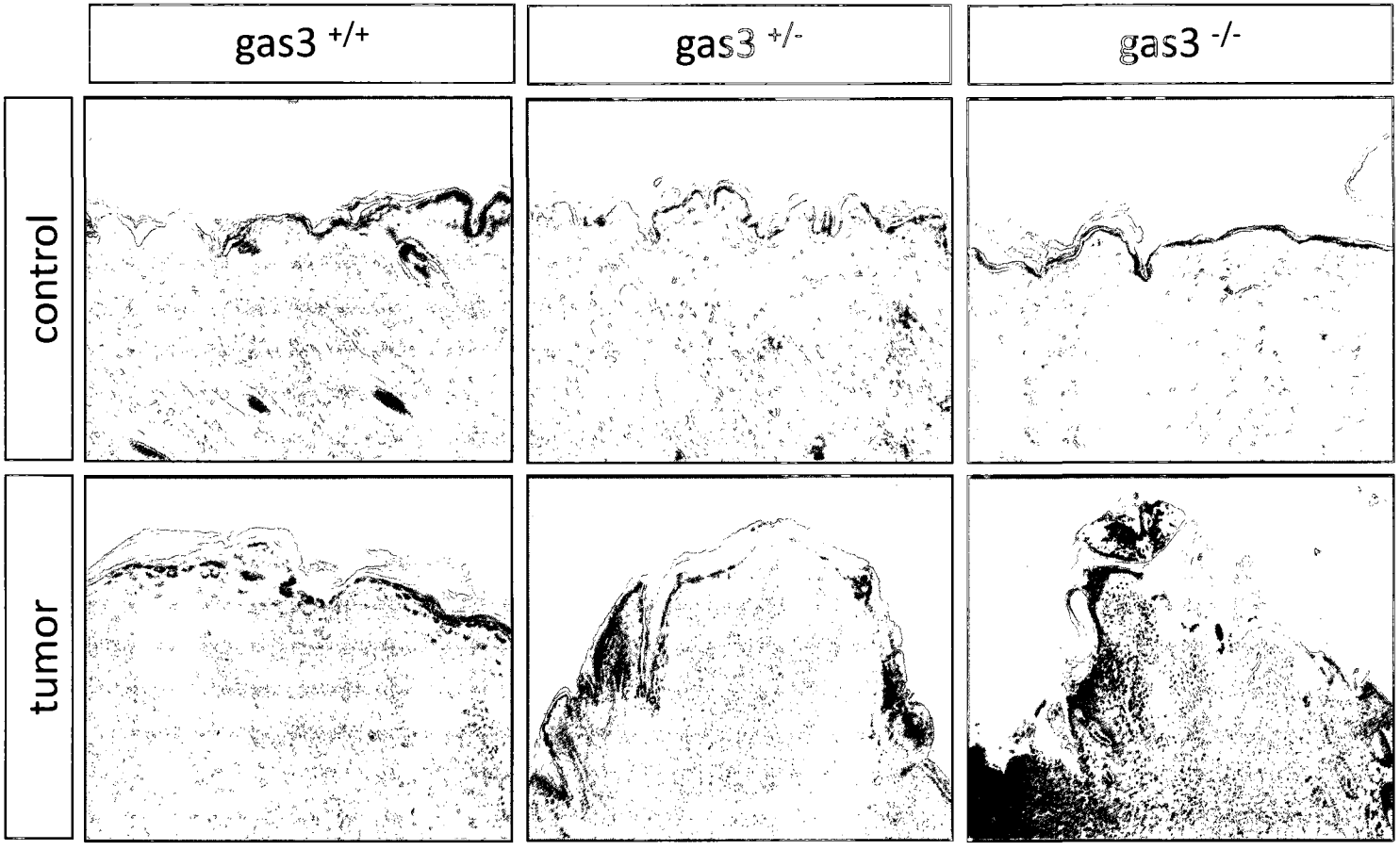

A)
% of mice with tumors
100
90
80
70
60
50
40
30
20
10
0
gas3+/+
gas3+/-
gas3-/-
B)
average tumor size
6
5
4
3
2
1
0
gas3+/+
gas3+/-
gas3-/-

Figure 3.5 Behavior of *gas3* in epidermal tumorigenesis (C57Bl/6 mice). Representative C57Bl/6 mice following completion of 20 week carcinogenesis study.

C57Bl/6 mice
gas3+/+
gas3+/-
gas3-/-

Figure 3.6 H&E staining of epidermal tumors. Representative tumors from wild type, *gas3*-heterozygous and *gas3*-null C57Bl/6 mice were collected at 20 weeks and subjected to H&E staining. All samples present as benign skin tumors and display massive epidermal hypertrophy and hyperkeratinization.


gas3 +/+
gas3 +/-
gas3 -/-
control
tumor

3.3 A Mechanism by which Gas3 impacts on Epidermal Tumorigenesis

The two-stage carcinogenesis study conducted on animals from an FVB/NJ background revealed that *gas3*-null mice evaded papilloma formation. In order to elucidate a potential mechanism leading to this outcome, we assessed proliferation and apoptosis of the epidermis of wild type and *gas3*-null mice following chemical mutagenesis. Three different sets of treatments were conducted; DMBA alone, DMBA and TPA, and TPA alone. This would allow us to infer which agent is responsible and at which stage of carcinogenesis (initiation or promotion) mechanistic changes are occurring that lead to the observed impact of gas3 status on tumor incidence.

In order to assess relative levels of proliferating and apoptotic cells, Ki67 and TUNEL analyses were conducted, respectively, comparing chemically treated skin of FVB/NJ wild type and *gas3*-null mice. First, a single topical application of DMBA was administered and a subset of these mice were sacrificed 24 hours later, while the other half were used for later treatment with TPA. The DMBA-treated mice were treated one week later with two separate applications of TPA per week for a total treatment period of 3 weeks. Finally, a set of mice were treated with TPA alone, twice a week for 2 weeks total. The dorsal skin was excised and utilized for analyses. For each sample, four arbitrary fields were chosen and the number of positive stained cells in the epidermis was recorded in a blind study.

Results from the Ki67 IHC revealed that wild type mice had an approximate two-fold increase in the number of proliferating cells in response to DMBA/TPA treatment compared to *gas3*-null mice (Figure 3.8A). Treatment with DMBA or TPA alone did not

lead to any differences with respect to proliferation between genotypes (Figures 3.7A and 3.9A). As wild type mice exhibited a high tumor burden relative to *gas3* mutants, this finding suggests that although a heightened proliferative response was exhibited by wild type mice in response to treatment with DMBA plus TPA, proliferation is not the primary mechanism that is causing the observed differences in tumor burden amongst the genotypes.

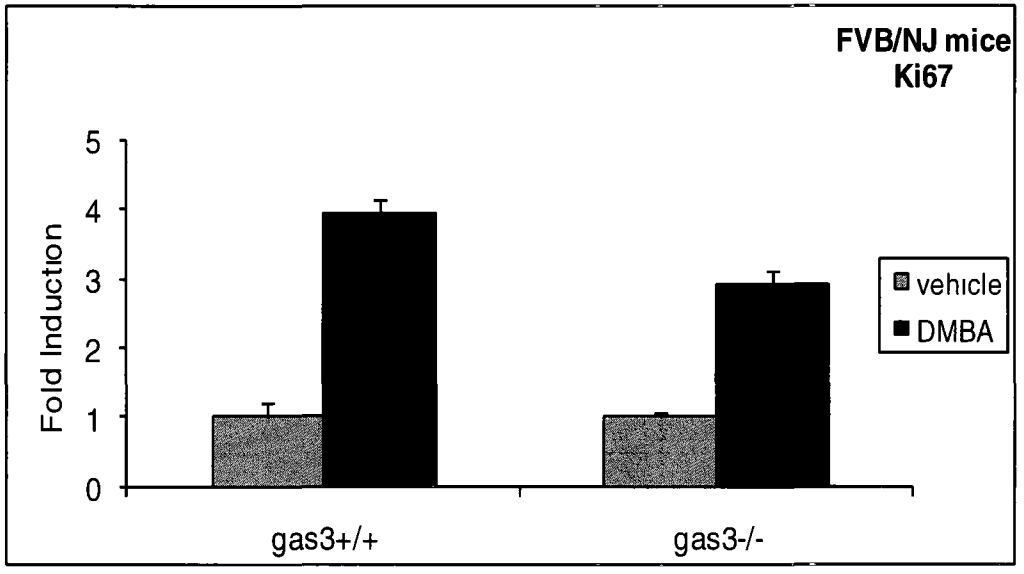
TUNEL data revealed that, in response to DMBA alone, and DMBA plus TPA, *gas3*-null mice exhibited approximately a two-fold increase in the number of apoptotic cells, while TPA treatment alone resulted in a three-fold increase relative to controls (Figures 3.10A, 3.11A and 3.12A). These results indicate that in response to all three treatments, *gas3*-null mice exhibit much higher apoptosis relative to wild type mice, and that this response commences at an early stage of carcinogenesis as depicted by the treatment with the initiating agent DMBA, and continues throughout subsequent treatments with TPA. This relative increase in apoptotic response suggests another possible basis by which gas3 mutants evade tumor formation.

The two-stage carcinogenesis study on C57Bl/6 mice did not reveal any differences in tumor onset, burden or size between genotypes, however to determine if apoptosis and/or proliferation also paralleled this outcome, we treated wild type and *gas3*-null mice with DMBA/TPA, or TPA alone, and conducted Ki67 and TUNEL analyses as per the FVB/NJ mice.

Ki67 IHC revealed that wild type and *gas3*-null mice on a C57Bl/6 background showed comparable changes in response to both treatments (Figure 3.13A and 3.14A).

Figure 3.7 Ki67 immunohistochemistry on DMBA treated skin (FVB/NJ mice). Wild type and *gas3*-null FVB/NJ mice were treated with a single dose of DMBA and sacrificed 24 hours later. Acetone was used as a control. A) Graphical representation of the number of Ki67 positive cells scored in the epidermis. B) Representative skin sections. Ki67 positive cells were identified as brown nuclear staining in the epidermis only. Error bars represent standard deviation of the mean.

A)


FVB/NJ mice
Ki67
Fold Induction
5
4
3
2
1
0
vehicle
DMBA
gas3+/+
gas3-/-

B)

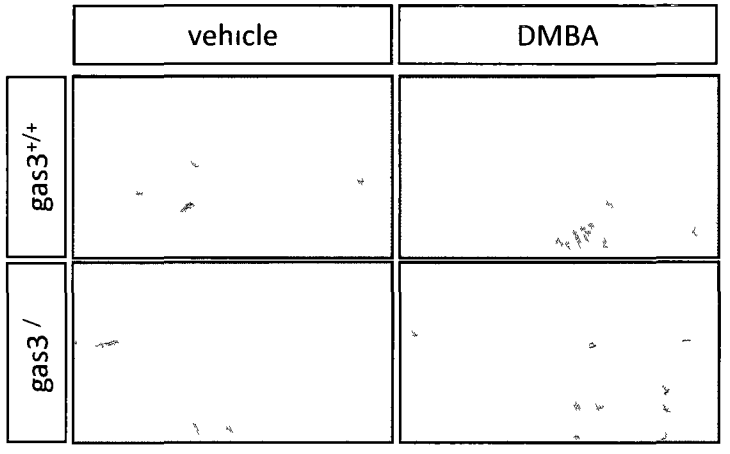
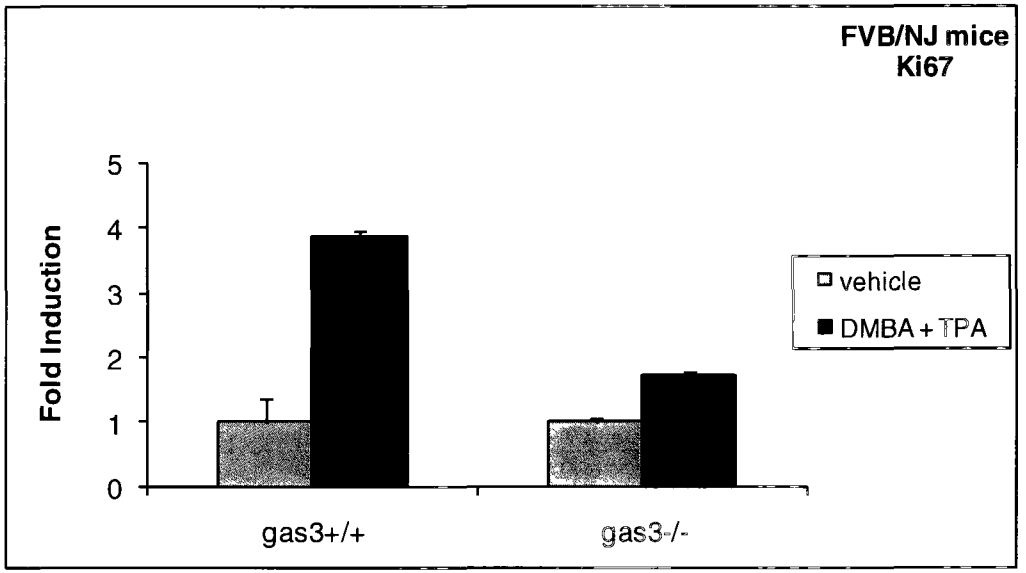

vehicle
DMBA
gas3+/+
gas3 /

Figure 3.8 Ki67 immunohistochemistry on DMBA/TPA treated skin (FVB/NJ mice). Wild type and *gas3*-null FVB/NJ mice were treated with DMBA, followed by two weekly TPA applications for a total treatment of 3 weeks. Acetone was used as a control. A) Graphical representation of the number of Ki67 positive cells scored in the epidermis. B) Representative skin sections. Ki67 positive cells were identified as brown nuclear staining in the epidermis only. Error bars represent standard deviation of the mean.

A)


FVB/NJ mice
Ki67
Fold Induction
5
4
3
2
1
0
vehicle
DMBA + TPA
gas3+/+
gas3-/-

B)

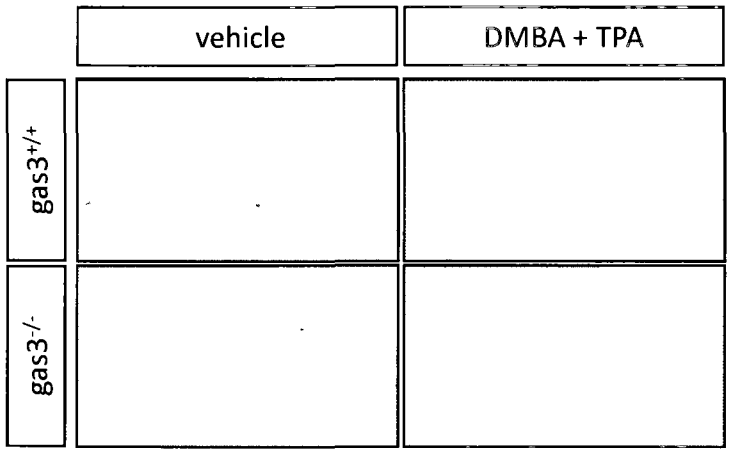
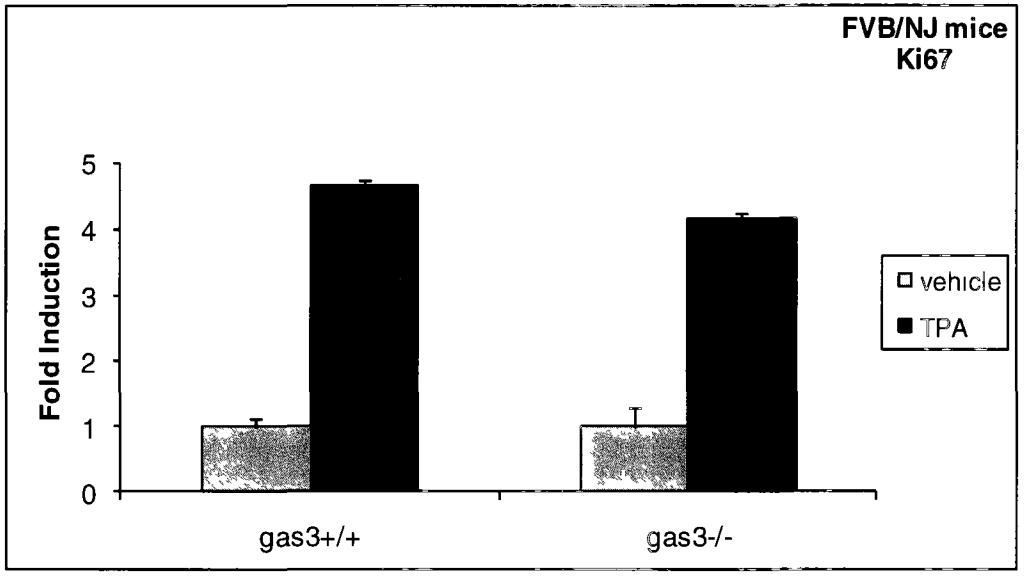

vehicle
DMBA + TPA
gas3+/+
gas3-/-

Figure 3.9 Ki67 immunohistochemistry on TPA treated skin (FVB/NJ mice). Wild type and *gas3*-null FVB/NJ mice were treated with two weekly TPA applications for a total treatment of 2 weeks. Acetone was used as a control. A) Graphical representation of the number of Ki67 positive cells scored in the epidermis. B) Representative skin sections. Ki67 positive cells were identified as brown nuclear staining in the epidermis only. Error bars represent standard deviation of the mean.

