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ABSTRACT

Skin carcinogenesis is a multistage process consisting of three distinct stages: initiation, promotion and malignant conversion. The multistep nature of cancer is characterized by progressive activation or loss of signaling pathways, which dictate the behavior of tumor cells, its interaction with neighboring cells, local microenvironment and organism. One of the experimental models that have been instrumental in investigating the stages of cancer formation is the mouse multistage skin carcinogenesis protocol. In this model, a carcinogen initiates cells by introducing a mutation in a Ras gene, and the initiated population expands under treatment with phorbol esters, typically 12-O-tetradecanoylphorbol-13-acetate (TPA). Eventually initiated cells outgrow the normal epidermal cells to form benign papillomas. Although phorbol esters are the prototypes of tumor promoters in skin, the nature of their interaction with Ras signaling in tumor promotion remains to be determined. RasGRP1 is a member of the guanine nucleotide exchange factor family for Ras that binds with high affinity to ultrapotent diacylglycerol analogs like the phorbol esters. The ability to bind to phorbol esters and to modulate Ras activity, make RasGRP1 an attractive candidate for an additional target of the phorbol esters in skin that could directly link phorbol ester signaling with Ras cascades and the tumorigenic process. Recent work from our lab has demonstrated expression of RasGRP1 in epidermal keratinocytes. Also, further work using a transgenic murine model that overexpresses RasGRP1 in basal keratinocytes has suggested that RasGRP1 participates in the action of tumor promoting phorbol esters like TPA in the skin. However, the relative contribution of RasGRP1 to Ras signals in keratinocytes and its role in tumor promotion and/or progression in response to the phorbol ester TPA in the absence of overexpression remains to be defined. Thus, the overall goal of this study is to investigate if RasGRP1 is a critical link to Ras activation in mouse epidermal
keratinocytes in response to tumor promoter TPA and also to ascertain the relative contribution of RasGRP1 in skin carcinogenesis.
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1 SPECIFIC AIMS

1.1 SPECIFIC AIM 1. Investigate the functional role of RasGRP1 in the actions of phorbol esters in keratinocytes

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2 BACKGROUND

2.1 Nonmelanoma skin cancer

Nonmelanoma skin cancer (NMSC) is the most common malignancy in the United States with an estimated 1.2 million new cases diagnosed every year accounting for 40 percent of all new cancer cases (Johnson et al., 1998; Parker et al., 1997). It is also among the most costly forms of cancers to treat (Housman et al., 2003). Approximately 80 percent of nonmelanoma skin cancers are basal-cell carcinomas (BCCs), and 20 percent are squamous-cell carcinomas (SCCs) (Kwa et al., 1992). Unlike almost all BCCs, SCCs are associated with a substantial risk of metastasis resulting in estimated 8,000 cases of nodal metastasis and 3,000 deaths in the United States annually (Brantsch et al., 2008; Rowe et al., 1992). Additionally, a high percentage of patients with a primary SCC develop a second primary skin cancer within 5 years of diagnosis (Frankel et al., 1992; Karagas et al., 1992). Many extrinsic and intrinsic factors are known to regulate the pathogenesis of SCC. Exposure to the sunlight UV is generally recognized as the most important extrinsic factor, as SCCs occur primarily on sun-exposed areas of the body and have been strongly associated with chronic sun exposure (Kwa et al., 1992). Other extrinsic factors that are related to the pathogenesis of SCCs are industrial carcinogens, such as pitch, tar, crude paraffin oil, fuel oil, creosote, lubricating oils, arsenic, and nitrosoureas (Marks, 1996). Intrinsic factors associated with squamous cell carcinoma include age, lighter skin pigmentation, scars, dermatoses associated with photosensitivity, and ulcerations (Lohmann and Solomon, 2001). Although the incidence of NMSC is widespread and increasing, its molecular pathogenesis has been poorly understood. Currently, there are no effective targeted therapies for NMSC and the majority of efforts rely primarily on early detection and surgical excision (Green and Khavari, 2004).
2.2 Skin structure

The skin is the largest organ in the body roughly accounting for 16 percent of body weight and an area of 1.8 m$^2$. There are three structural layers to the skin: the epidermis, the dermis and the subcutis. The epidermis is the outermost layer of the skin mainly comprising of keratinocytes but also containing melanocytes, Langerhans cells and Merkel cells. The dermis represents the area of supportive connective tissue between the epidermis and the underlying subcutis containing sweat glands, hair roots, nervous cells, and blood and lymph vessels while the subcutis forms layers of loose connective tissue and fat beneath the dermis (Freinkel and Woodley, 2001).

The epidermis is a stratified squamous epithelium. The five layers of epidermis, moving from the basal layer upwards towards the surface, are stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum (Figure 1). The homeostasis of epidermis is maintained by cell division in the basal layer and terminal differentiation in the suprabasal layers of the epidermis. Delamination of differentiating cells causes them to be displaced upwards through the epidermal layers. In the stratum corneum the cells become mitotically inactive, lose their nucleus and fuse to form squamous sheets, which are eventually shed from the surface thereby ensuring that the rate of production equals rate of loss in normal skin (Rook and Burns, 2004). It is noteworthy that most human cancers are epithelial in nature in which the homeostatic balance of cell production and cell loss is altered (Ehrenreiter et al., 2009; Hanahan and Weinberg, 2000).
2.3 Cellular origin of skin cancer

Skin cancers arise predominantly from the epidermis as a result of the transformation of keratinocytes. The epidermis functions to protect our bodies from a wide array of environmental assaults, including ultraviolet (UV) irradiation, chemical carcinogens, and entry of viruses and other pathogens. It is therefore not difficult to fathom that the epidermal keratinocytes are at a high risk of acquiring an oncogenic mutation. However, considering the magnitude of insults to the epidermis, relatively few skin malignancies develop, as most cells that acquire oncogenic mutations are lost through the process of terminal differentiation (Owens and Watt, 2003). It has been postulated that multiple events are required to transform a normal cell into a cancer cell in both humans and rodents (Hahn et al., 1999; Hahn and Weinberg, 2002) that led to
the idea that only long-term residents of the epidermis, or in other words the epidermal stem cells, have the ability to accumulate the number of genetic hits that are necessary to form tumors. A number of studies have revealed that the cells of the bulge region of the hair follicles display all of the biological characteristics of a stem cell population including multipotentiality and the ability to respond to morphogenetic signals to form hair follicles, sebaceous glands and the epidermis (Blanpain et al., 2004; Oshima et al., 2001). Although the bulge region of the hair follicle seems to contain the most important pool of self-renewing cells (Cotsarelis et al., 1990; Taylor et al., 2000), it is unlikely to be the only reservoir of the epidermal stem cells as different populations of stem cells have been identified in the skin (Ghazizadeh and Taichman, 2001; Kamimura et al., 1997; Potten and Booth, 2002). Previous studies using rodent models led to a proposal that while most malignant carcinomas arise from hair follicle region, low risk, benign papillomas arise from the interfollicular epidermal cells (Bailleul et al., 1990; Brown et al., 1998). Recent studies using elegant rodent models have further supported the notion that though the bulge stem cells and their immediate progenitors (hair germ and outer root sheath) are the primary cells of origin of skin carcinogenesis (White et al., 2011), the interfollicular epidermis can also serve as target sites for skin carcinogenesis, demonstrating thereby that initiation is not restricted to the hair follicle lineages (Lapouge et al., 2011).

2.4 Multistage carcinogenesis model

The ability to model human cancer in a laboratory setting has provided valuable insights into the mechanistic details of neoplasia thereby laying the ground work for development of new cancer therapies. Rodent models are commonly used as predictors of carcinogenic risk to humans and over the years have provided substantial mechanistic information applicable to human disease (Lynch et al., 2007). Over the years different
mouse models have been used to study cancer. Insertional mutagenesis have been used to screen for potential oncogenes and tumor suppressor genes, the xenograft model has been used extensively for pre-clinical studies of cancer, and the use of chemically-induced carcinogenesis as well as genetically engineered mouse models have also helped us improve our understanding of tumorigenesis quite significantly. Among these models, the mouse multistage carcinogenesis model has helped us to define cancer as a multistep process and is considered to be a paradigmatic model system to study tumorigenesis. This model defines the stages of carcinogenesis as initiation of the cells by an irreversible mutation in the Ras proto-oncogene caused by a single topical application of a carcinogen, typically 7,12-dimethylbenzanthracene (DMBA), at a sub-threshold level followed by clonal expansion of initiated cells by repeated topical application of a tumor promoter, typically 12-O-tetradecanoylphorbol-13-acetate (TPA), to form visible benign outgrowths known as papillomas, and finally malignant progression of the papillomas to cancer (Figure 2)(Yuspa, 1994; Yuspa et al., 1996). It is noteworthy that the papillomas that develop initially during initiation-promotion protocols are heterogeneous in that some can persist, some regress, while some can progress to SCC over time, depending on several factors such as papilloma risk-type and the genetic background of the mouse strain (DiGiovanni, 1992; Yuspa et al., 1990).

Based on analyses of this model, initiation is a rapid process with no apparent morphological changes in the epidermis (Scribner and Suss, 1978) and occurs due to the interaction of a reactive form of a carcinogen with the DNA of the epidermal target cells (Brookes and Lawley, 1964; Yuspa and Poirier, 1988). The discovery of chemical substances with low carcinogenic activity which were still able to induce the development of cancer under experimental conditions led to the concept of tumor
promotion (Berenblum and Shubik, 1947). Tumor promoting compounds do not directly interact with DNA but cause enhanced cell proliferation, alterations in genetic expression and cellular growth control (DiGiovanni, 1992). In stark contrast to initiation, treatment of mouse skin with a tumor promoting agent is characterized by dramatic morphological and biochemical effects, which are reversible in the absence of continued treatment (Aldaz et al., 1985; Argyris, 1981; Boutwell, 1974, 1976). Since tumor promotion accounts for most of the latent period in carcinogenesis and is a reversible process (Yuspa et al., 1996), it is an attractive stage from the perspective of chemoprevention and cancer therapeutics.

2.5 Oncogenes and tumor suppressors in skin cancer

The development of cancer is a reflection of stepwise activation or loss of signaling pathways that provide a selective growth advantage to the incipient cancer
cells. SCCs of the skin develop through a multistep process that involves activation of proto-oncogenes and/or inactivation of tumor suppressor genes in keratinocytes. Although the pathways required to convert normal epidermis to SCC is yet to be delineated completely, there is strong evidence indicating that activation of Ras signaling and mutations in p53 are critically involved in the transformation of normal human epidermis to SCC (Green and Khavari, 2004; Pierceall et al., 1991b). Recent studies using elegant mouse models have shown that although the mere expression of an oncogenic K-Ras in the bulge stem cells and interfollicular epidermis was sufficient to cause papilloma formation, combined expression of oncogenic K-Ras and p53 deletion was necessary to initiate SCC in the epidermis (Lapouge et al., 2011; White et al., 2011) thereby highlighting the requirement of both Ras and p53 in the pathogenesis of cutaneous SCC.

