The Intricacies of Skin Cancer: A *Tpl2* knockout enhances tumorigenesis and affects tumor phenotype during promotion

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The *Tpl2* gene encodes a serine/threonine kinase in the MAPK signal transduction cascade and has been implicated in both inflammation and tumorigenesis. The protein homolog in humans, MAP3K8, is downstream of Ras signaling. It activates transcription factors such as Activator Protein-1 (AP-1), a complex involved in growth, differentiation and apoptosis. AP-1 is regulated by a number of proteins including Protein Kinase C and Ras. Previous research from our laboratory showed that *Tpl2* knockout mice treated under the two-stage skin chemical carcinogenesis model have a significantly higher incidence of tumors, with a higher number of tumors per animal on average. These tumors included squamous cell carcinomas, papillomas and a number of sebaceous adenomas. It has been previously reported that AP-1 inhibition transdifferentiates squamous tumors into sebaceous adenomas. Therefore, we tested whether TPA-treated knockout primary keratinocytes have alterations in the protein expression of specific AP-1 family members. Additionally, we analyzed mRNA levels of various AP-1 family members by using real-time PCR. Data from *in vitro* experiments show a higher expression of c-Fos in the knockout genotype. The mRNA expression of this AP-1 family member concurs with its protein expression. This shows that AP-1 expression is regulated at the transcriptional and post-translational processing stages, resulting in its increased expression in the knockout. These results suggest a link between c-Fos expression and the higher number of sebaceous adenomas observed in the *Tpl2* knockout genotype.

Introduction

"Cancer" is a term assigned to more than 100 diseases where abnormal cell proliferation is observed. Causative genetic alterations include changes in chromosome number, translocation, gain-of-function mutations in proto-oncogenes and loss-of-function mutations in tumor-suppressor genes. An accumulation of these changes results in the continual division of a single progenitor cell to form a clone with one or more of the hallmarks of cancer: A self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replication potential, sustained angiogenesis, tissue invasion and metastasis (Weinberg, 2006). A majority of human tumors rise in epithelia such as the skin. Cancers originating in the squamous cell layer, basal cell layer and the melanocyte layer of the skin are called squamous cell carcinomas, basal cell carcinomas and melanomas respectively.

Although melanomas are more lethal, non-melanomas are more abundant with more than a million cases diagnosed each year according to the National Cancer Institute. Basal cell carcinomas are the most abundant non-melanomas. The American Cancer Society reported that squamous cell cancers occur less often in the United States (~200,000 to 300, 000 diagnosed per year). Although death from non-melanomas is uncommon, a majority of the affected elderly die.

The different stages of cancer progression are predictable in normal epidermal cells, from the benign squamous papilloma stage to the squamous cell carcinoma stage (Yuspa and Dlugosz, 1991). The stages of cancer between normal cells and malignant cancerous cells include hyperplasia with an excessive number of cells; dysplasia with cytologically abnormal cells; neoplasia with both benign and malignant tumors; and metaplasia, where the normal cell layer is displaced by another tissue type usually not encountered in that area (Weinberg, 2006). Growths in the epithelial layer, such as adenomas and papillomas, fall in neither of these categories. Adenomas contain dysplastic tissue but are benign, as they respect the boundary formed by the basement membrane (Weinberg, 2006).

STUDY OF SKIN CARCINOGENESIS

Skin carcinogenesis is modeled *in vivo* on mouse (murine) epithelium. Dorsal murine skin is painted with a tumor initiating agent, dimethyl benz [a]-anthracene (DMBA), followed by 20 weekly applications of a phorbol ester tumor promoter, such as 12-O-tetradecanoylphorbol-13-acetate (TPA) (Weinberg, 2006). This model usually generates benign papillomas, with a small percentage that progress to squamous cell carcinomas (Gerdes and Yuspa, 2005). In several genetically altered mouse models, sebaceous adenomas were shown to be another tumor phenotype following similar multistage carcinogenesis models (Gerdes and Yuspa, 2005). Genetic analysis of the tumors induced revealed a characteristic mutation in codon 61 of the *H-ras* proto-oncogene (Gerdes et al., 2006).

SEBACEOUS ADENOMAS

Sebaceous adenomas are composed of incompletely differentiated sebaceous nodules, with basaloid cells in the periphery and mature sebaceous elements with characteristic cytoplasmic vacuoles in the center (Ponti and Leon, 2005). Clinically, they are observed in regions with a high concentration of sebaceous glands, such as the eyelids, scalp and the nose (Banse-Kupin et al., 1984; Ponti and Leon, 2005). Although sebaceous adenomas are relatively rare, they are observed in Muir- Torre syndrome (Ponti and Leon, 2005). This autosomal dominant disease is characterized by tumors of the sebaceous gland (Ponti and Leon, 2005). Other typical skin tumors associated with this disease include carcinomas, keratocanthomas and basal cell carcinomas with sebaceous differentiation (Schwartz and Torre, 1995). This differentiation is also considered a strong phenotypic marker of the disease (Ponti and Leon, 2005). Reported genetic alterations that result in sebaceous adenoma phenotypes included a promoter-driven

Cycloxygenase-2 (COX-2) transgene, and an *Fhit* knockout (Muller-Decker et al., 2002; Fong et al., 2000). It was recently shown that inhibiting Activator Protein-1 (AP-1) transcription factor after the formation of squamous tumors caused them to transdifferentiate into sebaceous adenomas (Gerdes et al., 2006). Therefore, it is note-worthy that sebaceous adenomas were observed in a skin phenotype stimulated under the chemical carcinogenesis model.