A)


FVB/NJ mice
Ki67
Fold Induction
5
4
3
2
1
0
vehicle
TPA
gas3+/+
gas3-/-

B)

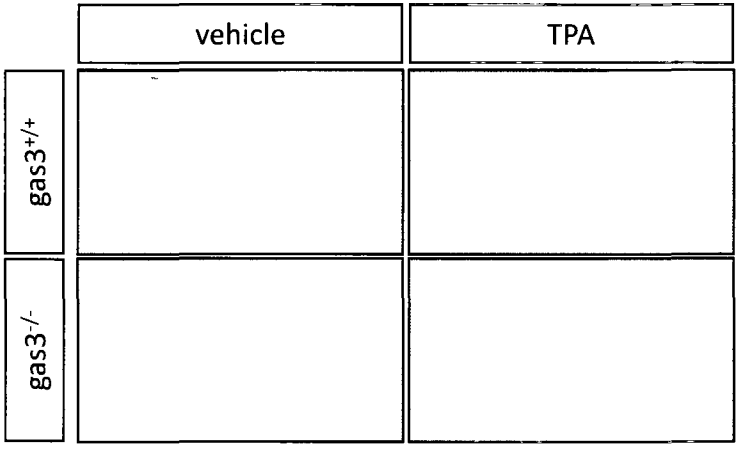
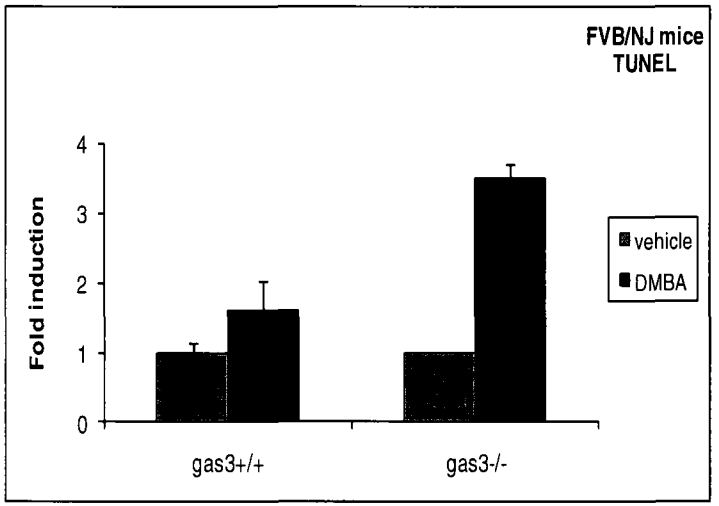

vehicle
TPA
gas3+/+
gas3-/-

Figure 3.10 TUNEL analyses on DMBA treated skin (FVB/NJ mice). Wild type and *gas3*-null FVB/NJ mice were treated with a single dose of DMBA and sacrificed 24 hours later. Acetone was used as a control. A) Graphical representation of the number of positive apoptotic cells scored in the epidermis. B) Representative skin sections. DAPI staining (blue) was used to counterstain nuclei; DNA fragmentation and stand breaks visualized using a rhodamine conjugated antibody (red); apoptotic cells represented in merged images (pink). Error bars represent standard deviation of the mean.

A)


FVB/NJ mice
TUNEL
Fold induction
4
3
2
1
0
vehicle
DMBA
gas3+/+
gas3-/-

B)

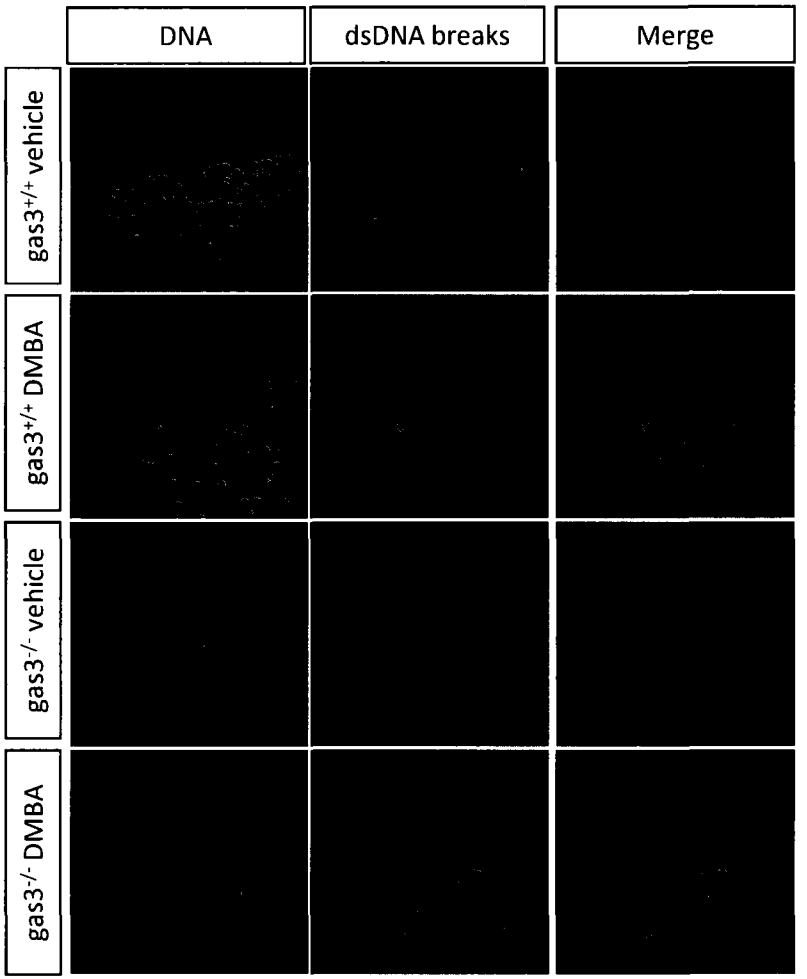
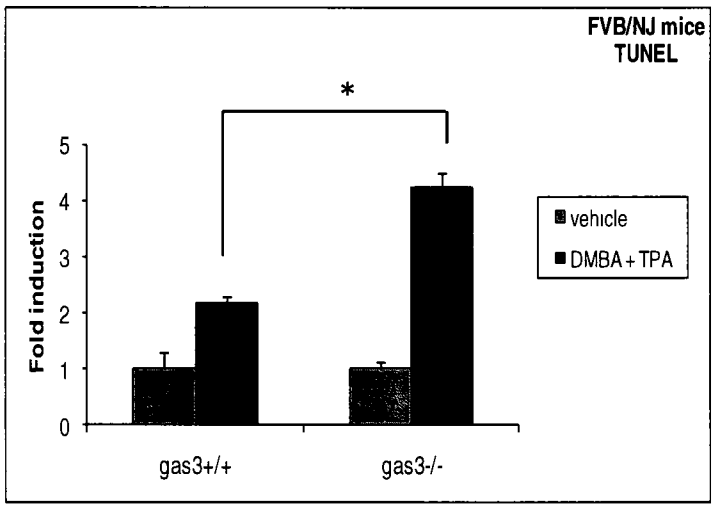

DNA
dsDNA breaks
Merge
gas3+/+ vehicle
gas3+/+ DMBA
gas3-/- vehicle
gas3-/- DMBA

Figure 3.11 TUNEL analyses on DMBA/TPA treated skin (FVB/NJ mice). Wild type and *gas3*-null FVB/NJ mice were treated with DMBA, followed by two weekly TPA applications for a total treatment of 3 weeks. Acetone was used as a control. A) Graphical representation of the number of positive apoptotic cells scored in the epidermis. B) Representative skin sections. DAPI staining (blue) was used to counterstain nuclei; DNA fragmentation and stand breaks visualized using a rhodamine conjugated antibody (red); apoptotic cells represented in merged images (pink). Error bars represent standard deviation of the mean. Asterisk denotes a statistically significant difference ($p<0.05$) determined by Student's *t* test.

A)


FVB/NJ mice
TUNEL
*
Fold induction
5
4
3
2
1
0
vehicle
DMBA + TPA
gas3+/+
gas3-/-

B)

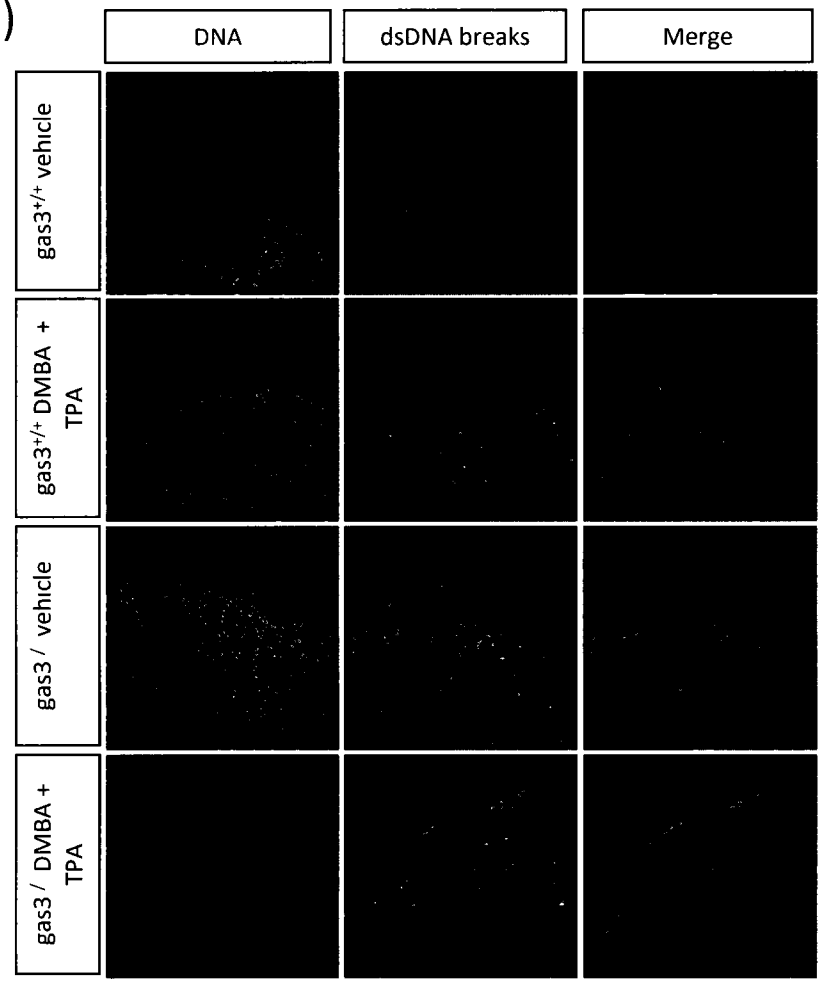
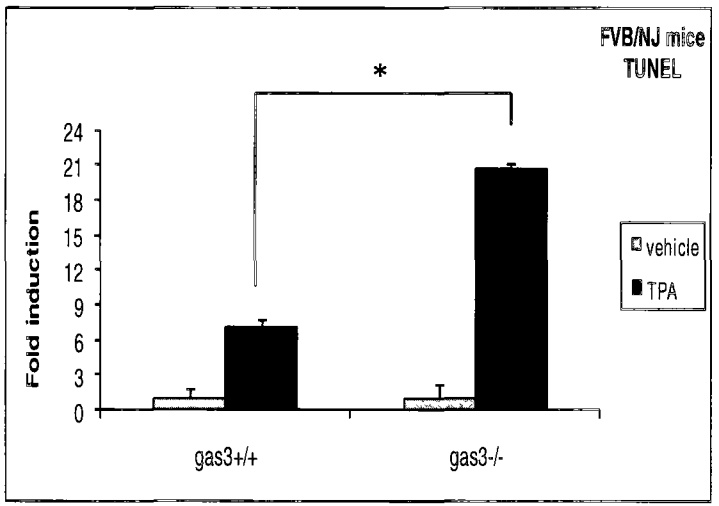

DNA
dsDNA breaks
Merge
gas3+/+ vehicle
gas3+/+ DMBA + TPA
gas3 / vehicle
gas3 / DMBA + TPA

Figure 3.12 TUNEL analyses on TPA treated skin (FVB/NJ mice). Wild type and *gas3*-null FVB/NJ mice were treated with two weekly TPA applications for a total treatment of 2 weeks. Acetone was used as a control. A) Graphical representation of the number of positive apoptotic cells scored in the epidermis. B) Representative skin sections. DAPI staining (blue) was used to counterstain nuclei; DNA fragmentation and stand breaks visualized using a rhodamine conjugated antibody (red); apoptotic cells represented in merged images (pink). Error bars represent standard deviation of the mean. Asterisk denotes a statistically significant difference ($p<0.05$) determined by Student's *t* test.

A)


FVB/NJ mice
TUNEL
*
Fold induction
24
21
18
15
12
9
6
3
0
vehicle
TPA
gas3+/+
gas3-/-

B)

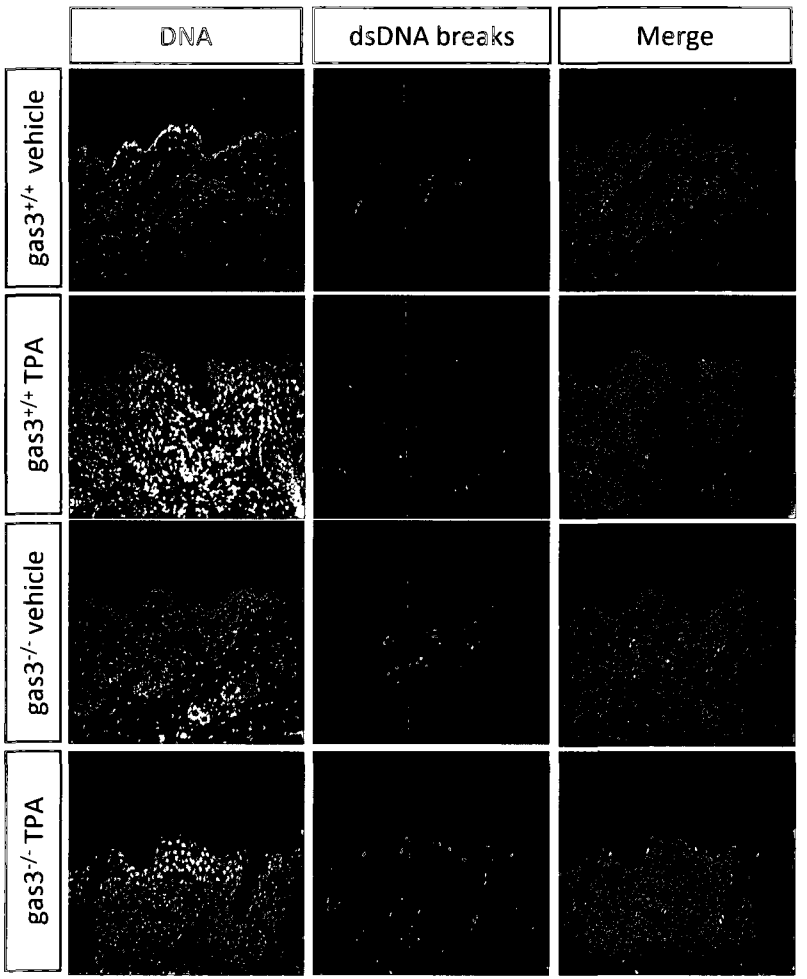
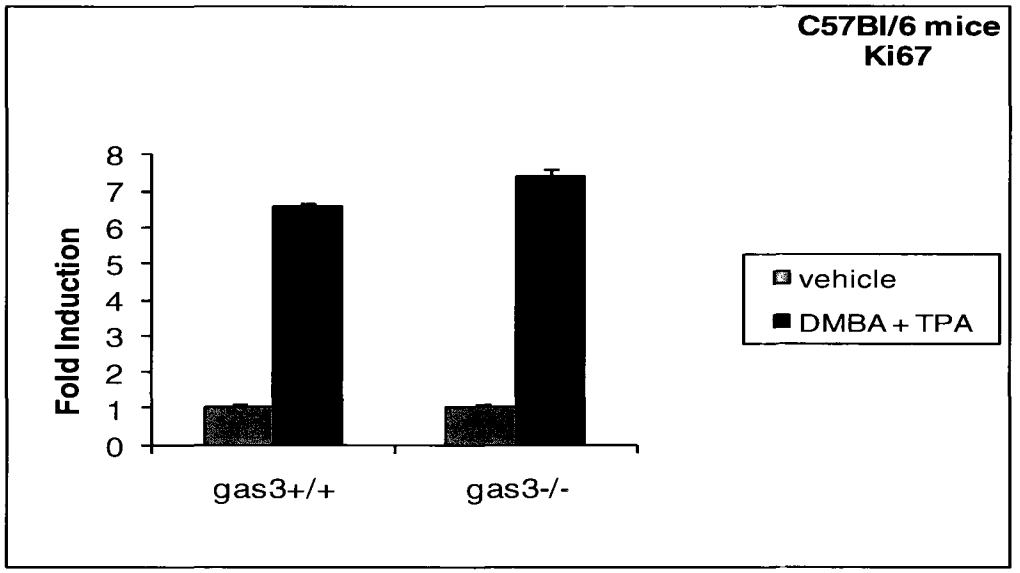

DNA
dsDNA breaks
Merge
gas3+/+ vehicle
gas3+/+ TPA
gas3-/- vehicle
gas3-/- TPA

Figure 3.13 Ki67 immunohistochemistry on DMBA/TPA treated skin (C57Bl/6 mice). Wild type and *gas3*-null C57Bl/6 mice were treated with DMBA, followed by two weekly TPA applications for a total treatment of 3 weeks. Acetone was used as a control. A) Graphical representation of the number of Ki67 positive cells scored in the epidermis. B) Representative skin sections. Ki67 positive cells were identified as brown nuclear staining in the epidermis only. Error bars represent standard deviation of the mean.