The prototypical tumor suppressor, p53, is mutated in various human cancers, including ~50 % of human skin cancers (Giglia-Mari and Sarasin, 2003). p53 mutations have also been observed in both chemically-induced and UV-induced mouse skin tumors (Ruggeri et al., 1993; van Kranen et al., 1995; Zhang et al., 1998). Interestingly, inactivation of p53 is not observed in papillomas but in SCC (Zhang et al., 1998) and the fact that deficiency of p53 promotes malignant progression but reduces the total number of tumors in the multistage carcinogenesis model (Kemp et al., 1993) suggests that p53 inactivation plays a role in tumor progression but not tumor promotion.

Activated Ras stimulates a multitude of downstream effectors, most of which have been implicated in skin carcinogenesis using the multistage carcinogenesis model. Different Ras effectors are known to modulate different aspects of tumorigenesis. While the PI3K pathway (Gupta et al., 2007) and Ral GDS (Gonzalez-Garcia et al., 2005) have
been implicated in cell survival, Tiam1-mediated Rac activation is needed for effective initiation of oncogenic Ras-induced epidermal tumors (Malliri et al., 2002).

2.6 Ras oncogene

In 1964 Jennifer Harvey observed that a preparation of a murine leukemia virus, taken from a leukemic rat, induced sarcomas in new-born rodents (Harvey, 1964), a finding that heralded the beginning of Ras research. The transforming principle of the murine sarcoma virus was later identified to be Ras oncogene, which was christened as H-Ras after Jennifer Harvey (Chang et al., 1982). Later, two additional Ras isoforms, K Ras and N Ras were identified (Kirsten and Mayer, 1967; Shimizu et al., 1983). Activating Ras mutations occur in ~ 30% of human cancers (Schubbert et al., 2007). Although Ras mutations are found in 10-30% of human skin cancers (Pierceall et al., 1991a; Spencer et al., 1995), majority of spontaneous human skin cancers display hyperactivation of Ras even in the absence of somatic Ras mutations, as other factors like overexpression of receptor tyrosine kinases are known to activate the pathway in SCC (Dajee et al., 2003; Uribe and Gonzalez, 2011).

Newly synthesized Ras isoforms, which are cytosolic proteins, require to be post-translationally modified to become membrane-associated and biologically active. Ras proteins are prototypical members of another class of proteins often referred to as CAAX proteins by virtue of a CAAX motif (C denotes cysteine, A represents any aliphatic amino acid and X may be any amino acid in their carboxyl terminus) (Konstantinopoulos et al., 2007). The CAAX motif serves as a substrate for a series of post-translational modifications that are critical for membrane targeting and biological function of Ras proteins. The first step is farnesylation of the cysteine residue catalyzed by farnesyltransferase, followed by removal of the AAX residues by Ras converting enzyme
(RCE) and finally methyl esterification of the carboxyl group of the terminal cysteine residue (Hancock et al., 1991; Hancock et al., 1989; Hancock et al., 1990; Laude and Prior, 2008).

Ras proteins are small GTPases that cycle between inactive guanosine diphosphate (GDP)-bound and active guanosine triphosphate (GTP)-bound conformations (RasGDP and RasGTP, respectively) (Boguski and McCormick, 1993) and serve to link membrane receptor signals to internal effector pathways. Ras can regulate a wide array of cellular processes including proliferation, differentiation, actin reorganization and apoptosis by its ability to engage multiple downstream effectors (Mitin et al., 2005; Repasky et al., 2004).

Raf-1 was the first identified downstream effector of Ras signaling, and it was believed then that the primary function of Ras was simply to facilitate Raf-1 activation (Moodie et al., 1993). Many other Ras effectors were subsequently identified implicating Raf-1 independent pathways in cancer. PI3K is another well-characterized downstream effector of Ras, which is known to play an important role in mediating pro-survival and proliferative functions of Ras in oncogenesis (Vivanco and Sawyers, 2002). RalGDS represents another well studied downstream effector of Ras (Kikuchi et al., 1994; Schubbert et al., 2007) known to play an important role in tumor formation in multistage skin carcinogenesis model by controlling survival of transformed cells (Gonzalez-Garcia et al., 2005). Tiam1, another direct downstream Ras effector, is known to mediate Ras activation of Rac 1 (Lambert et al., 2002) which is known to play a role in various aspects of tumorigenicity (Malliri et al., 2006; Malliri et al., 2002).

2.7 Tumor promotion and phorbol esters

The term 'phorbol' is used to describe the family of naturally occurring compounds that can be referred to as tigliane diterpenes. Phorbol esters are defined as
“polycyclic compounds in which two hydroxyl groups on neighboring carbon atoms are esterified to fatty acids” (Goel et al., 2007) (Figure 3). They are generally known for their ability to activate protein kinase C (PKC) and for their tumor promoting activity in the skin (Goel et al., 2007), although not all phorbol esters are skin tumor promoters and some are even reported to have apoptotic effects on tumor cells (Brodie and Blumberg, 2003; Gonzalez-Guerrico and Kazanietz, 2005). The phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) is the prototype of tumor promoters in skin (Hecker and Schmidt, 1974; Sorg et al., 1982). TPA does not induce tumors but promotes tumor growth following exposure to a subcarcinogenic dose of a carcinogen like 7,12-dimethylbenzanthracene (DMBA) (Slaga et al., 1995). Topical application of TPA induces a wide array of biological effects in skin, such as induction of cyclooxygenase-2 (COX-2) and inflammation, production of reactive oxygen species and induction of epidermal hyperplasia.

![Structure of tetradecanoyl phorbol-13-acetate (TPA)](Evans, 1986).

Several previous studies have investigated the role of inflammation in tumor promotion in mouse skin carcinogenesis (Furstenberger et al., 1989; Muller-Decker et al., 2002; Tiano et al., 2002). Transgenic mice overexpressing a pro-inflammatory COX-2, which is involved in the biosynthesis of prostaglandins (PGs) from arachidonic acid, in
the basal keratinocytes were reported to be sensitized for tumor promotion (Muller-Decker et al., 2002). On the other hand, depletion of cox-2 protects against chemically-induced mouse skin tumorigenesis (Tiano et al., 2002). Several studies have suggested that inflammatory mediators including the different prostaglandins are functionally related to mouse skin tumor promotion (Furstenberger et al., 1989; Millan et al., 2006). Topical application of TPA has been shown to induce the expression of COX-2 and its mRNA transcript in mouse skin (Chun et al., 2003).

The stimulation of DNA synthesis by TPA is one of the hallmarks of hyper proliferative action of TPA and is indispensable for TPA-induced tumor promotion (Furstenberger and Marks, 1978). There is a good correlation between promoting abilities of a series of phorbol esters, including TPA, and their ability to stimulate epidermal hyperplasia (Slaga et al., 1976).

TPA has been shown to increase production of reactive oxygen species (ROS) in keratinocytes both in vitro and in vivo (Fischer et al., 1986; Perchellet and Perchellet, 1989) resulting in oxidative stress, which has been associated with tumor promotion (Franco et al., 2008; Nishigori et al., 2004; Weitzman and Gordon, 1990).

However, many of the molecular mechanisms responsible for this impressive set of biological effects elicited by TPA on mouse skin are still elusive and rather poorly defined therefore making it difficult to determine which of the many phorbol ester mediated responses are essential components of the promotion process.

### 2.8 Molecular targets of phorbol esters

Early studies on phorbol esters revealed that they could interact with protein kinase C (PKC) and that they were structural mimetics of the second messenger diacylglycerol (DAG), the endogenous PKC ligand (Castagna et al., 1982; Kikkawa et al., 1983; Leach et al., 1983). In fact, the discovery of PKC as a phorbol ester target was
one of the first indications that PKC could play a role in transformation and cancer. PKC is a family of serine/threonine kinases that regulates a diverse set of cellular processes including proliferation, apoptosis, cell survival and migration. There are nine Pkc genes, which code for isozymes classified into three groups: classical PKCs (cPKCs: PKCα, PKCβI, PKCβII and PKCγ), novel PKCs (nPKCs: PKCδ, PKCε, PKCη and PKCθ) and atypical PKCs (aPKCs: PKCζ and PKCζ). cPKCs can be activated by calcium and phorbol esters or DAG, whereas nPKCs are calcium independent. aPKCs are unresponsive to either calcium or DAG (Newton, 1997; Nishizuka, 1995). PKC α, δ and ε are widely expressed, whereas expression of the other PKCs is largely cell-type specific (Griner and Kazanietz, 2007). PKCα, PKCβ, PKCδ, PKCε, PKCζ and PKCη are the isoforms expressed in human and mouse skin (Denning, 2004; Gschwendt et al., 1992; Rennecke et al., 1999; Wang et al., 1993). Although PKCs have a clear, indisputable role in tumorigenesis the relative contribution of individual isozymes in this process has been difficult to assess owing to isozyme and cell type specific variations (Griner and Kazanietz, 2007).

One of the approaches that have shed some light into the role of PKC in skin cancer has been the use of genetically engineered mouse models. For example, PKCδ overexpression in the epidermis by transgenic approach induces resistance to tumor promotion by TPA (Reddig et al., 1999). Transgenic mice overexpressing PKCε in the skin, show a reduction in the papilloma burden in response to multistage carcinogenesis, but display enhanced carcinoma formation (Reddig et al., 2000). Although overexpression of PKCα in the epidermis does not affect skin tumorigenesis in the standard DMBA/TPA protocol (Wang and Smart, 1999), when a low dose of TPA is used, tumor development and conversion to carcinomas is greatly enhanced (Cataisson et al., 2009). Paradoxically, PKCα null mice also show an increased susceptibility to skin
carcinogenesis even though TPA-induced epidermal hyperplasia is reduced (Hara et al., 2005). Thus, PKCα appears to be important for TPA induction of proliferation and inflammation, but it has contradictory roles in skin tumor promotion. Overall, the distinct roles that each individual PKC isoform plays in the signal transduction pathways to mouse skin tumor promotion by TPA remain ambiguous.

Since phorbol esters act as ultra potent mimetics of the endogenous second messenger DAG (Kazanietz et al., 1994), and DAG has additional molecular targets besides PKCs, one emerging question is whether non-PKC DAG receptors can also contribute to tumor promotion in skin. There are at least four other families of proteins that can bind to DAG and phorbol esters. These include chimaerins, Munc13 proteins, Diacylglycerol kinases (DGKs) and Ras guanyl nucleotide-releasing proteins (RasGRPs) (Figure 4) (Griner and Kazanietz, 2007). All of these receptors can respond to phorbol esters with sensitivities similar to PKC due to the presence of a C1 domain, which is the DAG binding site (Betz et al., 1998; Caloca et al., 1999; Carmena and Sardini, 2007; Lorenzo et al., 2000).

