Ei24-deficiency attenuates protein kinase Cα signaling and skin carcinogenesis in mice

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Etoposide–induced gene 24 (Ei24) is a p53 target gene that inhibits growth, induces apoptosis and autophagy, as well as suppresses breast cancer. To evaluate the role of Ei24 in in vivo tumorigenesis, we generated an Ei24-deficient mouse model. Here, we report that, although Ei24 homozygous knockout mice are embryonic lethal, Ei24 heterozygous null mice are attenuated to DMBA/TPA-induced carcinogenesis with regard to the number and size of tumors but not the incidence. Ei24 contains a functional consensus motif, named as an R motif that is highly analogous to amino acids 105–110 of RINCK1, an E3 ligase for protein kinase C (PKC) proteins. We found that Ei24 stabilizes PKCα via RINCK degradation and competition with RINCK for binding with the C1a domain of PKCα. We also found that Ei24 contributes to PKCα-mediated transactivation of EGFR by promoting PKCα membrane localization and interaction with EGFR. Finally, using Oncomine database we show that Ei24 and EGFR are upregulated in some subsets of human HNSCC. These results suggest that Ei24 is a regulator of the RINCK1-PKCα-EGFR signaling pathway in the development of skin-cancer.

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1. Introduction

p53 has been described as “the guardian of the genome” because it is mutated or deleted in approximately 50% of all of human cancers. It is one of the most important tumor suppressors that functions as a transcription factor (Polager and Ginsberg, 2009). Etoposide induced gene 24 [Ei24, also known as p53-induced gene 8 (PIG8)] was first identified as a p53-dependent, etoposide-inducible gene (Lehar et al., 1996). More recently, Ei24 was found to bind Bcl2 and to suppress breast cancer invasiveness (Zhao et al., 2005). Finally, Ei24 was identified as an important component of the autophagy pathway (Tian et al., 2010). However, the precise role of Ei24 in tumorigenesis in vivo is undefined and has not yet been studied using a mouse model.

Protein kinase C (PKC) is a family of serine/threonine kinases involved in diverse signaling pathways that regulate cell proliferation, differentiation, apoptosis, motility, and adhesion (Parker and Murray-Rust, 2004). PKCs have a putative role in tumorigenesis due to their ability to function as receptors for tumor-promoting phorbol esters (Castagna et al., 1982). However, the relative contribution of individual isoforms to this process has been difficult to establish, likely due to the redundancy of PKC isoforms among cell types (Griner and Kazanietz, 2007). Thus, a major question in PKC biology is how the functional specificity of each kinase is determined. It is widely accepted that the in vivo function of PKCs depends on the subcellular localization of both the kinases and their substrates, which is largely dependent on PKC-interacting proteins (Rosse et al., 2010). PKCα binds calcium and acts as an intracellular receptor for tumor promoting phorbol esters including 12-O-tetradecanoylphorbol-13-acetate (TPA). Therefore, PKCα is the major molecular target in DMBA/TPA-induced skin carcinogenesis (Rundhaug and Fischer, 2010). PKCα is ubiquitously expressed and its activation results in increased cell motility and invasiveness in several in vivo and in vitro cancer models (Martiny-Baron and Fabbro, 2007), and its inhibition might prevent epithelial cancers (Mullin et al., 2000). Along this line, studies unraveling the physiological importance of PKC regulators including scaffold proteins determining PKC function such as Discs Large Homolog 1 (DLC-1) (O’Neill et al., 2011), and E3 ubiquitin ligase of PKC such as the RING-finger protein that interacts with C kinase (RINCK) family (Chen et al., 2007) will provide us novel aspects of tumorigenesis and therapeutic.
Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase implicated in skin structure (Murillas et al., 1995) and epithelial carcinogenesis (Nicholson et al., 2001; Yarden, 2001). EGFR is reported to be upregulated in the mouse model of multistage carcinogenesis of the skin (Rho et al., 1994). Similarly, its overexpression and constitutively activating mutations are frequent in human tumors of epithelial and glial origins (Olayioye et al., 2000). EGFR deficiency and a hypomorphic allele of EGFR have been proven to reduce spontaneous skin tumorigenesis in K5-SOS-F transgenic mice. Thus, EGFR signaling is important in the development of skin cancer (Sibilia et al., 2000).