A)


C57Bl/6 mice
Ki67
Fold Induction
8
7
6
5
4
3
2
1
0
gas3+/+
gas3-/-
vehicle
DMBA + TPA

B)

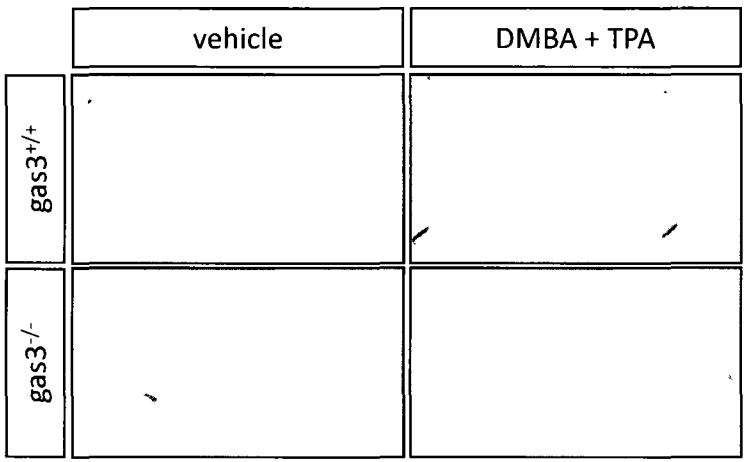
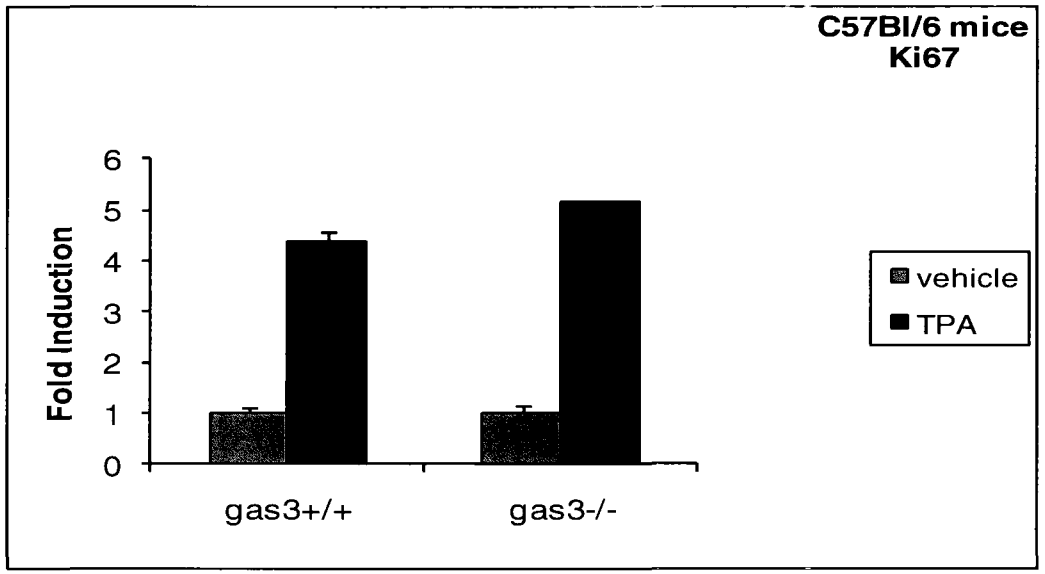
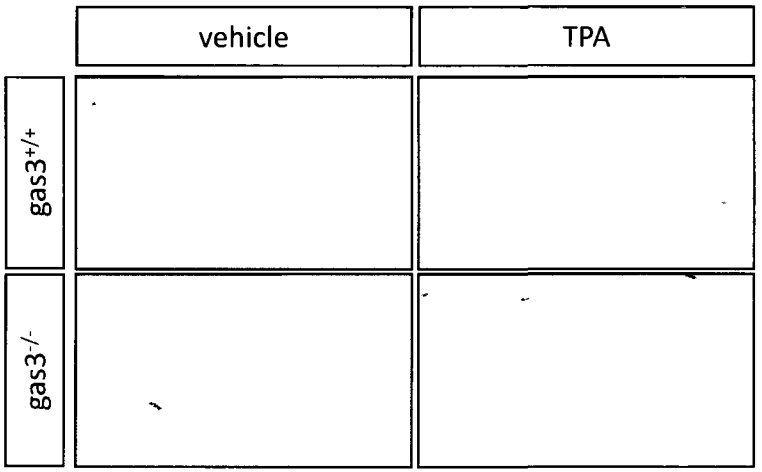

vehicle
DMBA + TPA
gas3+/+
gas3-/-

Figure 3.14 Ki67 immunohistochemistry on TPA treated skin (C57Bl/6 mice). Wild type and *gas3*-null C57Bl/6 mice were treated with two weekly TPA applications for a total treatment of 2 weeks. Acetone was used as a control. A) Graphical representation of the number of Ki67 positive cells scored in the epidermis. Ki67 positive cells were identified as brown nuclear staining in the epidermis only. B) Representative skin sections. Ki67 positive cells were identified as brown nuclear staining in the epidermis only. Error bars represent standard deviation of the mean.

A)


C57Bl/6 mice
Ki67
Fold Induction
6
5
4
3
2
1
0
gas3+/+
gas3-/-
vehicle
TPA

B)


vehicle
TPA
gas3+/+
gas3-/-

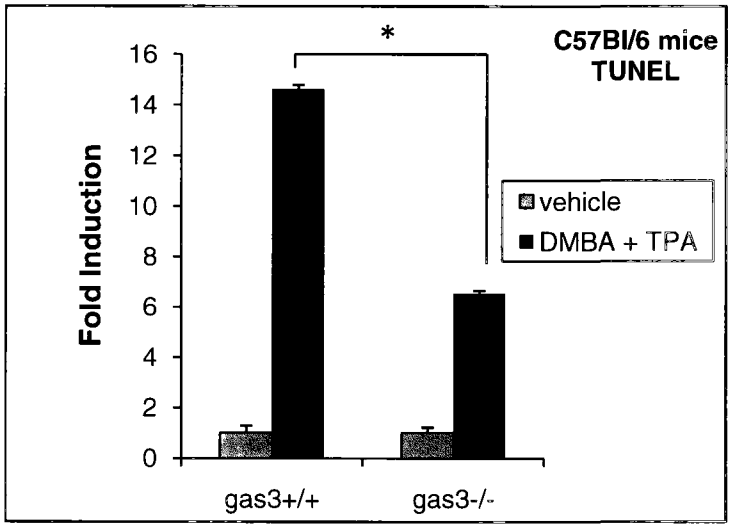
However, TUNEL analyses showed that, in response to DMBA/TPA treatment, wild type mice had a two-fold increase in the number of apoptotic cells compared to *gas3*-null mice (Figure 3.15A). Treatment with TPA alone did not lead to any difference in apoptosis between genotypes (Figure 3.16A). While there seems to be a two-fold increase in apoptosis observed in DMBA/TPA treated skin of wild type mice, it does not reflect the results from the two-stage carcinogenesis study as tumor formation did not differ between genotypes. It is possible that following DMBA/TPA treatments wild type mice attempted to eliminate the mutagenized cells, however the response was not adequate enough and resulted in the accumulation of damaged cells which developed into tumors. Overall, these results do not reveal any significant changes occurring between C57Bl/6 wild type and *gas3*-null mice in response to DMBA or TPA. In this regard, the latter results support our C57Bl/6 long term tumorigenesis study where there was no observed difference with tumor burden, onset or size across genotypes. Additionally, the FVB/NJ carcinogenesis study revealed that *gas3*-null animals displayed no tumors, and the TUNEL results illuminate that these animals experience a heightened apoptotic response, relative to wild type mice, reflecting their lack of tumor burden. Overall, the Ki67 and TUNEL data collected from both FVB/NJ and C57Bl/6 studies offers a mechanistic basis reflecting the different tumor phenotypes present in both strains.

3.4 Cellular Pathways affected by Gas3

There are several well documented signaling pathways that are known to be deregulated in, and contribute to, skin cancers. We focused on three such pathways: Akt,

Figure 3.15 TUNEL analyses on DMBA/TPA treated skin (C57Bl/6 mice). Wild type and *gas3*-null C57Bl/6 mice were treated with DMBA, followed by two weekly TPA applications for a total treatment of 3 weeks. Acetone was used as a control. A) Graphical representation of the number of positive apoptotic cells scored in the epidermis. B) Representative skin sections. DAPI staining (blue) was used to counterstain nuclei; DNA fragmentation and stand breaks visualized using a rhodamine conjugated antibody (red); apoptotic cells represented in merged images (pink). Error bars represent standard deviation of the mean. Asterisk denotes a statistically significant difference ($p<0.05$) determined by Student's *t* test.

A)


C57Bl/6 mice
TUNEL
Fold Induction
16
14
12
10
8
6
4
2
0
*
vehicle
DMBA + TPA
gas3+/+
gas3-/-

B)

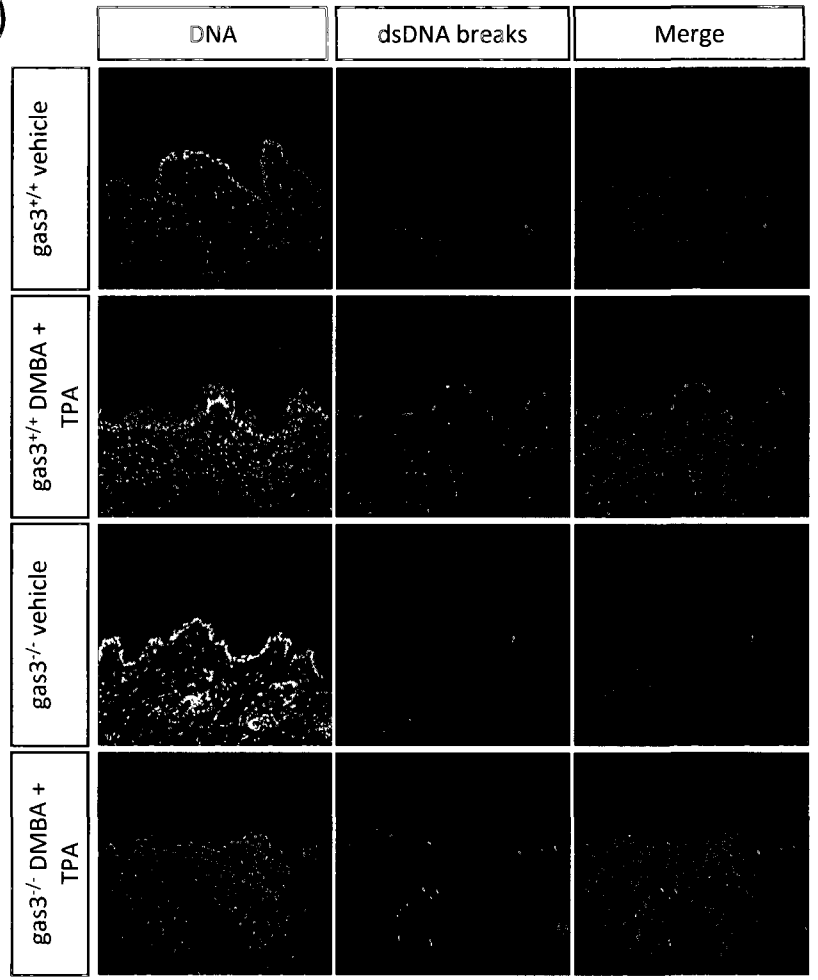
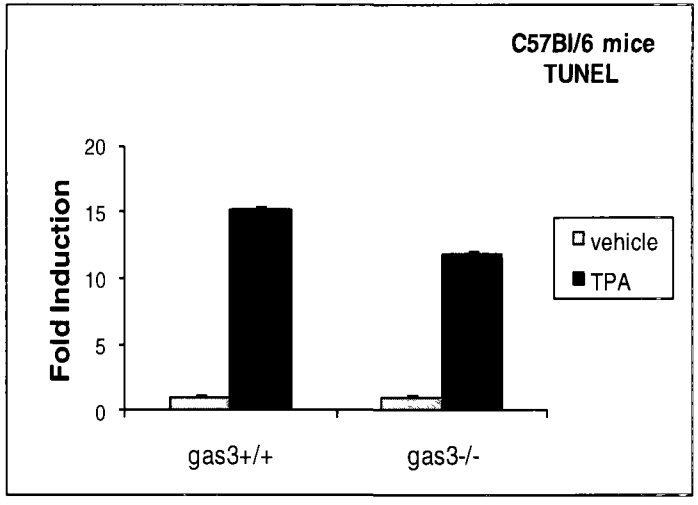
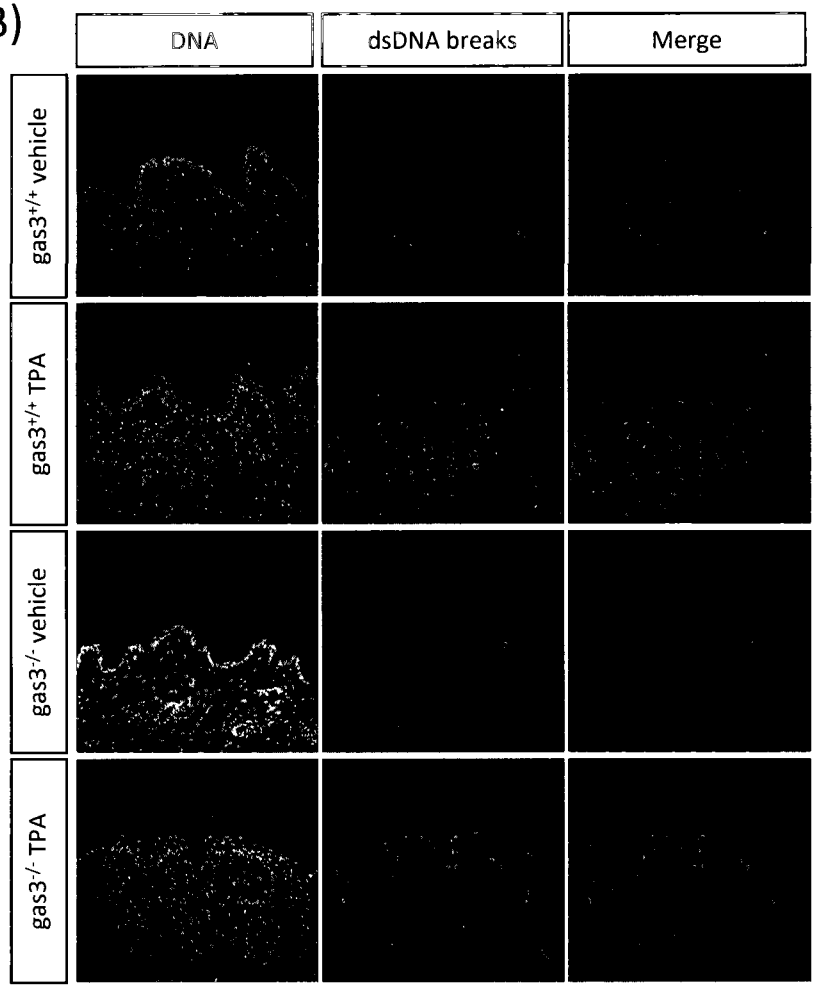

DNA
dsDNA breaks
Merge
gas3+/+ vehicle
gas3+/+ DMBA + TPA
gas3-/- vehicle
gas3-/- DMBA + TPA

Figure 3.16 TUNEL analyses on TPA treated skin (C57Bl/6 mice). Wild type and *gas3*-null C57Bl/6 mice were treated with two weekly TPA applications for a total treatment of 2 weeks. Acetone was used as a control. A) Graphical representation of the number of positive apoptotic cells scored in the epidermis. No significant difference is seen between wild type and *gas3*-null mice. B) Representative skin sections. DAPI staining (blue) was used to counterstain nuclei; DNA fragmentation and stand breaks visualized using a rhodamine conjugated antibody (red); apoptotic cells represented in merged images (pink). Error bars represent standard deviation of the mean.

A)


C57Bl/6 mice
TUNEL
Fold Induction
20
15
10
5
0
vehicle
TPA
gas3+/+
gas3-/-

B)


DNA
dsDNA breaks
Merge
gas3+/+ vehicle
gas3+/+ TPA
gas3-/- vehicle
gas3-/- TPA

transforming growth factor β (TGF-β), and nuclear factor κB (NF-κB). In order to investigate whether these cellular pathways are affected by *gas3*, we performed Western Blot analyses using protein extracts from DMBA, DMBA/TPA and TPA treated skin from wild type and *gas3*-null mice on a FVB/NJ background.

Akt is activated via extracellular signals that induce phosphatidylinositol 3-kinase (PI3K). PI3K activation leads to the phosphorylation of phosphoinositides (PIs) to generate PI(3,4,5)-triphosphate (PIP3) which recruits Akt to the plasma membrane where it is phosphorylated and activated (Skeen et al., 2006). Akt is a serine/threonine kinase involved in cell proliferation and survival (Chan et al., 1999; Stephens et al., 1998), and elevated Akt activity has been suggested to play an important role in skin tumor promotion, as well as contributing to tumorigenesis by inhibiting apoptosis (Lu et al., 2007).

The TGF-β signaling cascade commences when TGF-β ligands bind to the membrane-bound kinase type I and type II TGF-β receptors (TGF-βRI and TGF-βRII) complex. Upon ligand binding, TGF-βRI-TGF-βRII phosphorylates Smad2/3, which then associates with Smad4 forming a heterodimeric complex which then translocates to nucleus to activate TGF-β responsive genes (Li et al., 2005a). TGF-β has several regulatory roles including cell proliferation, differentiation, survival, adhesion, as well as maintaining normal tissue homeostasis. In addition, TGF-β possesses both growth suppressive and pro-oncogenic properties with respect to cancer pathogenesis; at early stages of carcinogenesis, TGF-β inhibits growth and induces apoptosis, however pathological forms of TGF-β have been shown to promote cell survival, immune

suppression, tumor cell invasion and metastasis (Massague, 2008; Roberts and Wakefield, 2003).