Chimaerins were identified in the early 1990s as ‘chimaeras’ between PKCs (for their C1 domain) and the BCR protein (for their GTPase-activating protein or GAP domain) (Hall et al., 1990). There are four known chimaerin isoforms (α1, α2, β1 and β2) that are splice variants of the α (CHN1) and β (CHN2) genes respectively. Chimaerins are GTPase-activating proteins for Rac, a member of the Ras superfamily involved in cytoskeletal rearrangements (Ahmed et al., 1993). The C-terminal GAP domain in chimaerins can accelerate GTP hydrolysis from Rac, leading to its inactivation. GPCRs and tyrosine-kinase receptors are known to activate chimaerins through the phospholipase C (PLC)–DAG pathway, leading to their translocation from cytosol to the plasma membrane and association with Rac (Buttery et al., 2006; Siliceo et al., 2006; Wang et
As expected from their Rac-GAP activity, chimaerins have been found to inhibit Rac-dependent functions, including actin-cytoskeleton reorganization, migration, cell-cycle progression and metastasis (Menna et al., 2003; Yang et al., 2005). There is emerging information that chimaerins are downregulated in cancer, such as in high-grade gliomas and breast cancer (Hoelzinger et al., 2005; Yang et al., 2005; Yuan et al., 1995), indicating that they might have tumour-suppressor properties. Although chimaerins are present in a variety of tissues with the highest expression level detected in human brain and pancreas (Yuan et al., 1995), expression in the epidermis has not been demonstrated.

**Figure 4 - The Diacylglycerol/phorbol ester receptors.** Classical and novel protein kinase Cs (cPKCs and nPKCs respectively) contain two C1 domains which mediate binding to DAG and
phorbol esters, a C2 domain which senses calcium in the case of cPKCs and a serine/threonine-kinase domain. The chimaerin proteins each contain a single C1 domain and a Rac GTPase activating protein (Rac-GAP) domain. The α2-chimaerin and β2-chimaerin isoforms also contain a SRC homology-2 (SH2) domain. The Munc 13 proteins have multiple C2 domains in addition to the DAG binding C1 domain. Ras guanyl nucleotide releasing proteins (RasGRPs) 1, 3 and 4 have a DAG-responsive C1 domain, a catalytic Ras guanine nucleotide exchange factor (GEF), a pair of atypical EF hands and a Ras exchange motif (REM). Diacylglycerol kinases β and γ (DGKβ, DGKγ) have a pair of EF hands, two C1 domains and lipid kinase domain.

The Munc13 family comprises mammalian proteins that participate in synaptic transmission. Three mammalian isoforms exist: Munc13-1, Munc13-2, and Munc13-3 (Kazanietz, 2002). These DAG receptors are mainly expressed in neurons (Betz et al., 1998). These proteins have been shown to play essential roles in neurotransmitter release, specifically by interacting with elements of the exocytotic machinery. Neurons of Munc13-1 knockout mice are unable to form mature synaptic vesicles (Augustin et al., 1999). Also, Munc13-1 has been identified as the main presynaptic DAG/phorbol ester receptor in hippocampal neurons (Betz et al., 1998). Like chimaerins, the expression of Munc13 proteins in the epidermis has not been demonstrated.

The diacylglycerol kinases (DGKs) are a family of signaling proteins that modulate levels of DAG by catalyzing its conversion to phosphatidic acid (Hokin and Hokin, 1959). Of the ten members of the diacylglycerol kinase (DGK) family identified to date, only two, DGKβ and DGKγ are DAG/phorbol ester responsive (Shindo et al., 2003). Interestingly, although all the DGK family members possess C1 domains, sequence analysis has revealed that except the first C1 of DGK β and DGK γ, the C1 domains in other DGK members lack the key residues that define phorbol ester-binding domain (Hurley and Misra, 2000). The negative regulatory function of DGKs in DAG mediated effects would suggest a suppressor role in the malignant transformation for this family. However, DGKs are reported to act both as tumor suppressors and positive
regulators of survival and proliferation in transformed cells (Filigheddu et al., 2007; Merida et al., 2008) although their role in tumorigenesis in the skin is obscure and not well defined.

Finally, RasGRP is a family of guanine-nucleotide exchange factors (GEFs) for Ras, highly expressed in hematopoietic cells and neurons (Ebinu et al., 1998). Studies from our lab have revealed expression of one of the RasGRP isoforms- RasGRP1- in epidermal keratinocytes; the cells transformed in skin carcinogenesis protocols (Rambaratsingh et al., 2003). Interestingly, RasGRP1 can both activate Ras and bind to phorbol ester, which makes it an attractive candidate for an additional target for phorbol esters in tumor promotion. This has lead to intensive investigation from our laboratory to address the potential role of RasGRP1 in cutaneous SCC.

2.9 Regulation of Ras activation

Ras GEFs greatly increase the rate of GDP release from Ras, allowing Ras to bind the relatively prevalent cellular GTP and assume an activated conformation (Boriack-Sjodin et al., 1998). Ras GAPs (GTPase-activating proteins) accelerate the rate of GTP hydrolysis, returning Ras$^{GTP}$ to its inactive state (Wittinghofer et al., 1997) (Figure 5). At least three different protein families exhibit GEF activity toward Ras in mammalian cells: Sos, RasGRF and RasGRP (Boguski and McCormick, 1993; Ebinu et al., 1998; Jones and Jackson, 1998).
Figure 5 - Ras activity cycle.

Sos family members are involved in the coupling of tyrosine kinase receptors to Ras-dependent mitogenic signaling pathways (Schlessinger, 1993). Following ligand binding to a receptor tyrosine kinase like epidermal growth factor receptor (EGFR), Sos translocates from cytoplasm to the activated receptor in a phosphotyrosine dependent manner through adapter proteins such as Grb2. Grb2 contains two SH3 domains and one SH2 domain. The two SH3 domains are constitutively bound to a carboxy terminal proline rich region of Sos, and the Grb2 – Sos complex is recruited to activated receptors by interactions between the SH2 domain of Grb2 and phosphotyrosine residues on the receptor bringing Sos in close proximity of the membrane bound Ras where it can mediate Ras activation by displacing GDP, thereby allowing Ras to bind to the relatively abundant cellular GTP (Schlessinger, 1993). RasGRFs are induced to activate Ras by some G-protein coupled receptors such as muscarinic receptors by a phosphorylation dependent mechanism (Mattingly and Macara, 1996). RasGRF are also involved in Ca^{2+} influx/calmodulin-dependent activation of Ras (Farnsworth et al., 1995). RasGRF can activate H-Ras but not N-Ras or K-Ras in vivo (Jones and Jackson, 1998).
RasGRP activates Ras through mechanisms regulated by Ca\(^{2+}\) and DAG (Ebinu et al., 1998).

### 2.10 RasGRP

RasGRP was discovered in 1998 in a screen for cDNAs that could complement a transformation-defective allele of v-Ha-ras in fibroblasts (Ebinu et al., 1998). Later, another three isoforms of RasGRP were identified: RasGRP2, RasGRP3 and RasGRP4 (Kawasaki et al., 1998; Rebhun et al., 2000; Yamashita et al., 2000; Yang et al., 2002). RasGRP1, formerly known as RasGRP is the prototypical member of the RasGRP family. It possesses a catalytic region comprising of a GEF (Guanine nucleotide exchange factor) domain and an REM (Ras exchange motif) domain. In addition to the catalytic domain, it also has a pair of calcium binding EF hands that are atypical in that the region between the calcium binding loops consists of only 15 residues rather than the 20 to 30 residues typically found. Most importantly, RasGRP1 also has a DAG binding C1 domain (Ebinu et al., 1998) (Figure 6).

![Linear Structure of Ras GRP1](image)

**Figure 6 - Linear structure of RasGRP1.** RasGRP1 has a DAG-responsive C1 domain, a catalytic Ras guanine nucleotide exchange factor (GEF), a pair of atypical calcium binding EF hands and a Ras exchange motif (REM)

Recent studies have shown the presence of additional regulatory domains in RasGRP1. In B cells, Robert Kay and colleagues have shown the presence of previously
unrecognized plasma membrane targeting (PT) domain and the adjacent suppressor of plasma membrane targeting (SuPT) domain, which attenuates plasma membrane targeting activity of the PT domain thereby ensuring that RasGRP1 is not constitutively localized to the plasma membrane (Beaulieu et al., 2007). Interestingly, a recent study, also performed on B cells has shown that a basic/hydrophobic cluster, comprising of a 17 amino acid long segment containing lysines, arginines, tryptophan and phenylalanine, in the PT domain can mediate plasma membrane targeting of RasGRP1 in response to PI3K mediated generation of phosphoinositides in the plasma membrane, thereby potentially placing RasGRP1 as an integrator for convergence of signals from PI3K and PLC pathways (Zahedi et al., 2011).

All of the RasGRP isoforms show a similar structure, although they differ in their substrate recognition and ability to bind to DAG analogs. The initial functional studies on RasGRP looked at RasGRP1 and its ability to transform fibroblast on the basis of its activation of Ras. Interestingly, deletion studies showed the importance of the C1 domain, as lack of this domain abrogated its transformative capacity (Ebinu et al., 1998). It was later demonstrated that binding of the C1 domain to DAG analogs like phorbol esters, was indispensable for the recruitment of RasGRP1 to the plasma membranes where it mediated activation of Ras (Tognon et al., 1998). Subsequently, RasGRP1 was identified as a high affinity DAG/phorbol ester receptor (Lorenzo et al., 2000).

Biological studies using mouse models have shown a critical role of RasGRP1 in T-cell biology. Specifically, RasGRP1 couples the T-cell receptor (TCR) to the Ras-ERK cascade (Ebinu et al., 2000) and plays an important role in the positive selection of thymocytes as RasGRP1 null mice display a severe deficiency of single CD4$^+$ or CD8$^+$ positive thymocytes (Stone, 2006). In B cells, both RasGRP1 and RasGRP3 link B-cell-receptor signaling to the ERK cascade (Coughlin et al., 2005), suggesting a crucial role
in B-cell mitogenesis. RasGRP4, identified in a cDNA screen for transforming genes in cytogenetically normal acute myeloid leukemia (AML) patients (Reuther et al., 2002) is highly expressed in myeloid leukemia and possibly contributes to leukemogenesis (Watanabe-Okochi et al., 2009). It is also expressed in mast cells, where it plays an important role in mast cell development and function (Yang et al., 2002).

2.11 RasGRP1 and Ras induced tumorigenesis

Although the role of Ras in cancer is well established, the participation of GEFs like RasGRP is not entirely defined. The first indication that RasGRP1 could have oncogenic effects came from insertional mutagenesis screens to identify potential oncogenes in mice (Kim et al., 2003; Suzuki et al., 2002). More recently, RasGRP1 was identified as a gene involved in the resistance of hyperactive Ras-driven leukemias to MEK inhibitors (Lauchle et al., 2009). All this information points at a possible oncogenic role of RasGRP1 through its ability to modulate Ras. In this regard, it should be noted that while most of the emphasis on Ras oncogenic effects has been put on activating mutations in the Ras proto-oncogenes, increased activation of Ras by loss of GAPs (Bollag et al., 1996; Weiss et al., 1999) or overexpression/hyperactivation of certain growth factor receptors (Gibbs et al., 1990; Satoh et al., 1993; Sawyers and Denny, 1994) can also contribute to a malignant phenotype. As discussed above, Ras plays an important oncogenic role in cutaneous SCC and both mechanism of Ras activation – mutations and biochemical stimulation – have been found in human samples as well as in mouse models like the multistage carcinogenesis protocol (Field and Spandidos, 1990; Yarbrough et al., 1994; Yuspa, 1994).