In this study, we investigate the role of Ei24 as a modulator of signal transduction. We report that Ei24 interacts with both RINCK and PKcα to competitively disrupt the RINCK-PKcα interaction. This results in the stabilization of PKcα and promotes EGFR trans-activation. Finally, loss of Ei24 attenuates DMBA/TPA-induced skin carcinogenesis, likely by disrupting the signaling crosstalk between RINCK1, PKcα and EGFR. The data presented here suggest Ei24 is an important molecular link required for the RINCK1-PKcα-EGFR signaling axis in the development of skin cancer.

2. Materials and methods

2.1. Generation of Ei24 knockout mouse line

A gene-trap embryonic stem (ES) cell clone (AZ0023) was obtained from Welcome Trust Sanger Institute and was used to generate germ-line transmitted mice as described previously (Lee et al., 1998). A Sphi restriction enzyme and a DNA fragment corresponding to exon 2 of Ei24 gene was used as a probe for Southern blot analysis as described previously (Lee et al., 1998). Three-primer PCR genotyping was routinely conducted to manage the mouse colony using the following primers: 5′-AGAGAGATGTTGGGCTGAGA-3′ as a common primer, 5′-CTCGATGTGGATCTCAGAATCTC-3′ for wild type-specific primer, 5′-CATATGGGTGTAAGTACCGGACA-3′ for null allele-specific primer. All mice were housed in individually ventilated microisolation cages (Three Shine Inc., Korea) in a specific pathogen-free (SPF) area of Yonsei Laboratory Animal Research Center (YLARC). The animal experiments were approved by Institutional Animal Care and Use Committee (IACUC) of Yonsei University.

2.2. RNA isolation, Northern blot, cDNA synthesis, and real time qPCR

RNA was isolated from tissues and cells using Trizol reagent (Invitrogen) following manufacturer’s instructions. Reverse-transcription and real-time qPCR (IQ5, Bio-rad) analyses were conducted as described previously (Sung et al., 2010). The primer pairs used for the analyses were 5′-CTTTCGACAGGACTTGGCAA-3′ and 5′-CTCTTCTTCTTGTGATCC-3′ for Ei24, 5′-ACATTCACTGCCTAACAGTCT-3′ and 5′-GGCCAGCCCCCTATGATGCA-3′ for PKcα, and 5′-ATACCTGCACCCAGGACACAG-3′ and 5′-GATAGCGAGGATGATC-3′ for GAPDH. Northern blot analysis was conducted as described previously (Sung et al., 2010) using a cDNA fragment complementary to mouse Ei24 (NM_007915, 448–1153) as a probe. Signals were detected using a Phospho-Imager (Bio-rad).

2.3. DMBA/TPA skin carcinogenesis

For skin carcinogenesis studies, 8 week-old male and female Ei24-wild type and Ei24 heterozygous null mice (in both FVB/NJ and 129 x C57BL/6 backgrounds) were shaved on the dorsal skin and a single dose of 100 μg DMBA (Sigma) was applied. Two weeks after DMBA application, tumorigenesis was promoted with 5 μg TPA (Sigma) applied twice weekly for 27 weeks. In DMBA/TPA skin carcinogenesis model, 7,12-dimethylbenz(a)anthracene (DMBA) acts as an tumor initiator by causing random mutations of which Ras mutations are selected for during tumorigenesis, and TPA acts as a promoter by triggering clonal expansion of initiated keratinocytes, thus mimicking clinical cases of skin cancer development (Abel et al., 2009; Loeb and Harris, 2008). Mice were scored for tumor multiplicity and size each week. H&E-stained tumor samples were classified into SCC, high grade dysplasia, and low grade dysplasia by the certified pathologist.

2.4. Statistical analysis

For statistical analysis, GraphPad Prism was used. Each assay was performed in triplicates. The data were expressed as mean ± SD and Student’s t-test was used to determine the significance when multiple comparisons were made. P < 0.05 was considered statistically significant.