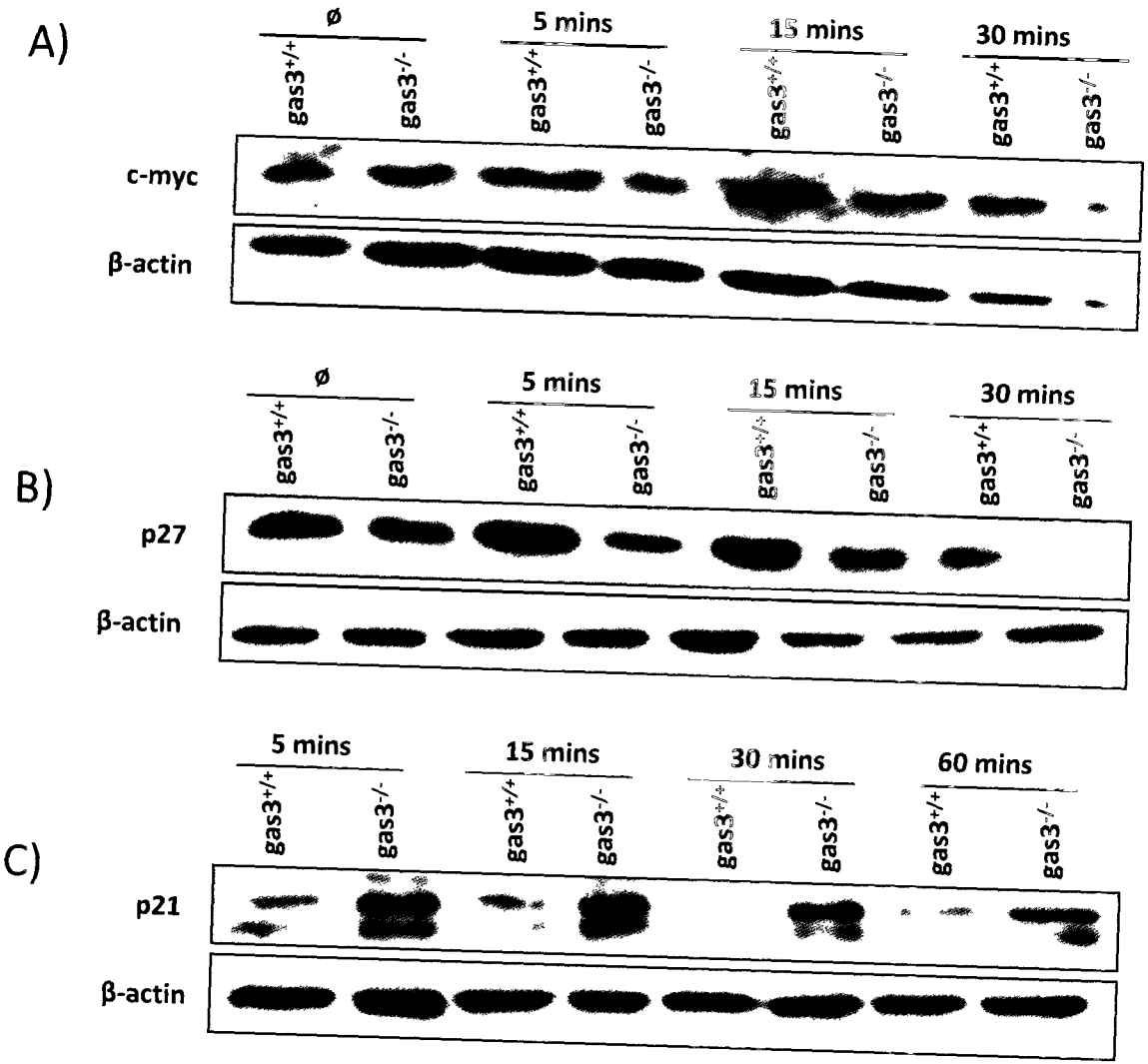
NF-κB transcription factors exist in the cytosol in an inactive state sequestered by inhibitors (IκB). Upon activation of this pathway by a plethora of cellular stresses, phosphorylation of the IκB kinase (IKK) complex, consisting of IKKα, -β and –γ, activates its kinase activity which in turn leads to the phosphorylation-induced degradation of IκB, releasing NF-κB and allowing it to translocate into the nucleus to regulate gene activity (DiDonato et al., 1997; Mercurio et al., 1997; Rothwarf et al., 1998; Zandi et al., 1997). NF-κB is a transcription factor that plays a prominent role in carcinogenesis as it promotes cell proliferation, regulates apoptosis, neoangiogenesis and can promote invasion and metastasis (Lee et al., 2007). In addition, IκB kinase α (IKKα) one of the two catalytic subunits involved in NF-κB activation, has been shown to play a critical role in the suppression of squamous cell carcinogenesis (Descargues et al., 2008). In order to test for Akt, TGF-β and NF-kB activation, we assessed expression levels of phosphorylated-Akt, phosphorylated-Smad2 and phosphorylated-IKKα, respectively

With the Western Blot analyses, our objective was to gain insight that would permit us to better comprehend how Gas3 impacts on epidermal tumorigenesis. When comparing DMBA treated skin of wild type and *gas3*-null mice, Western Blots probed for phosphorylated-Akt, phosphorylated-Smad2 and phosphorylated-IKKα did not reveal any observable differences (data not shown). Similar results were obtained when comparing wild type and *gas3*-null skin treated with DMBA/TPA and TPA, respectively (data not shown). The Akt, TGF-β or NF-κB pathways therefore do not appear to be influenced by *gas3* in skin tumorigenesis.

To study the effects of *gas3* loss in tissue culture models, we attempted to establish a *gas3*-null primary keratinocyte line, but were unsuccessful. As a corollary we established three *gas3*-null and wild type FVB/NJ mouse embryonic fibroblast (MEF) cell lines. This cell type seemed appropriate given that *gas3* was initially identified in growth arrested NIH 3T3 fibroblasts (Schneider et al., 1988). We conducted clonogenic assays in order to elucidate the impact of *gas3* on events related to cellular growth. Initial results indicated that *gas3*-null MEFs were growing faster and forming more colonies than wild type MEFs (data not shown), however this was not consistent throughout subsequent clonogenic assays, and as such we attribute this property to being specific to individual cell lines.

Given that *gas3* has been implicated in cell cycle regulation we conducted Western Blot analyses investigating whether the expression levels of cell cycle regulators, such as *c-myc*, p27 and p21, were affected by *gas3* loss. C-myc plays a crucial role in facilitating cell cycle entry and exit, as well as mediating several signal transduction pathways involved cell proliferation (Marcu et al., 1992). p27 and p21 are G1 cyclin-cyclin dependent kinase (CDK) inhibitors and function by halting cell cycle progression by blocking the kinase activity of CDKs (Abukhdeir and Park, 2008). To this end, we subjected MEFs to serum starvation overnight, and extracted for protein at 5, 15, 30 and 60 minutes following re-stimulation with serum. Preliminary Western Blot analyses indicated that gas3 status did not impact on *c-myc* or p27 expression levels (Figures 3.17A and 3.17B). However, p21 expression seemed to be increased at all time points in *gas3*-null MEFs relative to wild type (Figure 3.17C), suggesting that loss of gas3 induces expression of p21. Since p21 is involved in halting G1/S progression of the

Figure 3.17 Cell cycle regulators affected by gas3. Western Blot analyses conducted on protein extracts (50μg) from wild type and *gas3*-null MEFs probed with c-myc (A), p27 (B) and p21 (C) antibodies. MEFs were serum starved overnight, and time points (in minutes) represent extent to which cells were subjected to re-stimulation with serum.


A)
ø
5 mins
15 mins
30 mins
gas3+/+
gas3-/-
c-myc
β-actin
B)
p27
β-actin
C)
60 mins
p21
β-actin

cell cycle, further investigation into specific G1-associated cell cycle regulators such as D-type cyclins, CDK4 and CDK6, and G1 checkpoint proteins including phosphorylated and hyperphosphorylated forms of Rb, in addition to effectors of p21, could possibly reveal candidate proteins that are associated with or affected by gas3.

CHAPTER 4

Discussion

Retinoids display a multitude of effects associated with cancer prevention; they inhibit malignant transformation of cells in culture, prevent carcinogenesis in multiple organs in animal models and reduce premalignant epithelial lesions in humans (Sun and Lotan, 2002). The target genes by which retinoids mediate their chemopreventative effects remain largely unknown. To this end, we were interested in identifying genes that could mediate the anti-tumorigenic effects of RA, leading to the isolation of *gas3* as an RA-target gene in keratinocytes.

Given that *gas3* has been associated with growth arrest and cell cycle regulation, we investigated whether a relationship between *gas3* and epidermal tumorigenesis existed. Prior work revealed that *gas3* expression was extinguished in chemically induced papillomas in mice, suggesting that loss of this gene plays a crucial role in events related to skin tumor development. We then employed the two-stage carcinogenesis protocol on wild type, *gas3*-heterozygous and *gas3*-null mice on an FVB/NJ background, and found that *gas3*-null mice were refractory to tumor formation, suggestive of an oncogenic role for *gas3*, contradicting our original hypothesis. This finding is in contrast to a pilot study conducted using a mixed C57Bl/6-FVB/NJ background, which indicated that *gas3*-null mice exhibited a higher tumor burden compared to wild type controls.

In the present study, I first showed that RA-induced growth arrest in keratinocytes is mediated in part by *gas3*. In addition, I found that relative to wild type mice, *gas3*-null animals exhibited a heightened apoptotic response following treatment with DMBA and/or TPA; this increase in apoptosis may lead to tumor evasion in the *gas3* mutants relative to controls. Furthermore, to investigate if the impact of *gas3* on epidermal tumorigenesis is strain specific, I conducted a large scale two-stage carcinogenesis assay

on mice on a C57Bl/6 background, and found that wild type, *gas3*-heterozygous and *gas3*-null mice had similar tumor onset, burden and size. This suggests the existence of a modifier gene(s) that impact on gas3 function in FVB/NJ and C57Bl/6 strains. I also found that C57Bl/6 animals did not exhibit any changes in apoptosis or proliferation, consistent with the lack of a difference in tumor incidence in gas3 mutants in this background.

Western Blot analysis using extracts from chemically treated skin of FVB/NJ mice was used to investigate whether pathways known to influence skin cancer, such as Akt, TGF-β and NF-κB, were impacted by *gas3* loss; no changes in activity were observed. I also conducted Western Blot analyses using wild type and *gas3*-null MEF extracts to determine if certain cell cycle regulators, including c-myc, p27 and p21, were influenced by *gas3*, and found that, p21 expression was increased in *gas3*-null MEFs. These preliminary results may begin to elucidate how gas3 may impact on cell cycle regulation and tumorigenesis.

4.1 RA-mediated Growth Inhibition of Keratinocytes is Mediated in part by Gas3

Retinoids regulate cell growth, differentiation and apoptosis, and continue to be extensively researched as chemotherapeutic and chemopreventative agents against human cancers (Sun and Lotan, 2002). We previously demonstrated that RA induces growth arrest in transformed keratinocytes, an outcome primarily mediated by RARγ (Goyette et al., 2000), and identified *gas3* as a candidate target gene that may mediate these effects. Northern Blot analyses revealed that, in wild type keratinocytes, RA was able to induce

gas3 expression as early as 2 hours post-treatment (Laforest, 2004). Furthermore, we identified two DR2 RAREs in the *gas3* promoter, and demonstrated that these sequences are occupied by RARγ/RXRα in cells in culture (Foster-Hunt, 2007). Taken together, these data suggest that *gas3* is a direct RA-target gene, and may mediate RA-induced growth arrest observed in keratinocyte cultures.

Utilizing small inferring RNA (siRNA) targeted against *gas3* (siGas3), we transiently silenced its expression in wild type keratinocytes to determine if this protein is involved in conveying the growth inhibitory effects of RA. The siRNA reagent consisted of a pool of four individual siRNA duplexes targeted against the *gas3* mRNA transcript, subsequently causing a reduction of the targeted message. As a corollary to the above model, attempts were made to establish a *gas3*-null keratinocyte line. Due to technical issues, primary keratinocyte cultures were problematic, therefore siRNA-mediated knockdown was the route best suited for our studies.

Once an optimal working concentration was established to achieve maximal knockdown of gas3 expression, a time course revealed that *gas3* mRNA degradation occurred 3 days post-transfection and persisted until day 4. The effects of Gas3 on RA-mediated growth arrest was then examined during this window, and revealed that siGas3-treated keratinocytes were refractory to RA-induced growth arrest, while cells transfected with a scrambled siRNA sequence revealed a two-fold reduction of thymidine incorporation in response to RA. This finding suggests that gas3 mediates the effects of RA on mitosis.

RA-induced growth arrest in this keratinocyte line has been shown to be due to a halt in proliferation, and not due to apoptosis (Goyette et al., 2000). Furthermore, administration of RA to keratinocytes influences several cell cycle regulators. For instance, in wild type keratinocytes RA inhibits Rb hyperphosphorylation (Chen et al., 2004). D-type cyclins (D1, D2 and D3) and associated cyclin-dependent kinases (CDKs), CDK4 and CDK6, are largely responsible for the phosphorylation of pRB during G1, removing its repressor function and allowing cells to progress to S phase (Weinberg, 1995). $p21^{cip1}$ and $p27^{kip1}$ are G1-checkpoint CDK inhibitors, and function by interacting with CDKs obstructing their association with cyclins and essentially blocking their kinase activity (Abukhdeir and Park, 2008). Notably, RA, via $p21^{cip1}$ and $p27^{kip1}$, mediates growth arrest in keratinocytes by inhibiting G1 cyclin-CDK complexes (Mittnacht, 1998; Weinberg, 1995).

Although I found that RA-induced growth arrest in keratinocytes is partly mediated by gas3, the mechanism by which gas3 elicits this effect is unclear. *Gas3* has been implicated in negatively regulating cell proliferation, and elevated expression of this gene inhibits G0/G1 to S phase progression (Zoidl et al., 1995). As discussed above, in keratinocytes RA can inhibit the hyperphosphorylation of pRb, decrease cyclin D1 levels and increase $p21^{cip1}$ and $p27^{kip1}$ expression, all of which halt the G1 to S transition. Therefore Western Blot analyses on wild type and *gas3*-null MEFs were used to assess c-*myc*, p27 and p21 levels (Figures 3.17A, 3.17B, 3.17C) in serum stimulated cells. Although *c-myc* and p27 expression did not differ between wild type and *gas3*-null MEFs (Figures 3.17A and 3.17B), p21 levels appeared to be elevated in *gas3*-null MEFs, with highest expression at 5 and 15 minutes post re-stimulation with serum (Figure 3.17C).

Althought preliminary, these findings suggest that p21 may facilitate the actions of gas3 on cell cycle progression. Further work to assess effectors of p21, such as Sp1/Sp3, Ap2, BRCA1, and E2F-1/E2F-3 (Gartel and Tyner, 1999) may reveal potential mechanisms by which gas3 targets this pathway.

As *gas3* is a membrane bound protein, its induction by RA could trigger a downstream signaling cascade affecting cell cycle regulators. With the exception of α6β4 integrin (Amici et al., 2006), there are no known interacting partners of *gas3*. However, *gas3* could mediate downstream signaling by recruiting protein complexes to the plasma membrane to regulate signal transduction. A screen for interactors may reveal such partners, and begin to elucidate Gas3-dependent mechanisms influencing nuclear events.

4.2 Gas3 impact on Epidermal Tumorigenesis is subject to Strain-Specific Modifiers

Upon the isolation of *gas3* as a potential candidate gene for mediating the anti-tumorigenic effects of RA in keratinocytes, we became interested in examining the impact of *gas3* status on epidermal tumorigenesis. The role of *gas3* in tumorigenesis is not well understood, and its role in skin carcinogenesis has not been addressed. Initial observations revealed that *gas3* expression was extinguished in tumors from FVB/NJ mice subjected to the two-stage carcinogenesis protocol (Laforest, 2004). This finding, together with our discovery that *gas3* is a direct RA target, suggested that it may act as an RA-dependent tumor suppressor gene in epidermal carcinogenesis. If so, its loss would be expected to increase skin tumorigenesis and/or attenuate the inhibitory effects of RA

on tumor promotion. Indeed, *gas3*-heterozygous mice on an FVB/NJ background were refractory to RA-mediated tumor inhibition, using the two-stage carcinogenesis assay. However, *gas3*-heterozygous mice had a lower tumor burden than wild type controls, while *gas3* mutants were highly resistant to tumor induction (Foster-Hunt 2007). These results were not consistent with our hypothesis.

A pilot study repeating the two-stage carcinogenesis on mice of a mixed C57Bl/6-FVB/NJ background was conducted, and *gas3*-null mice appeared to exhibit an earlier tumor onset and higher tumor burden than wild type and *gas3*-heterozygous mice. These results not only suggested a dual functionality of *gas3* as both a RA-dependent tumor suppressor and oncogene in epidermal carcinogenesis, but also that its impact on tumor development was subject to strain-specific modifier effects.

These above results were obtained from a small scale pilot study; in order to acquire more quantitative proof of our findings, a large-scale experiment was conducted using mice on a C57Bl/6 background. Results from this study showed that mice from all three genotypes developed papillomas with similar tumor onsets and burden (Figure 3.3). Although the study conducted on C57Bl/6-FVB/NJ mice suggested that *gas3* was acting as a tumor suppressor, the results from this screen do not support this. Nonetheless, *gas3* clearly behaves differently in these two strains, although further analyses are required to understand the basis for this outcome.

Although the role of *gas3* in tumor biology is poorly understood, differing pathological roles for this gene have been suggested. In urethane-induced lung tumors in mice a downregulation of *gas3* expression is observed (Re et al., 1992), however, and in

contrast, elevated *gas3* expression is seen in osteosarcoma, osteoblastoma and glioblastoma tissues and cell lines (Huehne and Rautenstrauss, 2001; Huhne et al., 1999; van Dartel et al., 2002; van Dartel et al., 2003; van Dartel and Hulsebos, 2004). In addition, *gas3* has been postulated to play a role in the transformation of the pancreas to a cancerous state, as its expression in this tissue increases with increased malignancy (Li et al., 2005b). While limited, this literature nonetheless suggests the involvement of *gas3* in cancer pathogenesis.

Like Gas3, TGF-β signaling can function in a context-dependent manner as both a tumor suppressor and promoter (Bierie and Moses, 2006). At early stages of tumorigenesis, TGF-β functions as a tumor suppressor by exerting anti-proliferative effects (Akhurst and Derynck, 2001; Bakin et al., 2000; de Caestecker et al., 2000) downregulating c-Myc (Mulder and Brattain, 1988; Mulder et al., 1988; Zentella and Massague, 1992) and inducing the CDK inhibitors p15 and p21 (Datto et al., 1995; Iavarone and Massague, 1997; Moustakas and Kardassis, 1998; Reynisdottir and Massague, 1997). At later stages however, TGF-β contributes to invasion and metastasis by stimulating cell proliferation and growth (Shipley et al., 1985; Tucker et al., 1984), as well as promoting angiogenesis, immune suppression and epithelial to mesenchymal transitions (Akhurst and Derynck, 2001; Bakin et al., 2000; Lamouille and Derynck, 2007; Massague, 2008; Moustakas et al., 2002; Pepper, 1997; Roberts and Wakefield, 2003; Xu et al., 2009).

Gas3 exerts both tumor suppressive and promoting effects in a context-dependent manner. *Gas3*-null mice from an FVB/NJ background were resistant to chemically induced papillomas, whereas gas3 mutants on a C57Bl/6 strain developed a tumor burden

comparable to wild type controls. These data suggest that Gas3 is subject to modifier effects. Modifier genes can alter the expression or activity of another gene, while having no effect on their own (Fernandez-Piqueras and Hernandez, 2002). Modifiers can affect the penetrance, dominance, expressivity and pleiotropy of a given gene (Nadeau, 2001). As one example, mice homozygous deficient in the cystic fribrosis transmembrane conductance regulator gene (Ctfr) exhibit early post-natal death on most genetic backgrounds, but survive for several months on other backgrounds (Rozmahel et al., 1996). A modifier gene was identified that accounted for the survival of Ctfr-deficient mice; mice homozygous for the modifier gene survived several months longer relative to mice that lacked the modifier gene died almost immediately following birth (Rozmahel et al., 1996). Modifier genes can also affect tumor incidences such that mice may present different tumor susceptibilities depending on the genetic background (Nadeau, 2001). The adenomatous polyposis coli- multiple intestinal neoplasia (Apc^{Min}) mouse model is a classical example of this phenomenon. Apc^{Min} mice develop multiple polyps of the small intestine, an event that is strongly influenced by genetic background (Dietrich et al., 1993). For example, Apc^{Min} mice from a C57Bl/6 background present with several polyps, while mice from an AKR/J background are resistant to polyp formation; F1 hybrids show an intermediate number of polyps. The lack of tumor burden in the AKR/J strain was attributed to a modifier gene, identified as *modifier of Min* (*Mom1*) and subsequently mapped to a distal portion on chromosome 4 (Dietrich et al., 1993).