One of the main factors influencing Ras hyperstimulation in cutaneous SCC is the overexpression of the EGF receptor (EGFR) (Ch'ng et al., 2008; Shimizu et al., 2001). EGFR can activate a number of downstream effectors, including PI3K, Ras via
Sos activation, and PLCγ, which leads to DAG and inositol triphosphate (IP3) generation (Buday and Downward, 1993; Egan et al., 1993; Gale et al., 1993; Nishibe et al., 1990). The DAG generated via PLCγ is a way to activate PKCs (Griner and Kazanietz, 2007) but also non-PKC phorbol ester receptors like RasGRP1 (Blumberg et al., 2008).

Therefore, the possibility exists that EGFR hyperstimulation – or other growth factor receptors – could use RasGRP1 as a link to Ras hyperactivation during skin cancer (Figure 7). In the mouse model of skin carcinogenesis, TPA acts a mimetic of DAG, and although TPA and DAG do not have similar actions, it recapitulates several aspects of the DAG response (Goel et al., 2007; Mullin and McGinn, 1988). Then, it is possible that TPA could use RasGRP1 as a link to Ras activation in skin carcinogenesis.

**Figure 7 – Model of regulation of Ras by DAG receptors.** Stimulation of diacylglycerol (DAG)-generating receptors can potentially recruit Ras guanyl nucleotide-releasing protein (RasGRP) to the plasma membrane wherein RasGRP1 can mediate activation of Ras and the downstream effector pathways.
Previous studies from our lab examined in more detail the interaction between RasGRP1 and phorbol esters in keratinocytes and showed that in fact TPA can activate Ras in the epidermal cells (Rambaratsingh et al., 2003). Moreover this activation proceeds in a PKC-independent manner and is substantially increased by enforced expression of RasGRP1 (Rambaratsingh et al., 2003; Tuthill et al., 2006), suggesting that RasGRP1 is a novel link between TPA and Ras activation in epidermal keratinocytes. To further explore this finding and its relevance to the effects of TPA in tumorigenesis, the lab developed a transgenic murine model for overexpression of RasGRP1 in the epidermis (K5.RasGRP1 mice) (Oki-Idouchi and Lorenzo, 2007). Interestingly, the sole overexpression of RasGRP1 in mouse epidermis resulted in spontaneous tumors of the skin, which provided the first evidence for a role of RasGRP1 in skin cancer (Oki-Idouchi and Lorenzo, 2007). When the transgenic mice were exposed to multistage carcinogenesis, tumors generated were significantly larger and more aggressive than those from the Wild type mice, suggesting a participation of RasGRP1 in tumor progression (Luke et al., 2007). This is not an unexpected finding when one takes into account that those tumors generated in the multistage protocol carry a heterozygous H-Ras mutation induced by DMBA. Given the fact that RasGRP1 cannot modulate a Ras oncoprotein, RasGRP1 may be activating wild type Ras isoforms not mutated in the tumors (like N- or K-Ras, or even the non-mutated H-Ras). Since Ras hyperactivation plays a critical role in the malignant progression of skin tumors (Glick et al., 1999; Schubbert et al., 2007), the findings from our lab strongly suggest that RasGRP1 overexpression could contribute to the dosage of active Ras in transformed keratinocytes and thus play a role in cancer formation. It should be noted that K5.RasGRP1 mice also develop tumors in response to tumor promoting events (wounding or TPA) in absence of carcinogenic insults (Diez et al., 2009; Luke et al.,
suggesting that if overexpressed, RasGRP1 could also have a role in tumor initiation. Whereas the studies from the lab using the transgenic RasGRP1 model have provided a strong support to the hypothesis that RasGRP1 is an important pathway in skin carcinogenesis, this hypothesis needed to be validated with further testing in a different setting: that of the RasGRP1 knockout system (RasGRP1 KO). In this dissertation, we have investigated the impact of RasGRP1 depletion on Ras activation in keratinocytes and its effects on skin tumorigenesis. Our results show that lack of RasGRP1 severely abrogates Ras activation in the epidermal keratinocytes in response to TPA and further, mice lacking RasGRP1 exhibit remarkable resistance to skin tumor development in the multistage carcinogenesis protocol, thereby, highlighting the importance of RasGRP1 in skin tumorigenesis.
3 SIGNIFICANCE

Although many studies over the years have tried to define the nature of the interaction between Ras and phorbol esters during tumor promotion in skin, the molecular basis of this association still remains ill defined. Also, despite intensive efforts to identify pharmacological approaches to block Ras function for cancer treatments, a successful 'anti-Ras' strategy has eluded cancer researchers. Therefore, the current focus of cancer researchers is on indirect approaches to disrupt Ras function, and Ras GEFs are being looked at as tractable alternate targets for cancer therapy (Vigil et al., 2010). In this present study, we have explored a new link between Ras and phorbol esters in skin: RasGRP1, a novel non-PKC diacylglycerol and phorbol ester receptor that modulates Ras activation. We have used both in vitro and in vivo approaches to see if RasGRP1 contributes to Ras activation in mouse epidermal keratinocytes and to further delineate the role of RasGRP1 in skin carcinogenesis using the multistage skin carcinogenesis model. Overall, the information collected from the proposed studies is expected to advance our understanding of the molecular mechanisms by which RasGRP1 regulates skin tumorigenesis. Since the multistage carcinogenesis protocol also serves as a model of human cutaneous SCC, our studies could reveal novel pathways for therapeutic intervention in this disease.
4 RESULTS

4.1 SPECIFIC AIM 1. Investigate the functional role of RasGRP1 in the actions of phorbol esters in keratinocytes

4.1.1 Rationale

The ultimate goal of our study was to determine whether RasGRP1 is important in the tumor promoting effects of phorbol esters in keratinocytes. Previous work from the lab has shown that active Ras (Ras$^{GTP}$) levels are elevated in response to TPA in keratinocytes, and that RasGRP1 overexpression further increases it in a PKC-independent manner (Rambaratsingh et al., 2003). However, the biological consequence of this effect, as well as the degree of dependency of TPA on RasGRP1 in absence of overexpression, remained to be defined. In this aim, therefore, we investigated the signaling pathways downstream of the Ras-RasGRP1 axis, as this could provide information about the biological consequences of RasGRP1 activation in the epidermal cells. As a system model for our studies, we employed primary keratinocytes isolated from RasGRP1 KO mouse model (KO) and their wild type (Wt) counterparts. Further, we also complemented some of our studies with an alternative approach to suppress RasGRP1 expression via acute silencing through an shRNA approach.

4.1.2 Hypothesis

RasGRP1 is both necessary and sufficient to mediate Ras activation in response to the tumor promoter TPA in epidermal keratinocytes.
4.1.3 Determine the effect of RasGRP1 suppression on Ras activation in response to TPA

One important question that needed to be addressed was that of the contribution of RasGRP1 to TPA-induced Ras activation. To this end, we analyzed levels of Ras$^{\text{GTP}}$ induced by TPA in keratinocytes derived from KO mice and compared it to that of Wt cells using Ras$^{\text{GTP}}$ affinity precipitation or a pull down approach (Brymora et al., 2004). When primary KO keratinocytes were treated with TPA, the levels of active GTP-loaded Ras (Ras$^{\text{GTP}}$) were barely detectable even after 60 min of treatment, in clear contrast to the activation observed in Wt cells that showed a rapid stimulation of Ras declining after 30 min of treatment (Figure 8, A and B). Enforced expression of RasGRP1 in the KO keratinocytes significantly rescued the stimulation of Ras by TPA, suggesting that the effect on Ras was specific to RasGRP1 (Figure 8 C). As an independent test of a RasGRP1-specific effect, we used an shRNA approach to silence RasGRP1 in Wt primary keratinocytes. This approach resulted in ~ 90% RasGRP1 depletion from the cells and led to a significant reduction in the ability of TPA to stimulate Ras, similar to the one observed in the KO keratinocytes (Figure 8 D). Keratinocytes infected with a nontargeting shRNA vector (irrelevant shRNA) did not differ from uninfected, control epidermal cells in the activation of Ras in response to TPA (Figure 8 D).
Figure 8 - Depletion of RasGRP1 abrogates TPA-induced activation of Ras in mouse primary keratinocytes. A, mouse primary keratinocytes isolated from the epidermis of either Wt or KO mice, were serum-starved overnight and treated with 1µM TPA for the times indicated. Ras\textsuperscript{GTP} levels were pulled down as described under Materials and Methods. Total Ras and RasGRP1 were measured in a 50-µg aliquot of the total lysate used in the pulldown assay. The results shown are from a representative experiment of at least five independent experiments. B, densitometry analysis of Ras\textsuperscript{GTP} levels normalized by the total amount of Ras in each lane and plotted as the means ± S.E. White circles, Wt keratinocytes; black circles, KO keratinocytes. **, p
< 0.01; ***, \( p < 0.001 \), between Wt and KO values, two-way ANOVA followed by Bonferroni test (\( n = 5–7 \)). C, KO keratinocytes were infected for 48 h with adeno viral vectors encoding for either RasGRP1 (AdRGRP1) or the irrelevant protein LacZ (AdLacZ), serum-starved overnight, and treated for 15 min with 1 \( \mu \text{M} \) TPA (+) or control vehicle (DMSO; –). Ras\( ^\text{GTP} \) was precipitated by pulldown assay and run along with aliquots of the input to measure total Ras and RasGRP1. The results shown are representative of three independent experiments. D, mouse primary keratinocytes isolated from the epidermis of Wt mice were transduced with adeno viral vectors containing the short hairpin RNA sequence targeting mouse Rasgrp1 (shRNA-RGRP1) or a nontargeting, irrelevant sequence (shRNA-Irrel). Uninfected keratinocytes (control) were also included for comparison. 48 h later, the cells were serum-starved overnight and treated for 15 min with 1 \( \mu \text{M} \) TPA (+) or control vehicle (DMSO; –). Ras\( ^\text{GTP} \), total Ras, and RasGRP1 levels were measured as described above. The results shown are representative of three independent experiments.

4.1.4 Evaluate whether the Ras activation by RasGRP1 in response to TPA is specific for a particular Ras isoform

Previous studies have described RasGRP1 as a GEF for the classic Ras proteins H-, N-, and K-Ras (Ebinu et al., 1998; Kawasaki et al., 1998; Tognon et al., 1998). We therefore, sought to determine whether the potential contribution of RasGRP1 to TPA – induced Ras activation proceeds in a Ras isoform-specific manner, or affects the three isoforms equally. Using the pulldown approach, we compared individual levels of H-, N-, and K-Ras activation in response to a 15 min TPA treatment between KO and Wt keratinocytes using isoform specific antibodies in Western blotting. The three Ras isoforms could be detected in keratinocytes lysates, although the K-Ras total levels were lower than those of H- and N-Ras (data not shown). Under these conditions, activation of K-Ras could not be detected, whereas N-and H-Ras displayed 2-fold activation in response to TPA in the Wt keratinocytes (Figure 9, A and B). In contrast, TPA treatment had a negligible effect on the activation of either isoform in the KO keratinocytes (Figure 9, A and B), consistent with a dependence on RasGRP1 for stimulation. We conclude
that RasGRP1 mediates both H- and N-Ras activation in response to phorbol esters in keratinocytes.