3. Results

3.1. Ei24 stabilizes PKcα by promoting RINCK degradation

To elucidate the detailed molecular function of Ei24, we searched for proteins of known functions containing conserved Ei24 sequences (Fig. 1A). A Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) query using the full-length Ei24 amino-acid sequence could identify a 6 amino-acid sequence present in RINCK, but not in other RING domain E3 ligases (Fig. 1A). Since this 6 amino-acid sequence is present in RINCK, we named it the ‘R-motif’ (Fig. 1A). The Ei24 R-motif is highly conserved from fruit flies to humans, whereas the RINCK R-motif is conserved from mice to humans (Fig. 1A). Based on these clues, we hypothesized that Ei24 might regulate PKcα protein levels. The two homologues of RINCK (RINCK1 and RINCK2) show a similar molecular effect on PKcα degradation (Chen et al., 2007), but RINCK1 is the major form expressed in human tissues. Therefore, RINCK1 was used in this study. In fact, Whereas RINCK knockdown increases PKcα protein (Chen et al., 2007), Ei24 knockdown using short hairpin decreased PKcα protein in 293 T cells (Fig. 1B) without affecting mRNA level (Supplementary Fig. 1). Beforehand, Ei24 antibody was validated for immunoblotting and immunoprecipitation (Supplementary Fig. 2A–C). Interestingly, Ei24 loss resulted in the accumulation of RINCK protein (Fig. 1B). Furthermore, Ei24 overexpression resulted in RINCK degradation followed by PKcα stabilization (Fig. 1C), and the antagonistic effect of Ei24 on RINCK was dose-dependent (Fig. 1D).

Given that human and mouse Ei24’s share 98% sequence homology, we examined whether mouse Ei24 was also able to degrade human RINCK and determined that mouse Ei24 also degrades human RINCK in a dose-dependent manner (Supplementary Fig. 3A). Ei24 overexpression increased the stability of endogenous PKcα in H1299 cells (Fig. 1E) and both endogenous and exogenous PKcα in 293 T cells, respectively (Supplementary Fig. 3B and C). In addition, the half-life of RINCK decreased with Ei24 overexpression in 293 T cells (Supplementary Fig. 3D). Collectively, Ei24 promotes RINCK degradation which results in the PKcα stabilization.

3.2. Ei24 competes with RINCK for PKcα binding

As RINCK physically interacts with and ubiquitinates PKcα (Chen et al., 2007), we examined whether Ei24 could bind to both RINCK and PKcα. Immunoprecipitation assay revealed that both endogenous and exogenous Ei24 and PKcα proteins bind to each other (Fig. 2A; Supplementary Fig. 4A and B). Confocal microscopy also showed colocalization of PKcα-HA and Ei24-Flag in H1299

cells (Fig. 2B). To identify the region of PKCα that binds Ei24, we examined the interaction of several PKCα fragments (Raghunath et al., 2003) with full-length Ei24. We determined that Ei24 bound specifically to the C1a domain of PKCα, whereas the C2 domain was dispensable for the interaction (Fig. 2C). We also proved a physical association between Ei24 and RINCK (Fig. 2D). As RINCK also binds the C1a domain of PKCα (Chen et al., 2007), this finding suggested that Ei24 and RINCK may compete for the C1a domain of PKCα. Indeed, immunoprecipitation assays revealed the interactions among Ei24, RINCK, and PKCα (Fig. 2E). These data might explain our previous observation that Ei24 stabilizes PKCα. If Ei24 and RINCK bind the same domain in PKCα, Ei24 interaction with
Fig. 2. Ei24 binds to PKCα and RINCK1. (A) Endogenous Ei24 and PKCα bind to each other. 1 µg of Rabbit polyclonal antibody against Ei24 or Rabbit-IgG (rlgG, as a control) was used for immunoprecipitation. (B) Immunocytochemistry showing colocalization of Ei24 with PKCα in H1299 cells by confocal microscopy. (C) The depicted PKCo-GFP constructs (C-term tagged, FL = full length, RD = regulatory domain) were transfected with Ei24-Flag in 293 T cells and immunoprecipitation was performed 36 h later. Solid arrow heads represent the expected band size and an asterisk represents non-specific bands. (D) Ei24 binds to RINCK1. (E) Ei24, RINCK1, and PKCα bind to each other. Immunoprecipitation was performed with GFP antibody (Ei24), and immunoblot analysis was performed with HA to detect PKCα and Flag to detect RINCK1. (F) Ei24 competitively inhibits the binding between RINCK1 and PKCα as determined by IP-assay. (G) ΔR-Ei24 promotes the binding between RINCK1 and PKCα. Cell lysates were pulled down with GFP antibody, and Western-blot analysis was performed with Flag for detecting RINCK1 signal. (H) R-motif is the structural determinant for binding to PKCα. Compared to wild-type Ei24, ΔR-Ei24 displayed 85% reduced binding.
PKCα may hinder RINCK binding, thus contributing to the stability of PKCα. Reduced interaction of PKCα and RINCK1 upon Ei24 overexpression confirmed our hypothesis that Ei24 plays a negative role in PKCα-RINCK1 binding (Fig. 2F). Based on the presence of the R-motif in both Ei24 and RINCK, we speculated that this motif is required for binding to C1α domain of PKCα. For this purpose, we generated R-motif-deleted Ei24 (hereinafter referred to as ΔR-Ei24). Indeed, ΔR-Ei24 was excluded from the complex resulting in the enhanced binding of RINCK to PKCα (Fig. 2G). Furthermore, direct comparison of the binding efficiencies of wild-type and ΔR-Ei24 with C1α domain of PKCα revealed that the loss of the R-motif results in reduced binding by 85% compared to wild-type Ei24 as determined by the band intensity of immunoprecipitated Ei24-Flag and ΔR-Ei24-Flag (Fig. 2H). These data suggest that Ei24 stabilizes PKCα in two ways. First, Ei24 inhibits the binding of the E3-ligase RINCK to the C1α domain of PKCα, thereby allowing PKCα accumulation. In addition, Ei24 stabilizes PKCα by promoting the degradation of RINCK.