Modifier genes can cause enhanced or reduced phenotypes by either regulating a gene of interest or by direct interaction (Nadeau, 2001). In the FVB/NJ background

where *gas3*-null mice do not form papillomas, the modifier gene could be acting to reduce the tumor phenotype.

4.3 A Mechanism by which Gas3 impacts on Epidermal Tumorigenesis

TUNEL and Ki67 analyses were used to determine if the Gas3 impacted on growth or apoptosis following DMBA and/or TPA treatment. Ki67 studies revealed that DMBA or TPA alone did not lead to any differences in these parameters between wild type and *gas3*-null mice on a FVB/NJ background (Figures 3.7A and 3.9A). However, compared to *gas3*-null mice, wild type mice treated with both DMBA and TPA exhibited a two-fold increase in the number of proliferating cells in the epidermis (Figure 3.8A). *Gas3*-null mice still exhibit a growth increase in response to treatments with the tumor promoter, however their proliferative response is not as pronounced as wild type mice, which is reflected in their lack of tumor burden.

The TUNEL results revealed an additional mechanism that may also enable *gas3*-null mice to evade tumor formation. In response to treatments with DMBA alone, *gas3*-null mice exhibited a two-fold increase in the number of apoptotic cells in the epidermis relative to wild type controls (Figure 3.10A). This suggests that, at early stages of mutagenesis, *gas3*-null mice exhibit an elevation in pro-apoptotic mechanisms leading to the elimination of transformed cells. Additionally, in response to treatments with DMBA/TPA, *gas3*-null mice again exhibited a two-fold increase in the number of apoptotic cells in the epidermis (Figure 3.11A), and to TPA alone, exhibited a three-fold increase (Figure 3.12A).

Typical cellular responses to mutagens such as DMBA include DNA repair, chromatin remodeling, cell cycle arrest, senescence and apoptosis, all of which are regulated by p53 (Erb et al., 2008). However, the p53 gene itself can become mutated, as seen in a high percentage of SCC cases (Ziegler et al., 1994), and such cells can persist, undergo uncontrolled proliferation and losing the ability to undergo apoptosis (Erb et al., 2008). A similar mechanism is seen in keratinocytes exposed to ultraviolet B (UVB), referred to as sunburn cells (SBCs). SBCs trigger apoptosis in response to the irreversible DNA damage sustained from UVB exposure. If SBCs are not eliminated, the risk of malignant transformation is high, and can result in the development of non-melanoma skin cancer. In order to dispose of these mutagenized cells, pro-apoptotic pathways are triggered including both extrinsic and intrinsic caspase activation (Van Laethem et al., 2005). The extrinsic pathway involves ligand binding to death receptors, with subsequent recruitment and activation of downstream effector procaspases. The intrinsic apoptotic pathway entails the permeabilisation of the mitochondria causing the release of proteins such as cytochrome c, which triggers the formation of the apoptosome complex mediating cell death processes (Danial and Korsmeyer, 2004). The observed apoptosis in the *gas3*-null mice could be governed by the same signaling cascade as adopted by SBCs involving caspase activation, and it would be informative to investigate which caspases are involved in governing the apoptotic response. If we could identify specific caspases and their effectors affected by *gas3* it could reveal potential associations with other major signaling pathways involved in regulating apoptosis of mutagenized and non-mutagenized keratinocytes.

We also investigated changes in proliferation or apoptosis in the C57Bl/6 background, and found that Ki67 expression in wild type and *gas3*-null mice were comparable (Figures 3.13A and 3.14A). Treatment with DMBA/TPA caused a two-fold increase in apoptosis in wild type mice, compared to *gas3*-null mice (Figure 3.15A), although this did not result in a difference in tumor incidence. Treatments with TPA alone did not elicit any differential effects between genotypes (Figure 3.16A). Taken together, these results are consistent with the long term mutagenesis study, and indicate that of *gas3* did not have any significant effect on skin carcinogenesis in the C57Bl/6 background. Again, these observations may be attributed to modifier gene effects influencing the differential tumor phenotypes exhibited by *gas3*-null mice in both backgrounds.

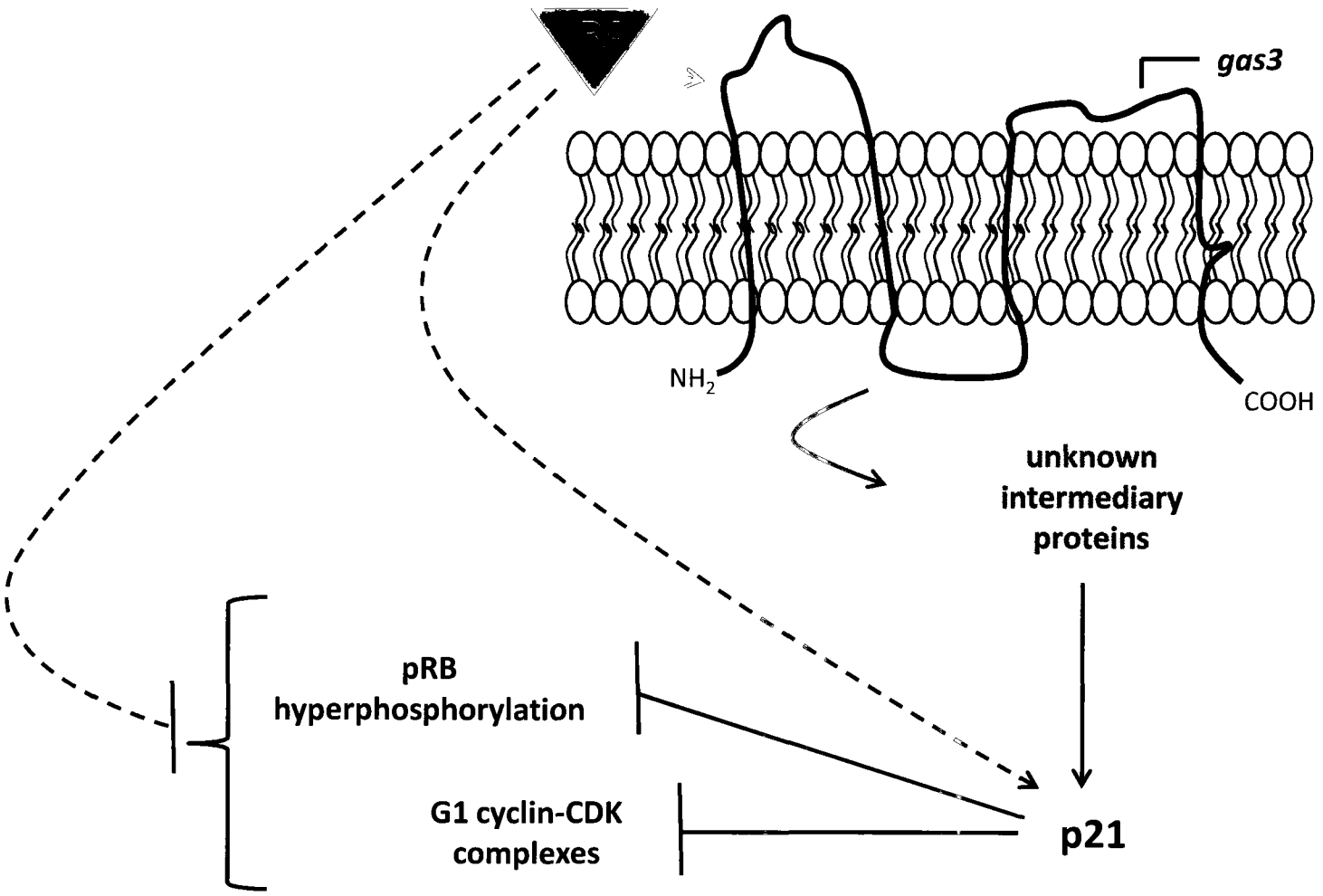
4.4 Summary and Future Directions

Work by our group has revealed that *gas3* is a direct RA-target gene and that it potentially exhibits dual roles in epidermal tumorigenesis as both an oncogene and tumor suppressor. My work consisted of investigating this multifaceted function. I have shown that RA-induced growth arrest in wild type keratinocytes is mediated in part by Gas3, in addition to elucidating a basis by which *gas3*-null mice on an FVB/NJ background evade tumor formation. I have also addressed the possibility that *gas3* impacts on skin tumorigenesis in a strain-specific manner and suggest that modifier genes may be responsible for modulating the different tumor phenotypes observed in *gas3*-null mice on FVB/NJ and C57Bl/6 backgrounds.

It would be informative to discover which pathway(s) might be involved in gas3-mediated RA-induced growth arrest in keratinocytes. Investigation into whether certain cell cycle regulators are influenced, such as $p21^{cip1}$ and its effectors including Sp1/Sp3, Ap2, BRCA1, and E2F-1/E2F-3, as well as G1 associated cyclin/CDK levels would certainly be instructive. Also, examining total Rb levels, as well as phorphorylated and hyperphorylated forms of Rb would reveal whether growth arrest is occurring at early or late stages of G1. The latter can be addressed by Western Blot analyses using protein extracts from siGas3-transfected keratinocytes treated with RA, or create a stable keratinocyte cell line using short hairpin RNA (shRNA) targeted against gas3. In addition, the established *gas3*-null MEF lines can be utilized, as well as wild type and *gas3*-null skin. We could subject these keratinocytes or MEFs (in the presence or absence of RA and/or serum) to flow cytometry in order to identify which phase of the cell cycle is affected. This would then allow focus on relevant cell cycle regulators. To this end, we could also investigate effectors of p21, as this is our most viable target at present.

We showed that RA-induced growth arrest in keratinocytes requires *gas3*, and suggested a possible mechanism involving *gas3* recruiting and/or interacting with proteins which subsequently mediate the downstream signaling to $p21^{cip1}$ (Figure 4.1). It should be noted that our group had previously conducted yeast-two-hybrid screen to identify gas3 interacting proteins which failed to provide informative results. Alternatively, stable isotope labeling with amino acids in cell culture (SILAC) could be performed in order to isolate candidate proteins. SILAC would entail culturing wild type and *gas3*-null MEFs, and growing each culture in medium containing heavy versus light

Figure 4.1 Proposed schematic model representing *gas3* signaling in keratinocytes. Although the mechanism why which *gas3* mediates RA-induced growth arrest in wild type keratinocytes is unknown, we suggest that it involves regulators of G1 progression of the cell cycle. In keratinocyte cultures, RA alone is able to inhibit G1/S transition by increasing $p21^{cip1}$ levels as well as inhibiting the hyperphosphorylation of pRB. It is possible that *gas3* is mediating this suppressive retinoid effect by acting as an intermediate effector subsequently triggering a downstream cascade involving the activation of $p21^{cip1}$, leading to G1 growth arrest (see text for details).


gas3
NH2
COOH
unknown
intermediary
proteins
pRB
hyperphosphorylation
G1 cyclin-CDK
complexes
p21

amino acids. Once the cells incorporate the respective labeled amino acids into all of their proteins, both cell populations can be combined, immunoprecipitated for Gas3, and interacting proteins analyzed via mass spectrometry. Once candidate proteins have been selected, immunofluorescence assays to detect for co-localization with gas3 can be conducted. If co-localization occurs, we could further examine whether these proteins interact, using bioluminescence resonance energy transfer (BRET) by constructing fusion proteins for gas3 and the protein of interest, tagged with Luciferase and green fluorescent protein (GFP), respectively. The identification of protein-protein interactions would enable us to better understand to role of *gas3* in mediating growth arrest in keratinocytes, and could reveal signaling pathways affected by this gene.

We demonstrated that on a FVB/NJ background, *gas3*-null mice escape tumor formation by an elevated apoptotic response relative to controls. It would be of interest to further investigate apoptotic markers involved in both the intrinsic and extrinsic caspase cascades, such as Bax and Bcl-2 family members, Fas ligands, and caspases 3, 6, 7, 8 and 9. Identifying specific effectors could disclose potential relationships between *gas3* and other major signaling pathways that employ similar apoptotic routes.

As mentioned previously, there are no pathways or genes known to be directly influenced by gas3, thus performing a microarray using skin tumor samples of FVB/NJ wild type and *gas3*-null mice could reveal potential signaling pathways affected by *gas3*. Equally, skin tumor samples of C57Bl/6 mice could also be used in a similar microarray analysis. In order to investigate a candidate gene from the microarray data, Western Blot analyses should be conducted using protein extract from wild type and *gas3*-null skin in order to evaluate whether the activity of the protein of interest increases or decreases as a

result of *gas3* loss. Following this, upstream effectors of the gene of interest could be assessed to deterimine if they are affected by the loss of *gas3*; this may potentially isolate specific signaling pathways. In addition, repeating the two-stage carcinogenesis study on C57Bl/6 mice with the administration of RA would allow us to better examine the role of *gas3* in this specific background, and also examine whether the inhibitory effects of RA are attenuated with the loss of *gas3*. Finally, to address the function of *gas3* in epidermal tumorigenesis being subjected to strain-specific modifications, Quantitative Trait Loci (QTL) mapping, which entails utilizing single nucleotide polymorphisms (SNPs) as markers to identify genomic regions that contribute to variation in traits, can be conducted to identify potential modifier loci.

In our study, we have shown that *gas3* is required for RA-mediated growth arrest in keratinocytes, in addition to elucidating that its functionality in skin tumorigenesis may be subject to strain-specific modifier effects. A topic that had not been previously investigated, we exposed novel roles for *gas3* in epidermal tumorigenesis. This field of research is unique and our work expands and contributes to the collective findings characterizing the function of *gas3* in cancer.

Reference List

Abel, E.L., Angel, J.M., Kiguchi, K., and DiGiovanni, J. (2009). Multi-stage chemical carcinogenesis in mouse skin: fundamentals and applications. Nat. Protoc. *4,* 1350-1362.

Abu-Abed, S., Dolle, P., Metzger, D., Beckett, B., Chambon, P., and Petkovich, M. (2001). The retinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures. Genes Dev *15,* 226-240.

Abu-Abed, S., MacLean, G., Fraulob, V., Chambon, P., Petkovich, M., and Dolle, P. (2002). Differential expression of the retinoic acid-metabolizing enzymes CYP26A1 and CYP26B1 during murine organogenesis. Mech. Dev. *110,* 173-177.

Abukhdeir, A.M., and Park, B.H. (2008). P21 and p27: roles in carcinogenesis and drug resistance. Expert Rev. Mol. Med. *10,* e19.

Akhurst, R.J., and Derynck, R. (2001). TGF-beta signaling in cancer--a double-edged sword. Trends Cell Biol. *11,* S44-51.

Aldaz, C.M., Trono, D., Larcher, F., Slaga, T.J., and Conti, C.J. (1989). Sequential trisomization of chromosomes 6 and 7 in mouse skin premalignant lesions. Mol. Carcinog. *2,* 22-26.

Alland, L., Muhle, R., H., H.,Jr, Potes, J., Chin, L., Schreiber-Agus, N., and DePinho, R.A. (1997). Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. Nature *387,* 49-55.

Altucci, L., and Gronemeyer, H. (2001). The promise of retinoids to fight against cancer. Nat Rev Cancer *1,* 181-193.

Amici, S.A., A., D.W.,Jr, Murphy, A.J., Adams, N.C., Gale, N.W., Valenzuela, D.M., Yancopoulos, G.D., and Notterpek, L. (2006). Peripheral myelin protein 22 is in complex with alpha6beta4 integrin, and its absence alters the Schwann cell basal lamina. J Neurosci *26,* 1179-1189.

Ang, H.L., and Duester, G. (1999). Retinoic acid biosynthetic enzyme ALDH1 localizes in a subset of retinoid-dependent tissues during xenopus development. Dev Dyn *215,* 264-272.

Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R.J., Rahmsdorf, H.J., Jonat, C., Herrlich, P., and Karin, M. (1987). Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. Cell *49,* 729-739.

Bach, H., and Goerttler, K. (1971). Morphological studies on the hyperplasiogenic effect of the biologically active phorbol ester A1. Virchows Arch. B Cell Pathol. *8,* 196-205.

Baechner, D., Liehr, T., Hameister, H., Altenberger, H., Grehl, H., Suter, U., and Rautenstrauss, B. (1995). Widespread expression of the peripheral myelin protein-22 gene (PMP22) in neural and non-neural tissues during murine development. J Neurosci Res *42,* 733-741.

Bai, Y., Zhang, X., Katona, I., Saporta, M.A., Shy, M.E., O'Malley, H.A., Isom, L.L., Suter, U., and Li, J. (2010). Conduction block in PMP22 deficiency. J. Neurosci. *30,* 600-608.

Bakin, A.V., Tomlinson, A.K., Bhowmick, N.A., Moses, H.L., and Arteaga, C.L. (2000). Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. J. Biol. Chem. *275,* 36803-36810.

Balkan, W., Klintworth, G.K., Bock, C.B., and Linney, E. (1992). Transgenic mice expressing a constitutively active retinoic acid receptor in the lens exhibit ocular defects. Dev. Biol. *151,* 622-625.

Balmain, A. (1976). The synthesis of specific proteins in adult mouse epidermis during phases of proliferation and differentiation induced by the tumor promoter TPA, and in basal and differentiating layers of neonatal mouse epidermis. J. Invest. Dermatol. *67,* 246-253.

Balmain, A., and Brown, K. (1988). Oncogene activation in chemical carcinogenesis. Adv. Cancer Res. *51,* 147-182.

Barbacid, M. (1987). Ras Genes. Annu. Rev. Biochem. *56,* 779-827.

Baron, J.M., Heise, R., Blaner, W.S., Neis, M., Joussen, S., Dreuw, A., Marquardt, Y., Saurat, J.H., Merk, H.F., Bickers, D.R., and Jugert, F.K. (2005). Retinoic acid and its 4-oxo metabolites are functionally active in human skin cells in vitro. J. Invest. Dermatol. *125,* 143-153.