Figure 9 - H-Ras and N-Ras depend on RasGRP1 for activation in response to TPA in mouse primary keratinocytes. A, mouse primary keratinocytes isolated from the epidermis of either Wt or KO mice were serum-starved overnight and treated for 15 min with 1µM TPA (+) or
control vehicle (DMSO; −). Ras\textsuperscript{GTP}, total Ras, and RasGRP1 levels were measured as described in the legend to Figure. 7. Immunoblotting for Ras\textsuperscript{GTP} and total Ras was performed with specific monoclonal antibodies against H-Ras (Santa Cruz) or N-Ras (Calbiochem). The results shown are from a representative experiment of at least three independent experiments. B, densitometry analysis of Ras\textsuperscript{GTP} levels normalized by the total amount of Ras in each lane and plotted as the means ± S.E. White bars, Wt keratinocytes; black bars, KO keratinocytes. *, p < 0.05, one-tailed Student’s unpaired t test (n = 3–4).

4.1.5 Determine whether RasGRP1 status influences the ERK-induced phosphorylation by TPA

Ras has multiple downstream effectors, and one of them is MAPK ERK (Vojtek and Der, 1998), which is also a downstream response element of TPA in various cell types, including keratinocytes (Li et al., 1999). Previously, the lab, reported that overexpression of RasGRP1 in keratinocytes is associated with increased ERK phosphorylation; however, further ERK stimulation with TPA could be entirely blocked by the PKC inhibitor GF103209X (Rambaratsingh et al., 2003). Whereas these data clearly suggested a PKC-independent effect of TPA on ERK activation, they did not rule out a role for RasGRP1 downstream of PKC. To assess the participation of RasGRP1, we compared levels of ERK activation in response to TPA treatment between Wt and KO keratinocytes. We exploited the fact that activation of ERK requires its phosphorylation and therefore levels of phosphothreonine/phosphotyrosine ERK can be evaluated by using specific antibodies in Western blotting. TPA rapidly induced ERK phosphorylation within 5 min of treatment in both Wt and KO keratinocytes, with no differences in response between the two cell genotypes during the 60-min time course performed (Figure 10 A). The total amount of ERK in the cells remained unchanged during TPA treatment (Figure 10 B). Together, the data suggest a RasGRP1-independent mechanism for ERK activation by TPA in mouse keratinocytes.
Figure 10 - TPA-induced ERK activation is independent of RasGRP1 in mouse primary keratinocytes. A, mouse primary keratinocytes isolated from the epidermis of either Wt or KO mice were serum-starved overnight before treatment with 1 µM TPA for the times indicated. Western blots were performed on 50 µg of total lysate protein, using antibodies specific for total p44/42 MAPK (Total ERK) or the phosphorylated form phospho-p44/42 MAPK Thr202/Tyr204 (pERK). The results shown are representative of six independent experiments. B, densitometry analysis of pERK normalized by the corresponding total ERK levels and plotted as the means ± S.E. of six independent experiments. White circles, Wt keratinocytes; black circles, KO keratinocytes. Two-way ANOVA was used. p < 0.39 for genotype, not significant. Total ERK values for each group (arbitrary units by densitometry) are shown in the right panel (means ± S.E.; n= 6).

4.1.6 Determine the contribution of additional Ras effectors in the potential RasGRP1-Ras axis stimulated by TPA

There are various effectors of Ras in keratinocytes; for this study, we focused our efforts on ERK (studied above) as well as on JNK, two critical components of the Ras
oncogenic pathways in skin carcinogenesis. JNK can be activated in response to Ras and phorbol esters and has been implicated in a myriad of effects on skin, from inflammatory responses to proliferation and skin cancer (Takahashi et al., 2002; Zhang et al., 2007; Zhang et al., 2004). In particular, JNK2 has been described as a key player having a dominant role in human epidermal neoplasia (Ke et al., 2010). To examine the activation status of JNK, in particular JNK2, we used phospho-JNK levels as a measurement of its activation, as like ERK, JNK activation also requires phosphorylation at threonine and tyrosine residues. For this, we compared levels of phospho-JNK between Wt and KO keratinocytes treated with TPA using phospho-JNK specific antibody in Western blotting. In contrast to the effect of TPA on ERK, JNK activation was altered in keratinocytes lacking RasGRP1 (Figure 11 A). Interestingly, phosphorylation of JNK1 and JNK2, the two isoforms present in keratinocytes, was differentially affected by depletion of RasGRP1. Specifically JNK2 phosphorylation was reduced in the KO keratinocytes compared with the Wt counterparts, whereas levels of phospho-JNK1 did not significantly differ between the groups (Figure 11 B). Ectopic expression of RasGRP1 in the KO keratinocytes rescued the response of JNK2 to TPA (Figure 12), further demonstrating a RasGRP1-specific effect. Taken together, these results position JNK2 as a potential downstream target of RasGRP1 in epidermal cells.
Figure 11 - JNK2 activation by TPA requires RasGRP1 in mouse primary keratinocytes. A, mouse primary keratinocytes isolated from the epidermis of either Wt or KO mice, were serum starved overnight before treatment with 1 μM TPA for the times indicated. Western blots were performed on 50 µg of total lysate protein, using specific antibodies against JNK or the phosphorylated form phospho-JNK Thr183/Tyr185 (pJNK). The arrows indicate the positions of the two phospho isoforms expressed in the keratinocytes (p54, pJNK2 and p46, pJNK1). The results are from a representative experiment of at least five independent experiments. B,
densitometry analysis of each pJNK isoform was normalized by the corresponding total JNK and plotted as the means ± S.E. *White circles*, Wt keratinocytes; **black circles**, KO keratinocytes. *, p < 0.05; **, p < 0.01, between Wt and KO values, two-way ANOVA followed by Bonferroni test (n = 6–7). C, the total JNK values for each group (arbitrary units by densitometry) are shown on the bottom panel (means ± S.E.; n = 5–7).

Figure 12 - Enforced expression of RasGRP1 rescues JNK2 phosphorylation induced by TPA in KO mouse keratinocytes. A, primary keratinocytes derived from KO mouse skin were infected for 48 h with adenoviral vectors encoding for either RasGRP1 (AdRGRP1) or the irrelevant protein LacZ (AdLacZ), then serum-starved overnight, and treated for 15 min with 1µM TPA (+) or control vehicle (DMSO, −). The results shown are representative of four independent experiments. B, densitometry analysis of pJNK2 normalized by the total JNK2 levels and plotted as the means ± S.E. *White bars*, DMSO control; **black bars**, TPA treatment. *, p < 0.05; **, p < 0.01, one-way ANOVA followed by Tukey’s test (n= 4).
4.1.7 Discussion

Previous studies from the lab have demonstrated expression of RasGRP1 in epidermal keratinocytes and suggested an important role for this exchange factor in mediating effects of ultrapotent diacylglycerol analogs like TPA in a PKC-independent manner (Rambaratsingh et al., 2003; Tuthill et al., 2006). Our experimental findings from Aim 1 provide the critical data that define RasGRP1 as an essential component in the pathway to Ras activation in response to TPA in these cells.

The dependence on RasGRP1 for Ras activation by TPA in keratinocytes was initially reminiscent of the effect seen in T-cells (Dower et al., 2000; Priatel et al., 2002). However, the effect in T-cells involves PKC through phosphorylation of RasGRP1 (Roose et al., 2005), a mechanism that we have not found in keratinocytes, at least when probing PKC participation with specific inhibitors. Moreover, whereas RasGRP1 induces ERK phosphorylation when overexpressed in keratinocytes (Rambaratsingh et al., 2003), ERK activation by TPA is a RasGRP1-independent event, in contrast with the requirements observed in T-cells. Although our results were initially surprising, given the established link between Ras and ERK (Howe et al., 1992; Nori et al., 1992; Wood et al., 1992), there are several examples in the literature of Ras-independent mechanisms of ERK activation (Burgering and Bos, 1995; Ueda et al., 1996; Wen-Sheng, 2006) suggesting that pathways that utilize ERK can substantially differ depending on cell type and stimuli.

Interestingly, another member of the MAPK family, JNK, required RasGRP1 for activation by phorbol esters in keratinocytes. Similarly, natural killer cells show reduced TPA-mediated activation of JNK when RasGRP1 was silenced by RNA interference approach (Lee et al., 2009). However, as in the case of ERK, the effect of RasGRP1 on JNK cannot be generalized. For example, thymocytes from RasGRP1 KO mice did not show any defect in JNK activation (Priatel et al., 2002), and although mutant Jurkat cells
depleted of RasGRP1 display a reduction in JNK phosphorylation upon TPA treatment, the defect in activation could not be rescued by RasGRP1 replenishment (Roose et al., 2005). One should note that there are differences in the concentration of TPA and time course employed among these studies. Thus, the discrepancies in response to RasGRP1 depletion may not only depend on the cell type but also on the experimental conditions utilized for the studies.

The association of JNK phosphorylation with Ras activation has been documented in various cell types, including keratinocytes (Byun et al., 2009; Li et al., 1996; Mainiero et al., 1997). Recent evidence suggests that Ras-induced JNK activation distinctively occurs in endomembranes such as endoplasmic reticulum and the Golgi apparatus (Chiu et al., 2002; Matallanas et al., 2006). This is particularly interesting if one considers that RasGRP1 preferentially activates Ras in endomembranes (Bivona et al., 2003; Caloca et al., 2003; Chiu et al., 2002). Previous work from the lab has shown that TPA is able to translocate RasGRP1 to both plasma and internal membranes in keratinocytes and that the fraction redistributed to internal membranes colocalizes with Golgi and partially with endoplasmic reticulum (Rambaratsingh et al., 2003). Together, the data generated in the keratinocytes support a model for RasGRP1 activation of Ras in endomembranes leading to JNK phosphorylation. However, the precise molecular pathway involved in this activation remains to be elucidated.

The fact that RasGRP1 preferentially signals to Ras activation in internal membranes also suggests that not all Ras GTPases can be substrates for RasGRP1 in vivo. For the classic Ras isoforms, this means that only H- and N-Ras, but not K-Ras (Apolloni et al., 2000; Choy et al., 1999), could be activated by RasGRP1, despite the fact that in vitro they all work as RasGRP1 substrates (Ebinu et al., 1998; Kawasaki et al., 1998; Ohba et al., 2000). Because TPA can translocate RasGRP1 to the plasma
membrane in keratinocytes (Rambaratsingh et al., 2003; Tuthill et al., 2006; Yuspa et al., 1982), we cannot rule out the stimulation of plasma membrane-bound Ras isoforms under our experimental conditions. However, we were unable to detect any activation of K-Ras in response to TPA in keratinocytes, which supports the idea that the main site of activation of Ras by RasGRP1 is in endomembranes or that the localization of the active RasGRP1 pool in plasma membrane differs from that of K-Ras. Alternatively, our results on K-Ras may just be a reflection of poor antibody sensitivity on Western blot, a situation that could only be resolved if new antibodies become available. Nevertheless, the results with JNK activation independently argue in favor of a TPA-RasGRP1 pathway for Ras activation in endomembranes.