3.3. Ei24 promotes PKCα-mediated transactivation of EGFR and is required for clonogenic potential of keratinocytes

We next elucidated the physiological meaning of the stabilizing effect of Ei24 on PKCα by promoting RINCK1 degradation using in vitro and in vivo systems. Since Protein Kinase C family of proteins is known to be involved in skin malignancies (Mullin et al., 2000), we focused on the role on Ei24 in skin tumorigenesis. For in vitro experiments, we established Ei24 knocked-down PAM 212 stable keratinocyte cell line (Fig. 3A). Knockdown efficiency of Ei24 was more than 90% using two independent shRNA sequences (Fig. 3A). Loss of Ei24 in these cells decreased the stability of PKCα (Fig. 3B) and TPA-induced localization of PKCα to the membrane was severely compromised (Fig. 3C). Ei24 knockdown also reduced the relative colony formation potential of PAM212 keratinocytes by almost 50% (Fig. 3D). Furthermore, the sizes of the colonies formed in Ei24 knock-down cells were much smaller compared to those of parental PAM 212 keratinocytes indicating the role of Ei24 in facilitating the single cell growth into a colony (Fig. 3D). This observation can be explained as the consequence of Ei24 loss to result in decreased PKCα, which is also known to reduce colony formation potential in other cell line (Huigsloot et al., 2003). Collectively, Ei24–PKCα interaction contributes to the tumorigenesis using this standard in vitro assay.

To explain the molecular mechanism of reduced clonogenic potential of PAM212 keratinocytes upon Ei24 loss, we sought to identify the downstream pathways affected by Ei24-RINCK1–PKCα axis. Since EGFR signaling is essential for epidermal tumorigenesis (Sibilia et al., 2000) and EGFR is regulated by PKCα (Stewart and O’Brien, 2005), we investigated whether Ei24 is essential for PKCα-mediated regulation of EGFR signaling. Immunoblotting revealed that TPA-mediated activation of ERK signaling is attenuated upon Ei24 loss in PAM 212 keratinocytes by reducing the protein level of phosphorylated ERK (pERK) (Fig. 3E). Since ERK is the downstream target of EGFR and TPA-mediated activation of EGFR is channeled through PKCα (Stewart and O’Brien, 2005), we concluded that Ei24 is necessary for PKCα-mediated activation of EGFR and ERK signaling. To directly verify the effect of transactivation of EGFR by PKCα is dependent on Ei24, we examined the phosphorylated EGFR (pEGFR) (Tyrrosine 1068) upon TPA treatment in parental and Ei24 knocked-down PAM 212 keratinocytes. Consistent with the previous findings in the prostate (Stewart and O’Brien, 2005), immunostaining of pEGFR (Tyrrosine 1068) was increased upon TPA treatment in the parental cell line of PAM212 keratinocytes (Fig. 3F, upper panel). However, there was significantly less signals detected in Ei24-silenced cells (Fig. 3F, lower panel) which is consistent with the attenuated ERK activation upon TPA application in knock-down cells (Fig. 3E). Thus, it can be concluded that since TPA is not a ligand for EGFR, activation of EGFR by TPA should be channeled through PKCα. Furthermore, the extent of EGFR activation by PKCα is dependent on Ei24 in PAM 212 keratinocytes.