Baudet, C., Perret, E., Delpech, B., Kaghad, M., Brachet, P., Wion, D., and Caput, D. (1998). Differentially expressed genes in C6.9 glioma cells during vitamin D-induced cell death program. Cell Death Differ *5,* 116-125.

Begemann, G., Schilling, T.F., Rauch, G.J., Geisler, R., and Ingham, P.W. (2001). The zebrafish neckless mutation reveals a requirement for raldh2 in mesodermal signals that pattern the hindbrain. Development *128,* 3081-3094.

Berggren, K., McCaffery, P., Drager, U., and Forehand, C.J. (1999). Differential distribution of retinoic acid synthesis in the chicken embryo as determined by immunolocalization of the retinoic acid synthetic enzyme, RALDH-2. Dev Biol *210,* 288-304.

Besa, E.C., Abrahm, J.L., Bartholomew, M.J., Hyzinski, M., and Nowell, P.C. (1990). Treatment with 13-cis-retinoic acid in transfusion-dependent patients with

myelodysplastic syndrome and decreased toxicity with addition of alpha-tocopherol. Am. J. Med. *89,* 739-747.

Bierie, B., and Moses, H.L. (2006). Tumour microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer. Nat. Rev. Cancer. *6,* 506-520.

Blomhoff, R., and Blomhoff, H.K. (2006). Overview of retinoid metabolism and function. J Neurobiol *66,* 606-630.

Bollag, W. (1971). Therapy of chemically induced skin tumors of mice with vitamin A palmitate and vitamin A acid. Experientia *27,* 90-92.

Bosse, F., Zoidl, G., Wilms, S., Gillen, C.P., Kuhn, H.G., and Muller, H.W. (1994). Differential expression of two mRNA species indicates a dual function of peripheral myelin protein PMP22 in cell growth and myelination. J Neurosci Res *37,* 529-537.

Bowden, G.T., Schneider, B., Domann, R., and Kulesz-Martin, M. (1994). Oncogene activation and tumor suppressor gene inactivation during multistage mouse skin carcinogenesis. Cancer Res *54,* 1882s-1885s.

Brancolini, C., Edomi, P., Marzinotto, S., and Schneider, C. (2000). Exposure at the cell surface is required for gas3/PMP22 To regulate both cell death and cell spreading: implication for the Charcot-Marie-Tooth type 1A and Dejerine-Sottas diseases. Mol Biol Cell *11,* 2901-2914.

Brancolini, C., Marzinotto, S., Edomi, P., Agostoni, E., Fiorentini, C., Muller, H.W., and Schneider, C. (1999). Rho-dependent regulation of cell spreading by the tetraspan membrane protein Gas3/PMP22. Mol Biol Cell *10,* 2441-2459.

Brancolini, C., Marzinotto, S., and Schneider, C. (1997). Susceptibility to p53 dependent apoptosis correlates with increased levels of Gas2 and Gas3 proteins. Cell Death Differ *4,* 247-253.

Brown, K., Buchmann, A., and Balmain, A. (1990). Carcinogen-induced mutations in the mouse c-Ha-ras gene provide evidence of multiple pathways for tumor progression. Proc. Natl. Acad. Sci. U. S. A. *87,* 538-542.

Calleja, C., Messaddeq, N., Chapellier, B., Yang, H., Krezel, W., Li, M., Metzger, D., Mascrez, B., Ohta, K., Kagechika, H., *et al.* (2006). Genetic and pharmacological evidence that a retinoic acid cannot be the RXR-activating ligand in mouse epidermis keratinocytes. Genes Dev *20,* 1525-1538.

Caulin, C., Bauluz, C., Gandarillas, A., Cano, A., and Quintanilla, M. (1993). Changes in keratin expression during malignant progression of transformed mouse epidermal keratinocytes. Exp. Cell Res. *204,* 11-21.

Chambon, P. (1996). A decade of molecular biology of retinoic acid receptors. FASEB J. *10,* 940-954.

Chan, K.S., Sano, S., Kataoka, K., Abel, E., Carbajal, S., Beltran, L., Clifford, J., Peavey, M., Shen, J., and Digiovanni, J. (2008). Forced expression of a constitutively active form of Stat3 in mouse epidermis enhances malignant progression of skin tumors induced by two-stage carcinogenesis. Oncogene *27,* 1087-1094.

Chan, T.O., Rittenhouse, S.E., and Tsichlis, P.N. (1999). AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. Annu. Rev. Biochem. *68,* 965-1014.

Chapellier, B., Mark, M., Messaddeq, N., Calleja, C., Warot, X., Brocard, J., Gerard, C., Li, M., Metzger, D., Ghyselinck, N.B., and Chambon, P. (2002). Physiological and retinoid-induced proliferations of epidermis basal keratinocytes are differently controlled. EMBO J *21,* 3402-3413.

Cheepala, S.B., Syed, Z., Trutschl, M., Cvek, U., and Clifford, J.L. (2007). Retinoids and skin: microarrays shed new light on chemopreventive action of all-trans retinoic acid. Mol. Carcinog. *46,* 634-639.

Chen, C.F., Goyette, P., and Lohnes, D. (2004). RARgamma acts as a tumor suppressor in mouse keratinocytes. Oncogene *23,* 5350-5359.

Chen, H., Namkung, M.J., and Juchau, M.R. (1995). Biotransformation of all-trans-retinol and all-trans-retinal to all-trans-retinoic acid in rat conceptal homogenates. Biochem Pharmacol *50,* 1257-1264.

Chen, J.G., Fleischer, A.B.,Jr, Smith, E.D., Kancler, C., Goldman, N.D., Williford, P.M., and Feldman, S.R. (2001). Cost of nonmelanoma skin cancer treatment in the United States. Dermatol. Surg. *27,* 1035-1038.

Danial, N.N., and Korsmeyer, S.J. (2004). Cell death: critical control points. Cell *116,* 205-219.

Darwiche, N., Scita, G., Jones, C., Rutberg, S., Greenwald, E., Tennenbaum, T., Collins, S.J., De Luca, L.M., and Yuspa, S.H. (1996). Loss of retinoic acid receptors in mouse skin and skin tumors is associated with activation of the ras(Ha) oncogene and high risk for premalignant progression. Cancer Res *56,* 4942-4949.

Datto, M.B., Li, Y., Panus, J.F., Howe, D.J., Xiong, Y., and Wang, X.F. (1995). Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. Proc. Natl. Acad. Sci. U. S. A. *92,* 5545-5549.

de Caestecker, M.P., Piek, E., and Roberts, A.B. (2000). Role of transforming growth factor-beta signaling in cancer. J. Natl. Cancer Inst. *92,* 1388-1402.

De Luca, L.M. (1991). Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. FASEB J. *5,* 2924-2933.

De Luca, L.M., Tarone, R., Huynh, M., Jones, C.S., and Chen, L.C. (1996). Dietary retinoic acid inhibits mouse skin carcinogenesis irrespective of age at initiation. Nutr Cancer *25,* 249-257.

de Vries, E., van de Poll-Franse, L.V., Louwman, W.J., de Gruijl, F.R., and Coebergh, J.W. (2005). Predictions of skin cancer incidence in the Netherlands up to 2015. Br J Dermatol *152,* 481-488.

Dellambra, E., Golisano, O., Bondanza, S., Siviero, E., Lacal, P., Molinari, M., D'Atri, S., and De Luca, M. (2000). Downregulation of 14-3-3sigma prevents clonal evolution and leads to immortalization of primary human keratinocytes. J. Cell Biol. *149,* 1117-1130.

Dellambra, E., Patrone, M., Sparatore, B., Negri, A., Ceciliani, F., Bondanza, S., Molina, F., Cancedda, F.D., and De Luca, M. (1995). Stratifin, a keratinocyte specific 14-3-3 protein, harbors a pleckstrin homology (PH) domain and enhances protein kinase C activity. J. Cell. Sci. *108 (Pt 11),* 3569-3579.

Descargues, P., Sil, A.K., and Karin, M. (2008). IKKalpha, a critical regulator of epidermal differentiation and a suppressor of skin cancer. EMBO J. *27,* 2639-2647.

DiDonato, J.A., Hayakawa, M., Rothwarf, D.M., Zandi, E., and Karin, M. (1997). A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. Nature *388,* 548-554.

Dietrich, W.F., Lander, E.S., Smith, J.S., Moser, A.R., Gould, K.A., Luongo, C., Borenstein, N., and Dove, W. (1993). Genetic identification of Mom-1, a major modifier locus affecting Min-induced intestinal neoplasia in the mouse. Cell *75,* 631-639.

DiGiovanni, J. (1992). Multistage carcinogenesis in mouse skin. Pharmacol. Ther. *54,* 63-128.

DiGiovanni, J., Prichett, W.P., Decina, P.C., and Diamond, L. (1984). DBA/2 mice are as sensitive as SENCAR mice to skin tumor promotion by 12-O-tetradecanoylphorbol-13-acetate. Carcinogenesis *5,* 1493-1498.

Dipple, A., Pigott, M.A., and Anderson, L.M. (1984a). 7,12-Dimethylbenz[alpha]anthracene-DNA adducts in cultured cells from mouse fetuses of different gestational ages. Cancer Lett. *21,* 285-292.

Dipple, A., Pigott, M.A., Bigger, C.A., and Blake, D.M. (1984b). 7,12-dimethylbenz[a] anthracene-DNA binding in mouse skin: response of different mouse strains and effects of various modifiers of carcinogenesis. Carcinogenesis *5,* 1087-1090.

Dlugosz, A.A., and Yuspa, S.H. (1993). Coordinate changes in gene expression which mark the spinous to granular cell transition in epidermis are regulated by protein kinase C. J. Cell Biol. *120,* 217-225.

Duester, G. (2000). Families of retinoid dehydrogenases regulating vitamin A function: production of visual pigment and retinoic acid. Eur J Biochem *267,* 4315-4324.

Duester, G., Farres, J., Felder, M.R., Holmes, R.S., Hoog, J.O., Pares, X., Plapp, B.V., Yin, S.J., and Jornvall, H. (1999). Recommended nomenclature for the vertebrate alcohol dehydrogenase gene family. Biochem. Pharmacol. *58,* 389-395.

Duffield-Lillico, A.J., and Begg, C.B. (2004). Reflections on the landmark studies of beta-carotene supplementation. J. Natl. Cancer Inst. *96,* 1729-1731.

Dyck, P.J., and Lambert, E.H. (1968). Lower motor and primary sensory neuron diseases with peroneal muscular atrophy. I. Neurologic, genetic, and electrophysiologic findings in hereditary polyneuropathies. Arch. Neurol. *18,* 603-618.

Edwards, L., and Jaffe, P. (1990). The effect of topical tretinoin on dysplastic nevi. A preliminary trial. Arch. Dermatol. *126,* 494-499.

Eferl, R., and Wagner, E.F. (2003). AP-1: a double-edged sword in tumorigenesis. Nat. Rev. Cancer. *3,* 859-868.

Elias, P.M., Fritsch, P.O., Lampe, M., Williams, M.L., Brown, B.E., Nemanic, M., and Grayson, S. (1981). Retinoid effects on epidermal structure, differentiation, and permeability. Lab. Invest. *44,* 531-540.

Erb, P., Ji, J., Kump, E., Mielgo, A., and Wernli, M. (2008). Apoptosis and pathogenesis of melanoma and nonmelanoma skin cancer. Adv. Exp. Med. Biol. *624,* 283-295.

Fabbretti, E., Edomi, P., Brancolini, C., and Schneider, C. (1995). Apoptotic phenotype induced by overexpression of wild-type gas3/PMP22: its relation to the demyelinating peripheral neuropathy CMT1A. Genes Dev *9,* 1846-1856.

Fernandez-Piqueras, J., and Hernandez J.S. (2002). Tumor modifier genes. Rev Oncol *7,* 349-357.

Fisher, G.J., and Voorhees, J.J. (1996). Molecular mechanisms of retinoid actions in skin. FASEB J *10,* 1002-1013.

Foster-Hunt, C., (2007). *Gas3*, a direct RA-target Gene That Conveys an RA-Mediated Tumor Suppressor Role on Chemically-Induced Skin Papillomas. A thesis submitted to the School of Graduate Studies and Research, Unversity of Ottawa.

Fuchs, E. (2007). Scratching the surface of skin development. Nature *445,* 834-842.

Fuchs, E. (1995). Keratins and the skin. Annu Rev Cell Dev Biol *11,* 123-153.

Fuchs, E., and Green, H. (1980). Changes in keratin gene expression during terminal differentiation of the keratinocyte. Cell *19,* 1033-1042.

Fuchs, E., and Raghavan, S. (2002). Getting under the skin of epidermal morphogenesis. Nat Rev Genet *3,* 199-209.

Fujii, H., Sato, T., Kaneko, S., Gotoh, O., Fujii-Kuriyama, Y., Osawa, K., Kato, S., and Hamada, H. (1997). Metabolic inactivation of retinoic acid by a novel P450 differentially expressed in developing mouse embryos. EMBO J *16,* 4163-4173.

Gagnon, I., Duester, G., and Bhat, P.V. (2003). Enzymatic characterization of recombinant mouse retinal dehydrogenase type 1. Biochem. Pharmacol. *65,* 1685-1690.

Garrett-Sinha, L.A., Eberspaecher, H., Seldin, M.F., and de Crombrugghe, B. (1996). A gene for a novel zinc-finger protein expressed in differentiated epithelial cells and transiently in certain mesenchymal cells. J Biol Chem *271,* 31384-31390.

Gartel, A.L., and Tyner, A.L. (1999). Transcriptional regulation of the p21((WAF1/CIP1)) gene. Exp. Cell Res. *246,* 280-289.

Gelboin, H.V. (1980). Benzo[alpha]pyrene metabolism, activation and carcinogenesis: role and regulation of mixed-function oxidases and related enzymes. Physiol. Rev. *60,* 1107-1166.

Gelfant, S. (1976). The cell cycle in psoriasis: a reappraisal. Br. J. Dermatol. *95,* 577-590.

Giguere, V. (1994). Retinoic acid receptors and cellular retinoid binding proteins: complex interplay in retinoid signaling. Endocr. Rev. *15,* 61-79.

Goyette, P., Feng, C.C., Wang, W., Seguin, F., and Lohnes, D. (2000). Characterization of retinoic acid receptor-deficient keratinocytes. J Biol Chem *275,* 16497-16505.

Green, H., Easley, K., and Iuchi, S. (2003). Marker succession during the development of keratinocytes from cultured human embryonic stem cells. Proc Natl Acad Sci U S A *100,* 15625-15630.

Grignani, F., De Matteis, S., Nervi, C., Tomassoni, L., Gelmetti, V., Cioce, M., Fanelli, M., Ruthardt, M., Ferrara, F.F., Zamir, I., *et al.* (1998). Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. Nature *391,* 815-818.

Grubbs, C.J., Eto, I., Juliana, M.M., Hardin, J.M., and Whitaker, L.M. (1990). Effect of retinyl acetate and 4-hydroxyphenylretinamide on initiation of chemically-induced mammary tumors. Anticancer Res. *10,* 661-666.

Gudas, L.J. (1994). Retinoids and vertebrate development. J. Biol. Chem. *269,* 15399-15402.

Hall, M., Parker, D.K., Grover, P.L., Lu, J.Y., Hopkins, N.E., and Alworth, W.L. (1990). Effects of 1-ethynylpyrene and related inhibitors of P450 isozymes upon benzo[a]pyrene metabolism by liver microsomes. Chem. Biol. Interact. *76,* 181-192.

Halprin, K.M. (1972). Epidermal "turnover time"--a re-examination. Br. J. Dermatol. *86,* 14-19.

Haselbeck, R.J., Hoffmann, I., and Duester, G. (1999). Distinct functions for Aldh1 and Raldh2 in the control of ligand production for embryonic retinoid signaling pathways. Dev Genet *25,* 353-364.

He, L.Z., Guidez, F., Tribioli, C., Peruzzi, D., Ruthardt, M., Zelent, A., and Pandolfi, P.P. (1998). Distinct interactions of PML-RARalpha and PLZF-RARalpha with co-repressors determine differential responses to RA in APL. Nat Genet *18,* 126-135.

He, L.Z., Merghoub, T., and Pandolfi, P.P. (1999). In vivo analysis of the molecular pathogenesis of acute promyelocytic leukemia in the mouse and its therapeutic implications. Oncogene *18,* 5278-5292.

Hearing, V.J. (2005). Biogenesis of pigment granules: a sensitive way to regulate melanocyte function. J Dermatol Sci *37,* 3-14.

Heinzel, T., Lavinsky, R.M., Mullen, T.M., Soderstrom, M., Laherty, C.D., Torchia, J., Yang, W.M., Brard, G., Ngo, S.D., Davie, J.R., *et al.* (1997). A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. Nature *387,* 43-48.

Heyman, R.A., Mangelsdorf, D.J., Dyck, J.A., Stein, R.B., Eichele, G., Evans, R.M., and Thaller, C. (1992). 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. Cell *68,* 397-406.

Hollemann, T., Chen, Y., Grunz, H., and Pieler, T. (1998). Regionalized metabolic activity establishes boundaries of retinoic acid signalling. EMBO J. *17,* 7361-7372.

Hong, W.K., Endicott, J., Itri, L.M., Doos, W., Batsakis, J.G., Bell, R., Fofonoff, S., Byers, R., Atkinson, E.N., and Vaughan, C. (1986). 13-Cis-Retinoic Acid in the Treatment of Oral Leukoplakia. N. Engl. J. Med. *315,* 1501-1505.

Hong, W.K., and Itri L.M. (1994). Retinoids and human cancer. In The retinoids, Sporn MB, Roberts AB, Goodman DS, ed. (New York: Raven Press), pp.597-658.

Hong, W.K., Lippman, S.M., Itri, L.M., Karp, D.D., Lee, J.S., Byers, R.M., Schantz, S.P., Kramer, A.M., Lotan, R., and Peters, L.J. (1990). Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. N. Engl. J. Med. *323,* 795-801.

Huehne, K., and Rautenstrauss, B. (2001). Transcriptional startpoints and methylation patterns in the PMP22 promoters of peripheral nerve, leukocytes and tumor cell lines. Int J Mol Med *7,* 669-675.

Huhne, K., Park, O., Liehr, T., and Rautenstrauss, B. (1999). Expression analysis of the PMP22 gene in glioma and osteogenic sarcoma cell lines. J Neurosci Res *58,* 624-631.