Previous studies from our laboratory demonstrated that transgenic animals overexpressing RasGRP1 in the epidermis are prone to develop skin tumors derived from keratinocytes and also respond to multistage carcinogenesis protocols in which TPA is used as a tumor promoter, with generation of larger and more aggressive carcinomas than the wild type mice (Diez et al., 2009; Luke et al., 2007). Together, the studies suggest that RasGRP1 is required during carcinogenesis in the skin, through its role as Ras activator. Both ERK and JNK have been linked to the effects of oncogenic Ras, and thus the participation of a RasGRP1-JNK kinase pathway in keratinocyte transformation is plausible. ERK is an essential pathway in Ras-induced keratinocytes transformation (Feith et al., 2005; Khavari and Rinn, 2007). The role of JNK is less evident, because this kinase has been associated with both pro- and anti-tumorigenic effects in various tissues, including skin (Gross et al., 2007; Kennedy et al., 2003; Nielsen et al., 2007; Wei et al., 2009; Zhang et al., 2007). However, recent carcinogenesis studies using genetically engineered mice have shed light on the action of JNK in skin cancer, revealing opposite functions of the two isoforms expressed in
keratinocytes, JNK1 and JNK2. Specifically, deletion of JNK1 enhances tumor promotion (She et al., 2002), whereas JNK2 knockout suppresses epidermal carcinogenesis (Chen et al., 2001), revealing a pro-tumorigenic role of JNK2 in the skin. These findings are particularly relevant to our present observations because we have found that TPA-induced activation of JNK2, but not JNK1, is susceptible to RasGRP1 depletion in the mouse epidermal cells. We speculate that RasGRP1 may represent a link between TPA and JNK2 activation in the skin, with the consequent implications for tumorigenesis. A model that emerges from our study is depicted in Figure 13. As explained in the Background section TPA is a structural analog of DAG. Therefore, RasGRP1 can translocate to the plasma membrane from cytosol in response to TPA, where it can mediate Ras activation. Our present study has shown that RasGRP1 is both necessary and sufficient to mediate Ras activation in response to TPA and also that TPA depends on RasGRP1 for activation of JNK2.

Figure 13 – Model of regulation of Ras by RasGRP1. TPA can recruit RasGRP1 to the plasma membrane wherein RasGRP1 can mediate activation of Ras and JNK2 in response to TPA.
4.2 SPECIFIC AIM 2. Assess the role of RasGRP1 in the development of cutaneous squamous cell carcinoma

4.2.1 Rationale
The multistage model of mouse skin carcinogenesis is an elegant system in which biochemical events unique to initiation, promotion and progression can be studied and related to cancer formation (DiGiovanni, 1992). Previous studies from our laboratory demonstrated that transgenic animals overexpressing RasGRP1 in the epidermis (K5.RasGRP1) are prone to develop skin tumors derived from keratinocytes and also respond to multistage carcinogenesis protocols in which TPA is used as a tumor promoter, with generation of larger and more aggressive carcinomas than the Wt mice (Diez et al., 2009; Luke et al., 2007). Together, the studies suggest that RasGRP1 is required during carcinogenesis in the skin, through its role as Ras activator. In this aim, we propose to determine the effect of loss of RasGRP1 on skin carcinogenesis by subjecting the KO mouse model to the classic multistage skin carcinogenesis protocol along with its Wt counterparts.

4.2.2 Hypothesis
Lack of RasGRP1 will confer resistance to skin tumor development in the multistage skin carcinogenesis protocol due to reduced response to TPA.

4.2.3 Evaluate the response of RasGRP1 KO mice to multistage carcinogenesis protocol
To ascertain the role of RasGRP1 in skin tumor development, we subjected KO and Wt control mice to the well-characterized multistage mouse skin carcinogenesis model using DMBA as an initiator and TPA as a promoter. Animals were monitored weekly for any changes in the skin and fur, and any tumors generated were then measured bi-weekly. At the end of the protocol, tumors were excised and biopsies
collected for histology, DNA and protein extractions. An expert Veterinarian Pathologist performed the histopathological assessments of the tumors. The technical details of the protocol are explained in the Materials and Methods section.

As shown in Figure 14, the KO mice subjected to the multistage carcinogenesis protocol developed significantly less number of tumors than their Wt counterparts. Also, the percentage of animals that developed tumors was significantly higher in the Wt group compared to the KO group. At the end of the protocol almost 90 % of the Wt animals had developed tumors compared to only 16 % in the KO group (Figure 14 B). Since tumor size often correlates with tumor grade (Hachiya et al., 1998; Henson et al., 1991) we measured tumor size in Wt and KO animals both at an early stage and late stage of tumor development. At 28 weeks post-initiation (late stage), animals from Wt group displayed significantly larger tumors compared to the KO group (Figure 14 D).

The number of tumors measuring 5 mm or larger in diameter, was significantly higher in the Wt mice as compared to the KO mice (Figure 15 A). Although there was no difference in the distribution of tumors by histology type between Wt and KO mice as most tumors were SCCs (Figure 15 B), a small percentage of carcinomas that developed in the Wt mice displayed extensive invasion into the dermis and subcutaneous tissue, while all the SCCs from the KO mice were microinvasive (Figure 15 C). Taken together, these results strongly suggest that lack of RasGRP1 represses formation and growth of DMBA/TPA- induced skin tumors.
Figure 14 - Skin tumor development in RasGRP1 KO mice subjected to two-stage carcinogenesis. A, tumor multiplicity (average number of tumor per mouse ± SE). **, p< 0.009; ***, p< 0.0001 (student’s t-test) and B, incidence (percentage of mice with tumors) in KO (●) and Wt (○) mice treated with TPA following initiation with DMBA. C, Wt and KO mice bearing tumors; pictures were taken at the end of the protocol. D, tumor size (diameter in millimeters) at 17 (early stage) and 28 weeks (late stage) post-initiation with DMBA in both Wt and KO mice. ***, p< 0.0001 (unpaired t-test with Welch’s correction)
Figure 15 – Tumor distribution by size and histology type. A, size distribution of tumors at the end of the protocol in Wt (white columns) and KO (black columns) mice. Columns, means of tumors in 17 to 18 mice. Bars, SE; *, p < 0.05 compared with the KO values (student’s t test). B, tumors were histologically assessed and classified into two types: papilloma (Pap) and squamous cell carcinoma (SCC). Percentages of total number of tumors in Wt or KO mice. Number of tumors of defined type versus the total number in the group assessed in parantheses. C, SCCs were evaluated on the basis of their invasiveness as either microinvasive (micro, one or few clusters of cells invading the dermis or extensive invasion (extensive, advancing fronts of invading cells into the dermis and subcutaneous tissue).

4.2.4 Determine whether lack of RasGRP1 affects the ability of the cells to respond to the carcinogen DMBA

The results obtained from the multistage carcinogenesis in the KO mice led us to consider two possibilities: 1. Lack of RasGRP1 impairs the carcinogenic effect of DMBA, 2. Lack of RasGRP1 impairs the ability of TPA to exert tumor-promoting effects on the
initiated cells. We then decided to first examine whether lack of RasGRP1 impairs the carcinogenic effect of DMBA.

As described in the Background section, the tumor initiation stage in the multistage carcinogenesis is an irreversible phenomenon characterized by somatic mutations in the H-Ras proto-oncogene (DiGiovanni, 1992; Slaga et al., 1995; Yuspa and Poirier, 1988). Over 90% of tumors initiated with DMBA have a specific A-T transversional mutation at codon 61 of H-Ras (Quintanilla et al., 1986). Therefore impairment of the ability of DMBA to cause this specific mutation could be one mechanism by which RasGRP1 null animals are rendered resistant to skin tumor development. Ideally, a quantitative comparison of the number of epidermal cells that harbor this specific mutation between DMBA treated Wt and KO epidermis before the formation of papillomas would reveal if, indeed, lack of RasGRP1 adversely affected the ability of DMBA to cause this specific mutation in the mouse epidermis. Since the frequency of mutations induced by DMBA in mouse skin is low ($\approx 10^{-5}$) as observed in the Muta™ mouse model (Brooks and Dean, 1996) then this approach would be both labor intensive and time consuming. Nevertheless, our analysis of the H-Ras mutational status in the KO tumors revealed the presence of the A-T transversion at codon 61 of H-Ras suggesting that the capacity of DMBA to alter the H-Ras gene is not entirely abrogated (Figure 16).
Figure 16 - Analysis of H-Ras mutations in KO tumor samples. Mutations in codon 61 of H-Ras (Mut Ha-Ras) were evaluated by a PCR approach as described in Materials and Methods. M, DNA marker; DNA samples KO 1 to KO 6 are derived from KO tumors; Wt 1 and Wt 2, DNA samples derived from Wt tumors; Sp-1, DNA from a keratinocyte cell line derived from papillomas generated using DMBA/TPA in SENCAR mice; Control, genomic DNA from wild-type mouse tail. Amplification of wild-type Ha-Ras was included for comparison. The lower bands in the gels represent primer dimers (PD).

There have been previous studies showing that increased susceptibility to DMBA induced apoptosis in multistage carcinogenesis model renders animals resistant to tumor initiation (Malliri et al., 2002) while reduced apoptosis contribute to survival of DNA-damaged keratinocytes and, therefore, to skin tumorigenesis (Parra et al., 2009).
We therefore, investigated if RasGRP1 deficiency renders cells more susceptible to DMBA induced apoptosis that could, in part, explain the resistance of the KO animals to tumor development. For the study we evaluated the level of apoptosis in epidermises of mice subjected to one topical application of DMBA for 24 hours. As shown in Figure 17, TUNEL assay revealed no difference in the number of apoptotic cells between the Wt and KO epidermis implying that lack of RasGRP1 did not alter the susceptibility of the keratinocytes to DMBA-induced apoptosis.
Figure 17 - Evaluation of Apoptosis in DMBA treated Wt and KO Epidermis. Wt and KO mice were treated with either acetone (control) or DMBA. Apoptotic cells (arrows) were analyzed 24 hours after treatment using Dermatacs, *In situ* Apoptosis Detection Kit from Trevigen (TUNEL staining) as explained in detail in *Materials and Methods*. Arrows indicate apoptotic cells. Pos
indicates positive control included in every experiment as explained in detail in Materials and Methods for comparison.

4.2.5 Determine if suppression of RasGRP1 in the epidermis desensitizes the skin to the acute hyperplastic effects of TPA

Topical application of TPA stimulates epidermal DNA synthesis (Marks et al., 1979) and induces hyperplasia. Although the role of epidermal hyperplasia in tumor promotion is still a matter of debate, the stimulation of DNA synthesis appears to be obligatory for TPA-induced tumor promotion (Furstenberger and Marks, 1978). We therefore examined if lack of RasGRP1 in the skin affects the acute hyperplastic response of the skin to TPA.

As expected, Wt mice skin exhibited acute hyperproliferation of the epidermis as evidenced by the significantly more epidermal thickening compared to KO mice (Figure 18). In contrast, lack of RasGRP1 in the epidermis of the KO mice severely abrogated the acute hyperplastic effect of TPA.