Since TPA-mediated EGFR activation via PKCα was attenuated upon Ei24 loss, Ei24 may act as a molecular bridge to connect PKCα and EGFR. In fact, PKCα and EGFR bind each other in the presence of endogenous Ei24 expression. However, this binding was significantly diminished when Ei24 expression was knocked-down (Fig. 3G). Consistent with this observation, Ei24 overexpression in 293 T cells enhanced the interaction between EGFR and PKCα (Fig. 3H). In addition, fractionation assays performed in 293 T cells revealed that Ei24 facilitates the co-localization of EGFR and PKCα in the membrane (Fig. 3I). These results suggest Ei24 contributes to EGFR activation by enhancing the physical interaction between PKCα and EGFR. Collectively, Ei24 could affect the functional outcome of RINCK1–PKCα–EGFR axis by its ability to stabilize PKCα and enhancing transactivation of EGFR.

3.4. Ei24 loss plays a protective role in DMBA/TPA induced skin carcinogenesis

In order to determine how Ei24 affects tumorigenesis in vivo, we generated Ei24-deficient mice (Supplementary Fig. 5A–D). Ei24 homozygous null mice were embryonic lethal at embryonic day (E) 7.5 (unpublished results). As Ei24 heterozygous null mice exhibited significantly reduced Ei24 expression in the majority of tissues including skins (Supplementary Fig. 5E), we expected haploinsufficient phenotypes in these mice. Moreover, there was no difference in the Ki67 expression between Ei24 wild-type and heterozygous null mice skin (Supplementary Fig. 5F). In the mouse skin, Ei24 loss resulted in accumulation of RINCK1 and PKCα degradation validating our in vitro observations (Fig. 4A). Then we employed the widely used DMBA/TPA carcinogenesis model to evaluate the effect of Ei24 in in vivo skin tumorigenesis (Abel et al., 2009; Loeb and Harris, 2008). In contrast to the previously reported putative tumor suppressive functions of Ei24 (Gentile et al., 2001; Lehar et al., 1996; Zhao et al., 2005), reduced Ei24 expression exhibited a protective effect on DMBA/TPA-induced skin carcinogenesis (Fig. 4B–D; Supplementary Fig. 5G–I). Although the latency and incidence of tumorigenesis were not significantly altered, the average number and size of tumors were considerably decreased in Ei24 heterozygotes compared to wild-type mice (Fig. 4B and D; Supplementary Fig. 5G and I). The decreased susceptibility of Ei24 heterozygous null mice to the development of tumors was also confirmed in the 129 × C57BL/6j mixed genetic background (Supplementary Fig. 5J). These results indicate that Ei24 loss attenuates the development of tumors in skin carcinogenesis. When the tumors obtained after the completion of DMBA/TPA experiment were histologically classified (Supplementary Fig. 6), Ei24 loss appears to have no effect on tumor initiation but rather, delays tumor progression to SCC. Out of the total Hematoxylin and Eosin (H&E)-stained sections classified (WT n = 21, heterozygous null n = 24), low-grade dysplasia contributed 42.8% of the total tumor burden in wild-type mice compared to 66.6% in heterozygous null mice (Fig. 4E). There was no difference in the high-grade dysplasia between the Ei24 genotypes. On the contrary, SCC constituted 23.8% of the total tumor burden in wild-type mice, compared to 8.3% in heterozygous null mice (Fig. 4E). These results suggest that Ei24 loss prevents the progression of low-grade dysplasia into more advanced SCC. In addition, in the SCC samples, the number of proliferating cells in Ei24 heterozygous null mice was almost half compared to that of wild-type mice (Fig. 4F and G). The severity of skin tumorigenesis was less in female mice compared to male mice (Fig. 4B; Supplementary Fig. 5G) which is consistent with previous report (Thomas-Ahner et al., 2007). Finally, since DMBA/TPA...