Hummerich, L., Muller, R., Hess, J., Kokocinski, F., Hahn, M., Furstenberger, G., Mauch, C., Lichter, P., and Angel, P. (2006). Identification of novel tumour-associated genes differentially expressed in the process of squamous cell cancer development. Oncogene *25,* 111-121.

Iavarone, A., and Massague, J. (1997). Repression of the CDK activator Cdc25A and cell-cycle arrest by cytokine TGF-beta in cells lacking the CDK inhibitor p15. Nature *387,* 417-422.

Ise, K., Nakamura, K., Nakao, K., Shimizu, S., Harada, H., Ichise, T., Miyoshi, J., Gondo, Y., Ishikawa, T., Aiba, A., and Katsuki, M. (2000). Targeted deletion of the H-ras gene decreases tumor formation in mouse skin carcinogenesis. Oncogene *19,* 2951-2956.

Kanitakis, J. (2002). Anatomy, histology and immunohistochemistry of normal human skin. Eur. J. Dermatol. *12,* 390-9; quiz 400-1.

Kato, S., Sasaki, H., Suzawa, M., Masushige, S., Tora, L., Chambon, P., and Gronemeyer, H. (1995). Widely spaced, directly repeated PuGGTCA elements act as promiscuous enhancers for different classes of nuclear receptors. Mol. Cell. Biol. *15,* 5858-5867.

Kemp, C.J. (2005). Multistep skin cancer in mice as a model to study the evolution of cancer cells. Semin. Cancer Biol. *15,* 460-473.

Kliewer, S.A., Umesono, K., Heyman, R.A., Mangelsdorf, D.J., Dyck, J.A., and Evans, R.M. (1992). Retinoid X receptor-COUP-TF interactions modulate retinoic acid signaling. Proc Natl Acad Sci U S A *89,* 1448-1452.

Koster, M.I., Kim, S., Mills, A.A., DeMayo, F.J., and Roop, D.R. (2004). p63 is the molecular switch for initiation of an epithelial stratification program. Genes Dev *18,* 126-131.

Koster, M.I., and Roop, D.R. (2007). Mechanisms Regulating Epithelial Stratification. Annu Rev Cell Dev Biol

Kraemer, K.H., DiGiovanna, J.J., Moshell, A.N., Tarone, R.E., and Peck, G.L. (1988). Prevention of skin cancer in xeroderma pigmentosum with the use of oral isotretinoin. N. Engl. J. Med. *318,* 1633-1637.

Kuhn, G., Lie, A., Wilms, S., and Muller, H.W. (1993). Coexpression of PMP22 gene with MBP and P0 during de novo myelination and nerve repair. Glia *8,* 256-264.

Laforest, B., (2004). Isolation and characterization of RA-target genes from keratinocytes potentially involved in epithelial tumorigenesis. A thesis submitted to the Faculty of Graduate Studies and Research, McGill University.

Lamouille, S., and Derynck, R. (2007). Cell size and invasion in TGF-beta-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway. J. Cell Biol. *178,* 437-451.

Lee, C.H., Jeon, Y.T., Kim, S.H., and Song, Y.S. (2007). NF-kappaB as a potential molecular target for cancer therapy. Biofactors *29,* 19-35.

Lemon, B.D., and Freedman, L.P. (1996). Selective effects of ligands on vitamin D3 receptor- and retinoid X receptor-mediated gene activation in vivo. Mol. Cell. Biol. *16,* 1006-1016.

Levine, N., and Meyskens, F.L. (1980). Topical vitamin-A-acid therapy for cutaneous metastatic melanoma. Lancet *2,* 224-226.

Li, A.G., Lu, S.L., Han, G., Kulesz-Martin, M., and Wang, X.J. (2005a). Current view of the role of transforming growth factor beta 1 in skin carcinogenesis. J Investig Dermatol Symp Proc *10,* 110-117.

Li, J., Kleeff, J., Esposito, I., Kayed, H., Felix, K., Giese, T., Buchler, M.W., and Friess, H. (2005b). Expression analysis of PMP22/Gas3 in premalignant and malignant pancreatic lesions. J Histochem Cytochem *53,* 885-893.

Lin, R.J., Egan, D.A., and Evans, R.M. (1999). Molecular genetics of acute promyelocytic leukemia. Trends Genet. *15,* 179-184.

Lin, R.J., Nagy, L., Inoue, S., Shao, W., Miller, W.H.,Jr, and Evans, R.M. (1998). Role of the histone deacetylase complex in acute promyelocytic leukaemia. Nature *391,* 811-814.

Lippman, S.M., Kessler, J.F., Al-Sarraf, M., Alberts, D.S., Itri, L.M., Mattox, D., Von Hoff, D.D., Loescher, L., and Meyskens, F.L. (1988). Treatment of advanced squamous cell carcinoma of the head and neck with isotretinoin: a phase II randomized trial. Invest. New Drugs *6,* 51-56.

Lotan, R., Xu, X.C., Lippman, S.M., Ro, J.Y., Lee, J.S., Lee, J.J., and Hong, W.K. (1995). Suppression of retinoic acid receptor-beta in premalignant oral lesions and its up-regulation by isotretinoin. N. Engl. J. Med. *332,* 1405-1410.

Lotan, Y., Xu, X.C., Shalev, M., Lotan, R., Williams, R., Wheeler, T.M., Thompson, T.C., and Kadmon, D. (2000). Differential expression of nuclear retinoid receptors in normal and malignant prostates. J. Clin. Oncol. *18,* 116-121.

Lowy, D.R., and Willumsen, B.M. (1993). Function and regulation of ras. Annu. Rev. Biochem. *62,* 851-891.

Lu, J., Rho, O., Wilker, E., Beltran, L., and Digiovanni, J. (2007). Activation of epidermal akt by diverse mouse skin tumor promoters. Mol. Cancer. Res. *5,* 1342-1352.

Lupski, J.R., Oca-Luna, R.M., Slaugenhaupt, S., Pentao, L., Guzzetta, V., Trask, B.J., Saucedo-Cardenas, O., Barker, D.F., Killian, J.M., Garcia, C.A., Chakravarti, A., and Patel, P.I. (1991). DNA duplication associated with Charcot-Marie-Tooth disease type 1A. Cell *66,* 219-232.

MacLean, G., Abu-Abed, S., Dolle, P., Tahayato, A., Chambon, P., and Petkovich, M. (2001). Cloning of a novel retinoic-acid metabolizing cytochrome P450, Cyp26B1, and comparative expression analysis with Cyp26A1 during early murine development. Mech. Dev. *107,* 195-201.

Maden, M., Blentic, A., Reijntjes, S., Seguin, S., Gale, E., and Graham, A. (2007). Retinoic acid is required for specification of the ventral eye field and for Rathke's pouch in the avian embryo. Int. J. Dev. Biol. *51,* 191-200.

Maden, M., Gale, E., Kostetskii, I., and Zile, M. (1996). Vitamin A-deficient quail embryos have half a hindbrain and other neural defects. Curr Biol *6,* 417-426.

Malumbres, M., and Barbacid, M. (2003). RAS oncogenes: the first 30 years. Nat. Rev. Cancer. *3,* 459-465.

Mangelsdorf, D.J., and Evans, R.M. (1995). The RXR heterodimers and orphan receptors. Cell *83,* 841-850.

Manzow, S., Brancolini, C., Marks, F., and Richter, K.H. (1996). Expression of growth arrest-specific (Gas) genes in murine keratinocytes: Gas2 is specifically regulated. Exp Cell Res 1996 Apr 10;224(1): *224,* 200-203.

Marcu, K.B., Bossone, S.A., and Patel, A.J. (1992). Myc Function and Regulation. Annu. Rev. Biochem. *61,* 809-860.

Marks, F., and Furstenberger, G. (1990). The conversion stage of skin carcinogenesis. Carcinogenesis 1990 Dec;11(12): *11,* 2085-2092.

Massague, J. (2008). TGFbeta in Cancer. Cell *134,* 215-230.

Matsunami, N., Smith, B., Ballard, L., Lensch, M.W., Robertson, M., Albertsen, H., Hanemann, C.O., Muller, H.W., Bird, T.D., and White, R. (1992). Peripheral myelin protein-22 gene maps in the duplication in chromosome 17p11.2 associated with Charcot-Marie-Tooth 1A. Nat Genet *1,* 176-179.

McCaffery, P., Wagner, E., O'Neil, J., Petkovich, M., and Drager, U.C. (1999). Dorsal and ventral retinal territories defined by retinoic acid synthesis, break-down and nuclear receptor expression. Mech Dev *82,* 119-130.

Mercurio, F., Zhu, H., Murray, B.W., Shevchenko, A., Bennett, B.L., Li, J., Young, D.B., Barbosa, M., Mann, M., Manning, A., and Rao, A. (1997). IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. Science *278,* 860-866.

Mic, F.A., Haselbeck, R.J., Cuenca, A.E., and Duester, G. (2002). Novel retinoic acid generating activities in the neural tube and heart identified by conditional rescue of Raldh2 null mutant mice. Development *129,* 2271-2282.

Mic, F.A., Molotkov, A., Benbrook, D.M., and Duester, G. (2003). Retinoid activation of retinoic acid receptor but not retinoid X receptor is sufficient to rescue lethal defect in retinoic acid synthesis. Proc. Natl. Acad. Sci. U. S. A. *100,* 7135-7140.

Missero, C., Calautti, E., Eckner, R., Chin, J., Tsai, L.H., Livingston, D.M., and Dotto, G.P. (1995). Involvement of the cell-cycle inhibitor Cip1/WAF1 and the E1A-associated p300 protein in terminal differentiation. Proc. Natl. Acad. Sci. U. S. A. *92,* 5451-5455.

Mittnacht, S. (1998). Control of pRB phosphorylation. Curr. Opin. Genet. Dev. *8,* 21-27.

Moon, R.C., Mehta, R.G., and Rao K.J. V.N. (1994). Retinoids and cancer in experimental animals. In The retinoids, Sporn MB, Roberts AB, Goodman DS, ed. (New York: Raven Press), pp.573–95.

Moustakas, A., and Kardassis, D. (1998). Regulation of the human p21/WAF1/Cip1 promoter in hepatic cells by functional interactions between Sp1 and Smad family members. Proc. Natl. Acad. Sci. U. S. A. *95,* 6733-6738.

Moustakas, A., Pardali, K., Gaal, A., and Heldin, C.H. (2002). Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. Immunol. Lett. *82,* 85-91.

Mulder, K.M., and Brattain, M.G. (1988). Alterations in c-myc expression in relation to maturational status of human colon carcinoma cells. Int. J. Cancer *42,* 64-70.

Mulder, K.M., Levine, A.E., Hernandez, X., McKnight, M.K., Brattain, D.E., and Brattain, M.G. (1988). Modulation of c-myc by transforming growth factor-beta in human colon carcinoma cells. Biochem. Biophys. Res. Commun. *150,* 711-716.

Nadeau, J.H. (2001). Modifier genes in mice and humans. Nat. Rev. Genet. *2,* 165-174.

Nagy, L., Kao, H.Y., Chakravarti, D., Lin, R.J., Hassig, C.A., Ayer, D.E., Schreiber, S.L., and Evans, R.M. (1997). Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. Cell *89,* 373-380.

Nagy, L., Thomazy, V.A., Shipley, G.L., Fesus, L., Lamph, W., Heyman, R.A., Chandraratna, R.A., and Davies, P.J. (1995). Activation of retinoid X receptors induces apoptosis in HL-60 cell lines. Mol. Cell. Biol. *15,* 3540-3551.

Nakshatri, H., and Chambon, P. (1994). The directly repeated RG(G/T)TCA motifs of the rat and mouse cellular retinol-binding protein II genes are promiscuous binding sites for RAR, RXR, HNF-4, and ARP-1 homo- and heterodimers. J. Biol. Chem. *269,* 890-902.

Napoli, J.L. (1996). Retinoic acid biosynthesis and metabolism. FASEB J. *10,* 993-1001.

Navarro, P., Gomez, M., Pizarro, A., Gamallo, C., Quintanilla, M., and Cano, A. (1991). A role for the E-cadherin cell-cell adhesion molecule during tumor progression of mouse epidermal carcinogenesis. J. Cell Biol. *115,* 517-533.

Neville, J.A., Welch, E., and Leffell, D.J. (2007). Management of nonmelanoma skin cancer in 2007. Nat Clin Pract Oncol *4,* 462-469.

Nguyen, B.C., Lefort, K., Mandinova, A., Antonini, D., Devgan, V., Della, G.G., Koster, M.I., Zhang, Z., Wang, J., Tommasi, d.V., *et al.* (2006). Cross-regulation between Notch and p63 in keratinocyte commitment to differentiation. Genes Dev *20,* 1028-1042.

Nguyen, T.H., and Ho, D.Q. (2002). Nonmelanoma skin cancer. Curr. Treat. Options Oncol. *3,* 193-203.

Niederreither, K., Abu-Abed, S., Schuhbaur, B., Petkovich, M., Chambon, P., and Dolle, P. (2002). Genetic evidence that oxidative derivatives of retinoic acid are not involved in retinoid signaling during mouse development. Nat Genet *31,* 84-88.

Niederreither, K., McCaffery, P., Drager, U.C., Chambon, P., and Dolle, P. (1997). Restricted expression and retinoic acid-induced downregulation of the retinaldehyde

dehydrogenase type 2 (RALDH-2) gene during mouse development. Mech Dev *62,* 67-78.

Notterpek, L., Roux, K.J., Amici, S.A., Yazdanpour, A., Rahner, C., and Fletcher, B.S. (2001). Peripheral myelin protein 22 is a constituent of intercellular junctions in epithelia. Proc Natl Acad Sci U S A *98,* 14404-14409.

Odland, G. (1991). Structure of the skin. In physiology, biochemistry, and molecular biology of the skin, L. Goldsmith, ed. (New York: Oxford University Press), pp. 3-62.

Omenn, G.S., Goodman, G.E., Thornquist, M.D., Balmes, J., Cullen, M.R., Glass, A., Keogh, J.P., Meyskens, F.L., Valanis, B., Williams, J.H., Barnhart, S., and Hammar, S. (1996). Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. N. Engl. J. Med. *334,* 1150-1155.

Parkinson, E.K. (1985). Defective responses of transformed keratinocytes to terminal differentiation stimuli. Their role in epidermal tumour promotion by phorbol esters and by deep skin wounding. Br. J. Cancer *52,* 479-493.

Pastorino, U., Soresi, E., Clerici, M., Chiesa, G., Belloni, P.A., Ongari, M., Valente, M., and Ravasi, G. (1988). Lung cancer chemoprevention with retinol palmitate. Preliminary data from a randomized trial on stage Ia non small-cell lung cancer. Acta Oncol. *27,* 773-782.

Patel, P.I., Roa, B.B., Welcher, A.A., Schoener-Scott, R., Trask, B.J., Pentao, L., Snipes, G.J., Garcia, C.A., Francke, U., Shooter, E.M., Lupski, J.R., and Suter, U. (1992). The gene for the peripheral myelin protein PMP-22 is a candidate for Charcot-Marie-Tooth disease type 1A. Nat Genet *1,* 159-165.

Pawson, B.A., Cheung, H.C., Han, R.J., Trown, P.W., Buck, M., Hansen, R., Bollag, W., Ineichen, U., Pleil, H., and Ruegg, R. (1977). Dihydroretinoic acids and their derivatives. Synthesis and biological activity. J. Med. Chem. *20,* 918-925.

Peck, G.L., DiGiovanna, J.J., Sarnoff, D.S., Gross, E.G., Butkus, D., Olsen, T.G., and Yoder, F.W. (1988). Treatment and prevention of basal cell carcinoma with oral isotretinoin. J. Am. Acad. Dermatol. *19,* 176-185.

Pelkonen, O., and Nebert, D.W. (1982). Metabolism of polycyclic aromatic hydrocarbons: etiologic role in carcinogenesis. Pharmacol. Rev. *34,* 189-222.

Pellegrini, G., Dellambra, E., Golisano, O., Martinelli, E., Fantozzi, I., Bondanza, S., Ponzin, D., McKeon, F., and De Luca, M. (2001). P63 Identifies Keratinocyte Stem Cells. Proc. Natl. Acad. Sci. U. S. A. *98,* 3156-3161.

Pepper, M.S. (1997). Transforming growth factor-beta: vasculogenesis, angiogenesis, and vessel wall integrity. Cytokine Growth Factor Rev. *8,* 21-43.

Petkovich, P.M. (2001). Retinoic acid metabolism. J Am Acad Dermatol *45,* S136-S142.

Pierceall, W.E., Kripke, M.L., and Ananthaswamy, H.N. (1992). N-ras mutation in ultraviolet radiation-induced murine skin cancers. Cancer Res. *52,* 3946-3951.

Potten, C.S., Saffhill, R., and Maibach, H.I. (1987). Measurement of the transit time for cells through the epidermis and stratum corneum of the mouse and guinea-pig. Cell Tissue Kinet. *20,* 461-472.

Qiu, H., Zhang, W., El-Naggar, A.K., Lippman, S.M., Lin, P., Lotan, R., and Xu, X.C. (1999). Loss of retinoic acid receptor-beta expression is an early event during esophageal carcinogenesis. Am. J. Pathol. *155,* 1519-1523.

Raick, A.N. (1973). Late ultrastructural changes induced by 12-O-tetradecanoyl-phorbol-13-acetate in mouse epidermis and their reversal. Cancer Res. *33,* 1096-1103.

Rastinejad, F., Wagner, T., Zhao, Q., and Khorasanizadeh, S. (2000). Structure of the RXR-RAR DNA-binding complex on the retinoic acid response element DR1. EMBO J *19,* 1045-1054.

Ray, W.J., Bain, G., Yao, M., and Gottlieb, D.I. (1997). CYP26, a novel mammalian cytochrome P450, is induced by retinoic acid and defines a new family. J Biol Chem *272,* 18702-18708.

Re, F.C., Manenti, G., Borrello, M.G., Colombo, M.P., Fisher, J.H., Pierotti, M.A., Della Porta, G., and Dragani, T.A. (1992). Multiple molecular alterations in mouse lung tumors. Mol. Carcinog. *5,* 155-160.

Rehman, I., Lowry, D.T., Adams, C., Abdel-Fattah, R., Holly, A., Yuspa, S.H., and Hennings, H. (2000). Frequent codon 12 Ki-ras mutations in mouse skin tumors initiated by N-methyl-N'-nitro-N-nitrosoguanidine and promoted by mezerein. Mol. Carcinog. *27,* 298-307.