![Figure 18- Effect of RasGRP1 deficiency on epidermal hyperplasia in response to TPA in RasGRP1 KO mice. TPA-induced hyperplasia in Wt and KO mice were evaluated after topical application](image.png)
application of TPA on shaved dorsal skin for two consecutive days. Acetone treatment was also done in place of TPA treatment. Left, H&E-stained sections from Wt and KO mice treated with acetone or TPA. Right, the thickness of the epidermis (in micrometers) was measured as described in Materials and Methods in Wt (white columns) and KO (black columns) mice. Columns, means of five independent experiments for each group, measured in triplicate; bars, SE. ***, p< 0.0001 (two-way ANOVA followed by Bonferroni test)

The lack of hyperplastic response to TPA treatment displayed by the RasGRP1 KO mice could not only be a result of less keratinocyte proliferation but also of more apoptosis in response to the effects of TPA. Utilizing the same samples used to assess the hyperplastic response to TPA, we evaluated proliferation in RasGRP1 KO epidermis and compared it to Wt epidermis by immunohistochemistry (IHC) using Ki-67 as a proliferation marker. Further, we also investigated if more cells in the KO epidermis were undergoing apoptosis, in response to TPA treatment, by using In situ Apoptosis Detection Kit from Trevigen (TUNEL staining). TUNEL assay on skin treated with TPA did not show significant differences in apoptotic levels between Wt and KO mice. On the contrary, expression of the proliferation marker Ki-67 was elevated in Wt epidermis but almost absent in KO skin upon TPA treatment (Figure 19). These findings indicate that lack of RasGRP1 leads to decreased epidermal proliferation in response to TPA without altering apoptosis.
Figure 19 - Proliferation and apoptosis in TPA treated skin from Wt and KO mice. Sections of epidermis from Wt and KO mice were stained for Ki-67 for proliferation (top panel [original magnification, x40]) and apoptosis using TUNEL staining (bottom panel [original magnification, x40]). Pos indicates positive control included in every experiment for TUNEL assay as explained in detail in Materials and Methods for comparison. Arrows in the upper panels represent Ki-67 positive cells. Arrows in the lower panel represent TUNEL positive cells in the positive control.

4.2.6 Evaluate if lack of RasGRP1 impairs Ras activation in response to TPA in mouse skin

The decreased epidermal proliferation in KO mice in response to TPA could be a consequence of abrogation of TPA-induced Ras activation in KO epidermis as Ras activation is known to induce keratinocyte proliferation (Khavari and Rinn, 2007). We have previously shown that lack of RasGRP1 severely impairs Ras activation in mouse
epidermal keratinocytes in response to TPA and both H- and N- Ras are dependent on RasGRP1 for TPA-induced activation (Sharma et al., 2010). We therefore, investigated if lack of RasGRP1 also abrogated Ras activation in an in vivo setting of the adult mouse epidermis in response to TPA. Two days after their dorsal backs were shaved, adult KO and Wt mice were treated with 6µg TPA for two consecutive days. Control treatments with acetone were also performed on separate mice. Twenty fours hours after the last treatment the mice were sacrificed, dorsal skin collected, and homogenized in lysis buffer. Since H-Ras antibody showed the highest sensitivity in western blot analysis of epidermal lysates, we measured levels of H-RasGTP in Wt and KO epidermal lysates. Five hundred micrograms of epidermal tissue lysate was used to measure Ras activation by RasGTP pulldown assay as explained in detail in Materials and Methods. Similar to the results obtained in keratinocytes, H-Ras activation was impaired in KO epidermis as compared to the Wt epidermis (Figure 20) which could explain, at least in part, the decreased epidermal proliferation and hence the lack of hyperplastic response of the KO epidermis to the acute TPA treatment.
Figure 20 - Lack of RasGRP1 abrogates TPA-induced Ras activation in adult mouse skin. Pull down assay of levels of GTP-bound H-Ras in KO and Wt epidermal tissue extracts in response to topical treatment with TPA. Levels of total Ras included as loading controls. The results shown are representative of two independent experiments.
4.2.7 Discussion

In this Aim we have shown that mice lacking RasGRP1 are remarkably resistant to skin chemical carcinogenesis compared with their Wt littermates, thereby suggesting a critical role for RasGRP1 in tumorigenesis. Our lab previously reported that mice overexpressing RasGRP1 (K5.RasGRP1) in the basal keratinocytes using a transgenic approach did not respond differently to TPA induced tumor promotion but the tumors generated were significantly larger and more invasive than the tumors generated in the Wt skin (Luke et al., 2007).

At first, these findings seem contradictory to our present results. However, the explanation to this apparent discrepancy may be in the dosage of active Ras required during the different stages of carcinogenesis. In this regard, various studies have suggested a selective pressure towards higher active Ras levels as tumors progress to malignancy (Larcher et al., 1996; Motojima et al., 1994; Ochieng et al., 1991; Portella et al., 1994). This could explain why tumors generated in the K5.RasGRP1 mice are significantly larger and more invasive than the Wt counterparts, since RasGRP1 dosage correlates with active Ras dosage (Luke et al., 2007; Oki-Idouchi and Lorenzo, 2007). In contrast, the Ras activation threshold needed for efficient tumor promotion appears substantially lower than for progression, potentially due to differential requirements for the magnitude and/or type of downstream effector pathway activation between the two steps of carcinogenesis. Therefore, only when RasGRP1 is absent, tumor promotion is visibly impaired in the multistage model.

Previous studies have shown that DMBA-induced apoptosis in keratinocytes can impact tumorigenesis in a major way as animals in which the keratinocytes were susceptible to DMBA-induced apoptosis were resistant to tumor formation (Malliri et al., 2002; Sterneck et al., 2006). Therefore, another mechanism that could explain the significant reduction in the incidence of tumors in KO animals was the possibility that in
the KO skin DMBA treatment was causing more cells to undergo apoptosis thereby reducing the pool of initiated cells. However, our present study did not find any discernible difference in the apoptotic rates of Wt and KO keratinocytes in response to DMBA treatment. Although we cannot exclude the possibility that the metabolism of DMBA or its uptake by keratinocytes could be affected by the lack of RasGRP1, the more likely scenario is that absence of RasGRP1 impairs the effect of phorbol esters in tumor promotion.

Tumor promoters, like TPA, produce many morphological, biochemical and molecular changes in the skin (Slaga, 1983; Yuspa et al., 1996) and although it is difficult to assess which of the many effects associated with the phorbol ester tumor promoters are in fact essential components of the promotion process, there are some phorbol ester associated effects that have a very good correlation with tumor promotion.

Induction of epidermal hyperplasia is one of those phorbol ester effects that have been postulated to correlate with tumor promotion (Slaga et al., 1976). There are two schools of thought regarding the role of epidermal hyperplasia in tumorigenesis. The first school of thought belongs to researchers who have long argued that epidermal hyperplasia is a necessary but not sufficient condition to cause tumor promotion in the multistage carcinogenesis model (Boutwell, 1974, 1976; Slaga et al., 1975). Such arguments were based upon the fact that many chemicals, like acetic acid and mezerein, could produce dramatic epidermal hyperplasia after a single topical application but these were rather poor tumor promoters (Raick and Burdzy, 1973; Slaga et al., 1975). The other school of thought puts forth a counter argument saying that these compounds were unable to maintain a potentiated epidermal hyperplasia and cell proliferation when applied repeatedly due, in part, to severe epidermal toxicity (Argyris, 1983a, b, 1989; Naito et al., 1987). It was further claimed that these observations
coupled with the fact that regenerative hyperplasia alone can promote skin tumors (Argyris, 1980, 1985) supports the hypothesis that epidermal hyperplasia and cellular proliferation of a specific type and duration is sufficient for skin tumor promotion, at least, in susceptible mouse strains (Argyris, 1985, 1989). What is even more interesting is the fact that epidermal hyperplasia produced by the application of TPA, the most powerful and widely used promoter of skin carcinogenesis, is preceded by damage to the epidermis thereby strongly suggesting that the epidermal hyperplasia which ensues is a regenerative hyperplasia (Argyris, 1985). As we have shown under Results, RasGRP1 KO mice are impaired in their response to TPA-induced hyperplasia. Considering the fact that they also display a significantly reduced tumorigenic response, it would be tempting to speculate that hyperplasia and tumor promotion in response to TPA correlate well under our experimental conditions. However, a previous study in our lab has shown no difference in the hyperplastic response of K5.RasGRP1 mice as compared to Wt mice in response to TPA (Luke et al., 2007). It should be noted that the TPA dose utilized for the hyperplasia experiments in the transgenic mice was higher than the dose used in the present studies (3 µg vs 2 µg). Additionally, the studies were performed with only one application of TPA, vs. the two consecutive applications performed for the KO protocol. Since it has been reported that the concentration regimen of TPA can dramatically influence the epidermal response to tumorigenesis (Cataisson et al., 2009), the analysis of hyperplasia experiments done with different regimens can be difficult to interpret, or worse, lead to confounding interpretations. We now know that when studies are carried out at a lower TPA dosage under the same regimen as the one used for our RasGRP1 KO studies, the K5.RasGRP1 skin developed a marked hyperplastic response, significantly higher than that of the wt mice (Sharma and Lorenzo, unpublished results). These results strongly argue in favor of a positive
correlation between RasGRP1 levels and TPA-induced hyperplasia. By the same token, they suggest that hyperplasia alone cannot predict tumor promotion, at least in our system, since the K5.RasGRP1 mice do not show an elevated tumorigenic response during TPA treatment (Luke et al., 2007).

The observation that a few animals in the KO group developed some tumors brings to light the possibility that RasGRP1 maybe important but not essential to skin tumorigenesis. One possible explanation for the weak tumorigenic response of the KO mice is that the lack of RasGRP1 triggers an alternate, Ras independent pathway for tumor development. For instance, loss of function of the tumor suppressor, Pten is associated with skin tumor development in the multistage mouse skin cancer model (Mao et al., 2004). However, mutations in Pten and H-Ras are apparently, mutually exclusive (Mao et al., 2004). Considering that all our KO tumors had the A-T transversion at codon 61 in the H-Ras, the possibility that the few KO tumors that developed had a loss of Pten function is unlikely. As discussed in the Background section, loss of p53 function is also associated with tumor development in the mouse skin cancer model. However, loss of function of p53 is associated with tumor progression rather than tumor promotion in this model (Kemp et al., 1993). Therefore, the possibility of the KO tumors having a loss of p53 function does not explain the tumor development in the KO mice. At this stage we do not know which alternative pathways could drive the weak tumorigenic response of the KO mice and further studies are warranted in this regard.