Fig. 3. Ei24 promotes PKCα-mediated transactivation of EGFR. (A) Generation of Ei24-knock down PAM212 keratinocyte stable cell line. PAM212 Ei24 knock-down stable cell lines were stabilized using lentiviral shRNA packaging system. The efficiency of Ei24 knock down was around 95%. (B) Ei24 knock down in PAM212 keratinocytes resulted in the decreased stability of PKCα upon cycloheximide treatment (10 μg/ml). (C) Immunocytochemistry showing increased PKCα degradation without or upon TPA treatment (500 ng/ml) in Ei24 knockdown keratinocytes. (D) Ei24 loss has a negative effect on the colony formation in PAM212 keratinocytes (n = 6 for each group). (E) Ei24 is essential for EGFR transactivation by PKCα. pERK was detected by immunoblot after treating cells with TPA (500 ng) for 1 h. (F) Ei24 promoted EGFR transactivation by PKCα. Parental and Ei24 knock down keratinocytes were treated with TPA, and pEGFR level (Tyrosine 1068) was detected using immunocytochemistry (red) using Axioplan fluorescence microscope (Nikon). (G) Ei24-mediated binding between PKCα and EGFR in PAM212 keratinocytes was reduced by Ei24 knockdown. (H) Immunoprecipitation in 293 T cells showed that Ei24 overexpression enhances PKCα-EGFR binding. (I) Ei24 facilitated the co-localization of EGFR and PKCα in the membrane fraction. The purity of membrane fractionation was determined using N-Cadherin as a membrane marker and GAPDH as a cytosolic marker.
Fig. 4. Ei24 loss exhibits protective effects in DMBA/TPA induced skin carcinogenesis. (A) RINCK1 and PKCa expression in Ei24 wild-type and heterozygous null mouse skin. Ei24 loss resulted in accumulation of RINCK1 resulting in the degradation of PKCa. (B) Number of papillomas in DMBA/TPA-treated wild-type and Ei24 heterozygous null FVB/NJ male mice (wild type n = 5, heterozygous null n = 6). *P < 0.05. (C) Gross morphology of the tumors in mice after the completion of DMBA/TPA skin carcinogenesis. (D) Spectrum of the size distribution of the tumors. (E) Contribution of each tumor type to the total tumor burden. (F) Ki67 staining in the tumors. (G) Quantitation for the percentage of the Ki67 positive cells (wild type n = 5, heterozygous null n = 6). (H) Ei24 and EGFR are upregulated in human HNSCC. Expression of Ei24 and EGFR was compared in normal and HNSCC samples using Oncomine database. Panel I, Estilo Head and Neck; panel II, Talbot Lung; and panel III, Vasko Thyroid.
mouse model mimics the human SCC (Abel et al., 2009; Loeb and Harris, 2008), we examined if Ei24 expression is increased in HNSCC compared to normal tissue. Using Oncomine, a cancer microarray database (Rhodes et al., 2004), we observed increased Ei24 expression in several HNSCC samples compared to normal tissues (Fig. 4H, panels I–III). Estilo Head and Neck (Fig. 4H, panel I), which compares the expression pattern of genes in normal tongue and tongue tumor samples (Estilo et al., 2009), Talbot Lung (Fig. 4H, panel II), which described the differential expression of genes in normal tongue and tongue SCC (Talbot et al., 2005), and Vasko Thyroid (Fig. 4H, panel III), which evaluated the pattern of gene expression between thyroid gland and thyroid gland papillary carcinoma (Vasko et al., 2007), all displayed statistically significant up regulation of Ei24 in HNSCC compared to normal tissues. We further show that this increase in Ei24 expression correlates with the increase in EGFR expression in each of these datasets (Fig. 4H). Thus, the ultimate activation of EGFR in our in vitro and in vivo mouse model is also relevant in the human HNSCC. Collectively, Ei24 loss has a protective effect in skin tumorigenesis by attenuation of RINCK1-PKCα-EGFR signaling.

4. Discussion

The data from the current study allow us to draw several important conclusions regarding Ei24 function. Previous studies have characterized Ei24 as a tumor suppressor primarily because Ei24 is a p53 target gene (Gu et al., 2000a,b; Lehar et al., 1996; Mork et al., 2007; Zhao et al., 2005). However, our current findings are seemingly contradictory and illustrate that Ei24 loss attenuates skin carcinogenesis in mice. However, we provide a detailed molecular map by identifying novel Ei24 binding partners and describing how these molecular interactions affect the downstream signaling of PKCα and EGFR. A similar observation was reported recently in which the authors show that although DLG1 is a reported tumor suppressor (Bilder, 2004; Humbert et al., 2008), the binding of DLG1 to PKCα is oncogenic (O’Neill et al., 2011). These findings suggest that rather than the generalized labeling of proteins as tumor suppressors or oncogenes, these terms should be used in context- and tissue-dependent manners. In this regard, the role of the PKCα-Ei24 interaction in other tumor types should be the focus of future studies. This study further confirms that PKCα’s role in tumorigenesis is coordinated by binding proteins (such as Ei24) and the activation of downstream pathways.