Repa, J.J., Hanson, K.K., and Clagett-Dame, M. (1993). All-trans-retinol is a ligand for the retinoic acid receptors. Proc. Natl. Acad. Sci. U. S. A. *90,* 7293-7297.

Reynisdottir, I., and Massague, J. (1997). The subcellular locations of p15(Ink4b) and p27(Kip1) coordinate their inhibitory interactions with cdk4 and cdk2. Genes Dev. *11,* 492-503.

Roa, B.B., Dyck, P.J., Marks, H.G., Chance, P.F., and Lupski, J.R. (1993a). Dejerine-Sottas syndrome associated with point mutation in the peripheral myelin protein 22 (PMP22) gene. Nat. Genet. *5,* 269-273.

Roa, B.B., Garcia, C.A., Pentao, L., Killian, J.M., Trask, B.J., Suter, U., Snipes, G.J., Ortiz-Lopez, R., Shooter, E.M., Patel, P.I., and Lupski, J.R. (1993b). Evidence for a recessive PMP22 point mutation in Charcot-Marie-Tooth disease type 1A. Nat. Genet. *5,* 189-194.

Roa, B.B., Garcia, C.A., Suter, U., Kulpa, D.A., Wise, C.A., Mueller, J., Welcher, A.A., Snipes, G.J., Shooter, E.M., Patel, P.I., and Lupski, J.R. (1993c). Charcot-Marie-Tooth

disease type 1A. Association with a spontaneous point mutation in the PMP22 gene. N. Engl. J. Med. *329,* 96-101.

Roberts, A.B., and Wakefield, L.M. (2003). The two faces of transforming growth factor beta in carcinogenesis. Proc. Natl. Acad. Sci. U. S. A. *100,* 8621-8623.

Robertson, A.M., Perea, J., McGuigan, A., King, R.H., Muddle, J.R., Gabreels-Festen, A.A., Thomas, P.K., and Huxley, C. (2002). Comparison of a new pmp22 transgenic mouse line with other mouse models and human patients with CMT1A. J. Anat. *200,* 377-390.

Romani, N., Lenz, A., Glassel, H., Stossel, H., Stanzl, U., Majdic, O., Fritsch, P., and Schuler, G. (1989). Cultured human Langerhans cells resemble lymphoid dendritic cells in phenotype and function. J Invest Dermatol *93,* 600-609.

Ross, S.A., McCaffery, P.J., Drager, U.C., and De Luca, L.M. (2000). Retinoids in embryonal development. Physiol Rev *80,* 1021-1054.

Rothwarf, D.M., Zandi, E., Natoli, G., and Karin, M. (1998). IKK-gamma is an essential regulatory subunit of the IkappaB kinase complex. Nature *395,* 297-300.

Roux, K.J., Amici, S.A., and Notterpek, L. (2004). The temporospatial expression of peripheral myelin protein 22 at the developing blood-nerve and blood-brain barriers. J Comp Neurol *474,* 578-588.

Rozmahel, R., Wilschanski, M., Matin, A., Plyte, S., Oliver, M., Auerbach, W., Moore, A., Forstner, J., Durie, P., Nadeau, J., Bear, C., and Tsui, L.C. (1996). Modulation of disease severity in cystic fibrosis transmembrane conductance regulator deficient mice by a secondary genetic factor. Nat. Genet. *12,* 280-287.

Ruggeri, B., Caamano, J., Goodrow, T., DiRado, M., Bianchi, A., Trono, D., Conti, C.J., and Klein-Szanto, A.J. (1991). Alterations of the p53 tumor suppressor gene during mouse skin tumor progression. Cancer Res. *51,* 6615-6621.

Samokyszyn, V.M., Gall, W.E., Zawada, G., Freyaldenhoven, M.A., Chen, G., Mackenzie, P.I., Tephly, T.R., and Radominska-Pandya, A. (2000). 4-hydroxyretinoic acid, a novel substrate for human liver microsomal UDP-glucuronosyltransferase(s) and recombinant UGT2B7. J. Biol. Chem. *275,* 6908-6914.

Sass, J.O., Tzimas, G., and Nau, H. (1997). 9,13-Dicis-retinoic acid as an isomerization product of 9-cis-retinoic acid. Drug Metab. Dispos. *25,* 1447-1448.

Schneider, C., King, R.M., and Philipson, L. (1988). Genes specifically expressed at growth arrest of mammalian cells. Cell *54,* 787-793.

Segre, J.A., Bauer, C., and Fuchs, E. (1999). Klf4 is a transcription factor required for establishing the barrier function of the skin. Nat Genet *22,* 356-360.

Sekiya, T., Fushimi, M., Hori, H., Hirohashi, S., Nishimura, S., and Sugimura, T. (1984). Molecular cloning and the total nucleotide sequence of the human c-Ha-ras-1 gene

activated in a melanoma from a Japanese patient. Proc. Natl. Acad. Sci. U. S. A. *81,* 4771-4775.

Shiohara, M., Dawson, M.I., Hobbs, P.D., Sawai, N., Higuchi, T., Koike, K., Komiyama, A., and Koeffler, H.P. (1999). Effects of novel RAR- and RXR-selective retinoids on myeloid leukemic proliferation and differentiation in vitro. Blood *93,* 2057-2066.

Shipley, G.D., Tucker, R.F., and Moses, H.L. (1985). Type beta transforming growth factor/growth inhibitor stimulates entry of monolayer cultures of AKR-2B cells into S phase after a prolonged prereplicative interval. Proc. Natl. Acad. Sci. U. S. A. *82,* 4147-4151.

Skeen, J.E., Bhaskar, P.T., Chen, C.C., Chen, W.S., Peng, X.D., Nogueira, V., Hahn-Windgassen, A., Kiyokawa, H., and Hay, N. (2006). Akt deficiency impairs normal cell proliferation and suppresses oncogenesis in a p53-independent and mTORC1-dependent manner. Cancer. Cell. *10,* 269-280.

Slaga, T.J. (1984). Multistage skin carcinogenesis: a useful model for the study of the chemoprevention of cancer. Acta Pharmacol Toxicol (Copenh) *55 Suppl 2,* 107-124.

Snipes, G.J., Suter, U., Welcher, A.A., and Shooter, E.M. (1992). Characterization of a novel peripheral nervous system myelin protein (PMP-22/SR13). J Cell Biol *117,* 225-238.

Soprano, D.R., Qin, P., and Soprano, K.J. (2004). Retinoic acid receptors and cancers. Annu Rev Nutr *24,* 201-221.

Sporn, M.B., Dunlop, N.M., Newton, D.L., and Smith, J.M. (1976). Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids). Fed. Proc. *35,* 1332-1338.

Spreyer, P., Kuhn, G., Hanemann, C.O., Gillen, C., Schaal, H., Kuhn, R., Lemke, G., and Muller, H.W. (1991). Axon-regulated expression of a Schwann cell transcript that is homologous to a 'growth arrest-specific' gene. EMBO J. *10,* 3661-3668.

Stephens, L., Anderson, K., Stokoe, D., Erdjument-Bromage, H., Painter, G.F., Holmes, A.B., Gaffney, P.R., Reese, C.B., McCormick, F., Tempst, P., Coadwell, J., and Hawkins, P.T. (1998). Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. Science *279,* 710-714.

Studer, U.E., Biedermann, C., Chollet, D., Karrer, P., Kraft, R., Toggenburg, H., and Vonbank, F. (1984). Prevention of recurrent superficial bladder tumors by oral etretinate: preliminary results of a randomized, double blind multicenter trial in Switzerland. J. Urol. *131,* 47-49.

Sun, S.Y., and Lotan, R. (2002). Retinoids and their receptors in cancer development and chemoprevention. Crit Rev Oncol Hematol *41,* 41-55.

Suter, U., and Snipes, G.J. (1995). Peripheral myelin protein 22: facts and hypotheses. J Neurosci Res *40,* 145-151.

Suter, U., Snipes, G.J., Schoener-Scott, R., Welcher, A.A., Pareek, S., Lupski, J.R., Murphy, R.A., Shooter, E.M., and Patel, P.I. (1994). Regulation of tissue-specific expression of alternative peripheral myelin protein-22 (PMP22) gene transcripts by two promoters. J Biol Chem *269,* 25795-25808.

Suter, U., Welcher, A.A., Ozcelik, T., Snipes, G.J., Kosaras, B., Francke, U., Billings-Gagliardi, S., Sidman, R.L., and Shooter, E.M. (1992). Trembler mouse carries a point mutation in a myelin gene. Nature *356,* 241-244.

Swindell, E.C., and Eichele, G. (1999). Retinoid metabolizing enzymes in development. Biofactors *10,* 85-89.

Tachibana, T. (1995). The Merkel cell: recent findings and unresolved problems. Arch Histol Cytol *58,* 379-396.

Taimi, M., Helvig, C., Wisniewski, J., Ramshaw, H., White, J., Amad, M., Korczak, B., and Petkovich, M. (2004). A novel human cytochrome P450, CYP26C1, involved in metabolism of 9-cis and all-trans isomers of retinoic acid. J Biol Chem *279,* 77-85.

Tallman, M.S., Andersen, J.W., Schiffer, C.A., Appelbaum, F.R., Feusner, J.H., Ogden, A., Shepherd, L., Willman, C., Bloomfield, C.D., Rowe, J.M., and Wiernik, P.H. (1997). All-trans-retinoic acid in acute promyelocytic leukemia. N. Engl. J. Med. *337,* 1021-1028.

Taylor, V., Welcher, A.A., Program, A.E., and Suter, U. (1995). Epithelial membrane protein-1, peripheral myelin protein 22, and lens membrane protein 20 define a novel gene family. J Biol Chem *270,* 28824-28833.

Testoni, B., and Mantovani, R. (2006). Mechanisms of transcriptional repression of cell-cycle G2/M promoters by p63. Nucleic Acids Res. *34,* 928-938.

Thompson, J.N., Howell, J.M., Pitt, G.A., and McLaughlin, C.I. (1969). The biological activity of retinoic acid in the domestic fowl and the effects of vitamin A deficiency on the chick embryo. Br. J. Nutr. *23,* 471-490.

Timmerman, V., Raeymaekers, P., Nelis, E., De Jonghe, P., Muylle, L., Ceuterick, C., Martin, J.J., and Van Broeckhoven, C. (1992). Linkage analysis of distal hereditary motor neuropathy type II (distal HMN II) in a single pedigree. J. Neurol. Sci. *109,* 41-48.

Tucker, R.F., Shipley, G.D., Moses, H.L., and Holley, R.W. (1984). Growth inhibitor from BSC-1 cells closely related to platelet type beta transforming growth factor. Science *226,* 705-707.

Valentijn, L.J., Baas, F., Wolterman, R.A., Hoogendijk, J.E., van den Bosch, N.H., Zorn, I., Gabreels-Festen, A.W., de Visser, M., and Bolhuis, P.A. (1992). Identical point mutations of PMP-22 in Trembler-J mouse and Charcot-Marie-Tooth disease type 1A. Nat. Genet. *2,* 288-291.

van Dartel, M., Cornelissen, P.W., Redeker, S., Tarkkanen, M., Knuutila, S., Hogendoorn, P.C., Westerveld, A., Gomes, I., Bras, J., and Hulsebos, T.J. (2002).

Amplification of 17p11.2 approximately p12, including PMP22, TOP3A, and MAPK7, in high-grade osteosarcoma. Cancer Genet Cytogenet *139,* 91-96.

van Dartel, M., and Hulsebos, T.J. (2004). Amplification and overexpression of genes in 17p11.2 ~ p12 in osteosarcoma. Cancer Genet Cytogenet *153,* 77-80.

van Dartel, M., Leenstra, S., Troost, D., and Hulsebos, T.J. (2003). Infrequent but high-level amplification of 17p11.2 approximately p12 in human glioma. Cancer Genet Cytogenet *140,* 162-166.

Van Itallie, C.M., and Anderson, J.M. (2006). Claudins and epithelial paracellular transport. Annu Rev Physiol *68,* 403-429.

Van Laethem, A., Claerhout, S., Garmyn, M., and Agostinis, P. (2005). The sunburn cell: regulation of death and survival of the keratinocyte. Int. J. Biochem. Cell Biol. *37,* 1547-1553.

Verma, A.K., and Boutwell, R.K. (1977). Vitamin A acid (retinoic acid), a potent inhibitor of 12-O-tetradecanoyl-phorbol-13-acetate-induced ornithine decarboxylase activity in mouse epidermis. Cancer Res. *37,* 2196-2201.

Verma, A.K., Garcia, C.T., Ashendel, C.L., and Boutwell, R.K. (1983). Inhibition of 7-bromomethylbenz[a]anthracene-promoted mouse skin tumor formation by retinoic acid and dexamethasone. Cancer Res. *43,* 3045-3049.

Verma, A.K., Shapas, B.G., Rice, H.M., and Boutwell, R.K. (1979). Correlation of the inhibition by retinoids of tumor promoter-induced mouse epidermal ornithine decarboxylase activity and of skin tumor promotion. Cancer Res *39,* 419-425.

Verma, A.K., Slaga, T.J., Wertz, P.W., Mueller, G.C., and Boutwell, R.K. (1980). Inhibition of skin tumor promotion by retinoic acid and its metabolite 5,6-epoxyretinoic acid. Cancer Res *40,* 2367-2371.

Wang, Y., and Steinbeisser, H. (2009). Molecular basis of morphogenesis during vertebrate gastrulation. Cell Mol. Life Sci. *66,* 2263-2273.

Watt, F.M. (2002). The stem cell compartment in human interfollicular epidermis. J. Dermatol. Sci. *28,* 173-180.

Weinberg, R.A. (1995). The retinoblastoma protein and cell cycle control. Cell *81,* 323-330.

Weinberg, R.A. (1985). The action of oncogenes in the cytoplasm and nucleus. Science *230,* 770-776.

Welcher, A.A., Suter, U., De Leon, M., Snipes, G.J., and Shooter, E.M. (1991). A myelin protein is encoded by the homologue of a growth arrest-specific gene. Proc. Natl. Acad. Sci. U. S. A. *88,* 7195-7199.

Wellik, D.M., and DeLuca, H.F. (1995). Retinol in addition to retinoic acid is required for successful gestation in vitamin A-deficient rats. Biol Reprod *53,* 1392-1397.

Westfall, M.D., Mays, D.J., Sniezek, J.C., and Pietenpol, J.A. (2003). The Delta Np63 alpha phosphoprotein binds the p21 and 14-3-3 sigma promoters in vivo and has transcriptional repressor activity that is reduced by Hay-Wells syndrome-derived mutations. Mol. Cell. Biol. *23,* 2264-2276.

White, J.A., Guo, Y.D., Baetz, K., Beckett-Jones, B., Bonasoro, J., Hsu, K.E., Dilworth, F.J., Jones, G., and Petkovich, M. (1996). Identification of the retinoic acid-inducible all-trans-retinoic acid 4-hydroxylase. J. Biol. Chem. *271,* 29922-29927.

Willy, P.J., Umesono, K., Ong, E.S., Evans, R.M., Heyman, R.A., and Mangelsdorf, D.J. (1995). LXR, a nuclear receptor that defines a distinct retinoid response pathway. Genes Dev *9,* 1033-1045.

Xu, J., Lamouille, S., and Derynck, R. (2009). TGF-beta-induced epithelial to mesenchymal transition. Cell Res. *19,* 156-172.

Xu, X.C., Sneige, N., Liu, X., Nandagiri, R., Lee, J.J., Lukmanji, F., Hortobagyi, G., Lippman, S.M., Dhingra, K., and Lotan, R. (1997). Progressive decrease in nuclear retinoic acid receptor beta messenger RNA level during breast carcinogenesis. Cancer Res. *57,* 4992-4996.

Xu, X.C., Wong, W.Y., Goldberg, L., Baer, S.C., Wolf, J.E., Ramsdell, W.M., Alberts, D.S., Lippman, S.M., and Lotan, R. (2001). Progressive decreases in nuclear retinoid receptors during skin squamous carcinogenesis. Cancer Res *61,* 4306-4310.

Yuspa, S.H. (1994). The pathogenesis of squamous cell cancer: lessons learned from studies of skin carcinogenesis--thirty-third G. H. A. Clowes Memorial Award Lecture. Cancer Res *54,* 1178-1189.

Yuspa, S.H., Ben, T., Hennings, H., and Lichti, U. (1982). Divergent responses in epidermal basal cells exposed to the tumor promoter 12-O-tetradecanoylphorbol-13-acetate. Cancer Res *42,* 2344-2349.

Yuspa, S.H., Kilkenny, A., Cheng, C., Roop, D., Hennings, H., Kruszewski, F., Lee, E., Strickland, J., and Greenhalgh, D.A. (1991). Alterations in epidermal biochemistry as a consequence of stage-specific genetic changes in skin carcinogenesis. Environ. Health Perspect. *93,* 3-10.

Yuspa, S.H., Lichti, U., Morgan, D., and Hennings, H. (1980). Chemical carcinogenesis studies in mouse epidermal cell cultures. Curr Probl Dermatol *10,* 171-191.

Yuspa, S.H., and Poirier, M.C. (1988). Chemical carcinogenesis: from animal models to molecular models in one decade. Adv. Cancer Res. *50,* 25-70.

Zandi, E., Rothwarf, D.M., Delhase, M., Hayakawa, M., and Karin, M. (1997). The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. Cell *91,* 243-252.

Zentella, A., and Massague, J. (1992). Transforming growth factor beta induces myoblast differentiation in the presence of mitogens. Proc. Natl. Acad. Sci. U. S. A. *89,* 5176-5180.

Ziegler, A., Jonason, A.S., Leffell, D.J., Simon, J.A., Sharma, H.W., Kimmelman, J., Remington, L., Jacks, T., and Brash, D.E. (1994). Sunburn and p53 in the onset of skin cancer. Nature *372,* 773-776.

Zoidl, G., Blass-Kampmann, S., D'Urso, D., Schmalenbach, C., and Muller, H.W. (1995). Retroviral-mediated gene transfer of the peripheral myelin protein PMP22 in Schwann cells: modulation of cell growth. EMBO J *14,* 1122-1128.

Zoidl, G., D'Urso, D., Blass-Kampmann, S., Schmalenbach, C., Kuhn, R., and Muller, H.W. (1997). Influence of elevated expression of rat wild-type PMP22 and its mutant PMP22Trembler on cell growth of NIH3T3 fibroblasts. Cell Tissue Res *287,* 459-470.