Taken together, our experimental findings position RasGRP1 as a critical component of the Ras activation machinery in the keratinocytes, the target cells of skin carcinogenesis. Furthermore, we have shown that lack of RasGRP1 in the skin abrogates skin tumor development in the classic multistage carcinogenesis assay driven
by the activation of Ras. This discovery could potentially lead to development of molecule-targeted therapy in skin cancer treatment. At this stage we do not know precisely what mode of therapy could RasGRP1 be amenable to. However, RasGRP1 interference or allosteric inhibitors specific enough to disrupt the RasGRP1-Ras complex could be looked at. We are well aware of the fact that the data generated using a mouse model cannot be directly extrapolated in terms of human malignancies and studies are currently underway in our lab investigating the role of RasGRP1 in skin tumor development using human skin samples. The findings from this work could further validate our hypothesis and help us move closer to the design of effective molecule based therapy for cutaneous SCC that has eluded us for so long.
5 MATERIALS AND METHODS

5.1 Mice

The RasGRP1 KO mice, produced by inserting the Escherichia coli $\beta$-galactosidase gene and a neomycin cassette in exon 2 of Rasgrp1 (Dower et al., 2000), were originally obtained in 129/J-B6 mixed background and backcrossed for 10 generations to the FVB/N background. Wt mice of FVB/N background were bred in house. The mice were housed and utilized according to Institutional Animal Care and Use Committee guidelines at the University of Hawaii Animal Facility.

5.2 Primary keratinocyte culture

Mouse keratinocytes were isolated from Wt or KO newborn mice by the trypsin flotation method (Hennings et al., 1980) with modifications as described before (Diez et al., 2009). The cells were plated on 60-mm dishes coated with collagen I (Coating Matrix; Invitrogen) and incubated overnight in Eagle’s minimum essential medium containing 1.2 mM CaCl$_2$, antibiotics, antimycotics, and 8% fetal bovine serum. Afterward, the cells were washed with Dulbecco’s phosphate buffered saline and cultured in 154 medium (Invitrogen) supplemented with 50 $\mu$M CaCl$_2$, antibiotics, antimycotics, 2% calcium-free fetal bovine serum, and a human epidermal growth factor supplement containing epidermal growth factor, pituitary extract, insulin, transferrin, and hydrocortisone (Invitrogen). Keratinocytes were used within 5–6 days after plating.

5.3 Adenoviral vectors

Recombinant adenoviral vectors encoding rat RasGRP1 were generated with the Transpose-Ad system (Qbiogene, Irvine, CA) as described elsewhere (Rambaratsingh et al., 2003). Adenoviral vectors for expression of bacterial $\beta$-galactosidase (LacZ) were purchased from Qbiogene. Infection with the adenoviral vectors was performed as
described before (Tuthill et al., 2006). The cells were utilized 48–72 h post-infection. For the shRNA approach, an RNAi-Ready pSiren shuttle shRNA-RasGRP1 vector was generated using a 19-mer sequence corresponding to base pairs 1584 –1602 of mouse Rasgrp1 (accession number NM_011246). The adenoviral shRNA constructs were prepared with the pSiren-compatible Adeno-X System according to the manufacturer's instructions (Clontech). A nontargeting shRNA adenoviral vector (irrelevant shRNA) was utilized as control for nonspecific effects in the silencing experiments. Purification of the recombinant adenoviruses was done using the AdEasy virus purification kit (Stratagene, La Jolla, CA). Keratinocyte infections were done following similar protocols described before for the Transpose-based recombinant adenoviruses.

5.4 Ras activation assay and western blot
Levels of active, GTP-loaded Ras (Ras\textsuperscript{GTP}) were measured using the glutathione S-transferase-tagged Ras-binding domain of Raf-1 as a probe in a Ras affinity precipitation or pulldown assay. For the assay, keratinocytes were harvested on ice in lysis buffer containing 15 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl\textsubscript{2}, 1% Igepal, 5% glycerol, and protease inhibitors (Mini Complete with EDTA; Roche Applied Science). The lysates were vortexed and incubated on ice for 5 min followed by centrifugation at 13,000 rpm for 5 min at 4 °C. Five hundred µg of protein from the supernatant was then incubated with glutathione S-transferase-tagged Ras-binding domain conjugated to glutathione- Sepharose 4B beads (GE Healthcare) for 1 h with rotation in the cold. The affinity complexes were washed thrice with lysis buffer and then resuspended in 2X Laemmli buffer, boiled, and resolved on 15% acrylamide gels. Fifty µg of the total lysate protein was run in parallel as measurement of input of total Ras in the assay. The proteins were blotted onto nitrocellulose membranes, and immunostaining was done using the pan anti- Ras clone RAS10 antibody (Calbiochem, San Diego, CA) or
antibodies specific for either H-Ras (C-20; Santa Cruz Biotechnology, Santa Cruz, CA) or N-Ras (Calbiochem). Samples to evaluate RasGRP1 levels were run on 8% acrylamide gels, blotted, and immunostained with a monoclonal anti-RasGRP1 antibody (m199; Santa Cruz Biotechnology). For measurement of active GTP-loaded Ras (Ras\text{GTP}) in epidermal lysates, shaved dorsal backs of Wt and KO animals were treated with 6µg of TPA for 2 consecutive days. Control treatments with acetone were performed on separate Wt and KO mice. Twenty-four hours after the last treatment, the animals were sacrificed, dorsal skin collected, homogenized in lysis buffer and subjected to Ras pulldown assay as explained above. The Analysis of ERK and JNK forms was done on 12% acrylamide gels, followed by blotting on nitrocellulose membranes and immunostaining with one of the following antibodies: p44/42 MAPK (ERK), phospho-p44/42 MAPK Thr202/Tyr204 ERK1, Thr185/Tyr187 ERK2 (p-ERK), p56/44-SAPK/JNK (JNK), and phospho-p56/44 SAPK/JNK Thr183/Tyr185 (p-JNK) (Cell Signaling Technology, Danvers, MA). All of the primary antibody incubations were done overnight at 4 °C; incubations with the secondary anti-body conjugated to horseradish peroxidase were performed for 1 h at room temperature. Chemiluminescence was generated by using ECL Advance (GE Healthcare).

5.5 Skin carcinogenesis experiments

For the skin carcinogenesis experiments, the back of 6- to 8-week-old mice, both males and females, were shaved with electric clippers 2 days before the beginning of the protocol. The mice in each group, KO and Wt, were divided into two cohorts of 17-18 animals each of mixed gender to be treated with DMBA plus TPA or acetone plus TPA. The DMBA treatment consisted of a single topical application of 26 µg of DMBA in 200 µL of acetone on the shaved dorsal skin; TPA treatment was initiated 2 weeks after DMBA initiation by topical application of 2 µg of TPA in 200 µL of acetone twice a week.
for 20 weeks. Animals were monitored at the time of TPA applications, and tumors were counted and measured with a caliper at least once a week. After the end of the TPA treatment, mice were followed for an additional 8 weeks and then euthanized by CO₂ asphyxiation. Tumor samples were collected and fixed for histology.

5.6 Analysis of Ras mutations

A mutation-specific PCR assay developed by Nelson et al. (Nelson et al., 1992) was employed to determine the presence of Ha-Ras mutations in codon 61 in the tumors. Briefly, DNA was extracted from a minimum of four 10-µm sections of paraffin-embedded tumors using the QIAamp DNA FFPE tissue kit (Qiagen) according to the manufacturer’s instructions. Deparaffinization was done following standard histology procedures, and proteinase K treatment of the deparaffinized samples was done for an hour. Two hundred nanograms of DNA were used for the PCR reaction with the following primers: upstream ras primer, 5’-CTA AGC CTG TTG TTT TGC AGG AC-3’; downstream mutant ras primer, 5’-CAT GGC ACT ATA CTC TTC TA-3’. This primer combination produced a 110-bp band. Wild-type Ha-Ras was also amplified as a control (downstream wild-type Ras primer: 5’-CAT GGC ACT ATA CTC TTC TT-3’), and also generated a 110-bp PCR product.

5.7 Apoptotis assay

Apoptotic cells were detected using a modified terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay kit (DermaTACS; Trevigen Inc). Briefly, slides were treated with Proteinase K (1 µg in 50 µL of DNase-free H₂O) to ensure that the DNA fragments were accessible to the labeling enzymes, and the activity of endogenous peroxidase was quenched using 3% H₂O₂ in methanol. DNA fragmentation in individual apoptotic cells was then detected by labeling with terminal deoxynucleotidyl transferase (TdT) and the thymidine analog bromodeoxyuridine (BrdU).
Incorporated BrdU was detected by a specific and sensitive biotinylated anti-BrdU antibody in combination with a streptavidin–peroxidase conjugate (Trevigen Inc), according to the manufacturer’s instructions. Briefly, cells were incubated with the anti-BrdU antibody at 37 °C for 30 minutes, followed by three washes in PBS/Tween 20. The slides were then incubated with the streptavidin–peroxidase conjugate at room temperature for 10 minutes, followed by two washes in PBS and one wash in H2O. Labeled cells were visualized with TACS Blue Label (Invitrogen Inc). In each experiment, one slide sample was treated with TACS nuclease (to generate fragmentation of DNA in all cells, i.e., a positive control), and one slide sample was used in which TdT had been omitted (i.e., a negative control).

5.8 Histopathology and immunohistochemistry

Skin tumors were fixed in 4% paraformaldehyde for 24 h and maintained in 70% ethanol until paraffin-embedded. H&E-stained slides were used for descriptive histopathology. Immunohistochemistry was performed on sections derived from skin tumors. Briefly; deparaffinized sections were subjected to heat-induced epitope retrieval. After blocking, tissues were incubated with Ki-67 antibody (GeneTex Inc) followed by ImmPRESS anti-rabbit Ig reagent (Vector Labs). ImmPACT NovaRED peroxidase solution was used as a substrate (Vector labs). Tissues were counterstained with QS hematoxylin (Vector Labs).

5.9 Epidermal hyperplasia

TPA-induced acute hyperplasia were evaluated in both KO and Wt mice of 6 to 8 weeks of age after two treatments with TPA (2x2 μg of TPA in 200 μL of acetone, 24h apart) applied to the dorsal skin. The skin area to be treated was shaved with electric clippers 2 days before TPA treatment. The epidermal thickness was determined by microscopic examination of H&E-stained skin samples. Microphotographs were taken.
with a CoolSnap CDD camera (Roper Scientific) at 40X magnifications and the thickness of the epidermis were measured in micrometers using MetaMorph (Molecular Devices Corporation). The grid of a hemacytometer was used for calibration of the MetaMorph software. Each sample was measured at five different locations before calculating the average thickness within each treatment group.

5.10 Data analysis

Semi-quantitative densitometry was done on scanned films using Image J 1.42q. The data were analyzed with Prism 5 (GraphPad Software Inc., La Jolla, CA), and the selection of the statistical test for analysis of significance was done according to the experimental design. Briefly, Student’s unpaired t-test was employed to compare the means between two independent groups. One-way ANOVA was use to compare the means of three independent groups. When two independent variables (genotype and time) needed to be compared between two independent groups, two-way ANOVA was the test performed. If a significant difference was detected by ANOVA, Tukey’s or Bonferroni’s post-test was applied to specifically find which means were different from each other. The statistical significance level was set at p values of < 0.05.


glioblastoma multiforme invasive phenotype points to new therapeutic targets. Neoplasia 7, 7-16.


keratinocyte proliferation in normal and neoplastic mouse epidermis and in cell culture. Int J Cancer 80, 98-103.