This study identifies Ei24 as a PKCα binding partner, with the Ei24-PKCα interaction stabilizing PKCα. Ei24 provides PKCα stabilization in two ways. First, Ei24 promotes the degradation of RINCK, an E3 ligase for PKC proteins. Future work should focus on elucidating the mechanism of RINCK degradation by Ei24. In addition, Ei24 and RINCK share an R-motif that binds to the C1a domain of PKCα.
the presence of Ei24, RINCK is likely sterically hindered from binding PKCα, thereby stabilizing PKCα and allowing protein levels to accumulate. We suggest the R-motif might be useful as a structural component for identifying novel PKCα binding partners as well as for a possible peptide for drug development in diseases where PKCα expression needs to be regulated.

Ei24 homozygous null mice were embryonic lethal, suggesting an essential role of Ei24 during embryonic development. Although we utilized Ei24 heterozygous null mice as our Ei24-deficient mouse model, the generation of conditional Ei24 knockout mice will provide more precise information regarding Ei24 function. Based on the fact that the loss of just one allele of Ei24 in mice has a protective effect, we can speculate that genetic models comprising of total Ei24-deficiency could have further significant protection against skin cancer. As a preliminary study, using Oncomine cancer microarray database, we have shown that Ei24 and EGFR are upregulated in some subsets of human HNSCC compared to normal tissues. Thus, the next task in this study is to validate this observation in human skin malignancies. We also show that though Ei24 loss has no effect in the tumor initiation, it inhibits progression of low grade dysplasia into malignant SCC. Thus, limiting Ei24 expression could be used as a strategy to inhibit the progression of skin cancer into malignant forms.

Even though it is known that PKCα binds EGF (Gauthier et al., 2003), there is no consensus opinion regarding the outcome of this binding. It is well established that PKC-mediated phosphorylation of Threonine-654 at the juxtamembrane region of EGFR mediates the feedback inhibition of G-protein coupled receptor (GPCR)-induced EGFR transactivation (Davis and Czech, 1985; Santiskulvong and Rozengurt, 2007; Wang et al., 2007). On the contrary, several groups have reported the crosstalk between PKCα and EGFR signaling pathways resulting in the activation of subsequent downstream pathways. PKCα has been reported to act downstream of the epidermal growth factor receptor (EGFR) to activate the mammalian target of rapamycin (mTOR) signaling in glioma (Fan et al., 2009). PKCα also activates the metalloprotease ADAM17, which cleaves the EGFR ligand such as heparin binding-EGF (HB-EGF) to activate EGFR signaling (Kveiborg et al., 2011).

In the human prostate cancer cell line PC-3, PKCα activation is required for EGFR transactivation and subsequent ERK1/2 activation, highlighting the central role of PKCα as a ligand-independent activator of EGFR (Stewart and O’Brien, 2005). In this particular study, the authors have shown that the tumor-promoting phorbol esters transactivate EGFR in PKCα-dependent manner with the total increase in the phospho-tyrosine content of EGFR. But the authors are silent about the specific amino-acid in EGFR involved in this process. Our results from PAM212 Keratinocyte cell lines followed the same pattern of EGFR activation by PKCα as described in PC-3 cells. However, we have further pinpointed that phorbol ester-mediated activation of EGFR occurs by phosphorylation at Tyrosine-1068 and this process is dependent upon Ei24. Still several questions need to be addressed regarding regulation of EGFR by PKCα and the role of Ei24 in this process. Since PKCα can inhibit EGFR by phosphorylation on Threonine-654 and also activate EGFR signaling by phosphorylating its Tyrosine 1068, how and why this specificity is achieved should be determined. At least in PAM212 keratinocytes, Ei24 appears to direct PKCα signaling for EGFR activation by phosphorylation of Tyrosine-1068. Thus, Ei24 is required for maintaining signaling in the RINCK1-PKCα-EGFR axis for the development of skin carcinogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2012.06.034.

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