THE MAP3K8 GENE/ Tpl2

The *Tpl2*/*Cot* gene initially isolated from Moloney leukemia virus-induced rat thymomas codes for a dual specificity serine/threonine protein kinase of the MAP kinase kinase kinase (MAP3K) family (Patriotis et al., 1993; Salmeron et al., 1996). Its overexpression was implicated in epithelial transformation in mouse mammary gland cells (Erny et al., 1996; Patriotis et al., 1993). In addition to breast cancer, *Tpl2/ Cot* overexpression has been implicated in thymomas, lymphomas, Hodgkin's disease, nasopharyngeal carcinomas, invasive endometroid carcinomas and in a subset of T-cell neoplasms known as Large Granular Lymphocyte Proliferative Disorders (Krcova et al., 2008; Alves et al., 2006; Christoforidou et al., 2004; as cited in Clark et al., 2004). Mutations in the *MAP3K8* gene have been shown in lung cancer (Clark et al., 2006). Thus, this gene is important in the study of various cancers. It has also been implicated in inflammatory pathways and modulates T_{H1} differentiation (Eliopoulos et al., 2006; Stafford et al., 2006; Dumitru et al., 2000; Cismasiu et al., 2009; Sugimoto et al., 2004).

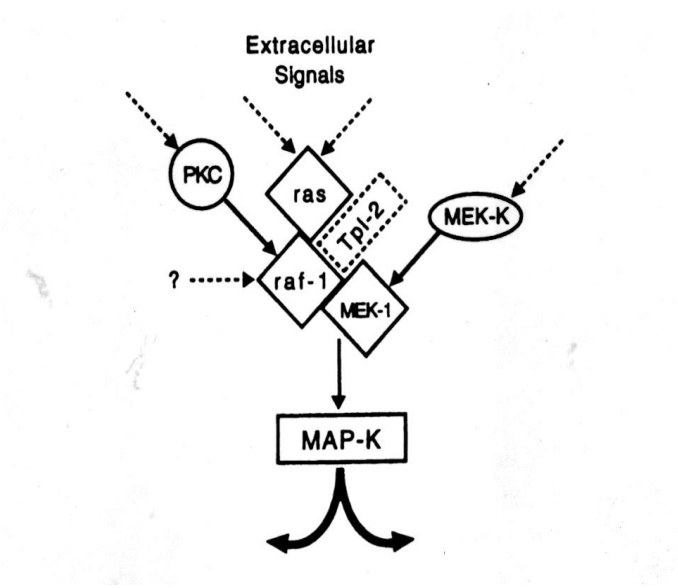

Figure 1- Model for MAPK activation by Tpl2. On activation by extracellular signals, a mulltimolecular complex of Tpl2, Ras and Raf induces MAPK activation[1].

Cot activation has been shown to activate the p38-MAPK, JNK, ERK 1/2 and NF-κB pathways in a cell-type and stimulus-specific manner (Dumitru et al., 2000; Das et al., 2005). The encoded kinase functions in the MAPK/ ERK kinase-1 (MEK-1) signaling pathway among others (Reviewed by Clark et al., 2004). MAPK activity is regulated by signals from activated receptor tyrosine kinases and Protein Kinase C (PKC), which in turn activates Raf-1, a MEK-1 kinase (as cited in Patriotis et al., 1994). Ras, a protein upstream of Raf, interacts with Raf-kinase in its Ras-GTP form. Now, Raf is more prone to activation by other upstream signaling factors when tethered to the plasma membrane.

Separate dominant negatives of Ras, Raf and Tpl2 inhibited MAPK activation in COS-1 cells (Patriotis et al., 1994). This suggests the formation of a multimolecular complex between Tpl2, Ras and Raf to induce MAPK activation, rather than the sequential signaling pathway mentioned earlier (Figure 1). Once activated by upstream Ras-Raf signaling, MEK-1 phosphorylates serine and threonine residues on Extracellular Regulated Kinase-1 and 2 (Erk-1 and Erk-2). Erks can translocate to the nucleus and phosphorylate transcription factors. MAPK can directly phosphorylate transcription factors such as Rsk, Elk-1, c-Myc and c-Fos (Patriotis et al., 1994). Other transcription factors include members of the Fos and Jun protein families, which associate to form Activator Protein- 1 (AP-1).

ACTIVATOR PROTEIN-1 (AP-1)

AP-1 regulates basal-level and inducible transcription of genes containing the AP-1 site (consensus sequence 5'-TGAG/CTCA-3') (Reviewed by Hess et al., 2004). These sites are also known as TPA Responsive Elements (TREs) (Angel and Karin, 1991). AP-1 contains a basic DNA-binding domain and a leucine zipper which is responsible for dimerization of the Jun and Fos proteins (Wagner, 2001). Members of the Jun protein family that make up AP-1 include c-Jun, JunB and JunD, initially identified as c-Jun collectively (Reviewed by Wagner, 2001). Members of the Fos protein family that dimerize with members of the Jun protein family are FosB, Fra-1 and Fra-2, collectively identified as c-Fos (Reviewed by Wagner, 2001). The JNK pathway results in the transcription of c-Jun, which is phosphorylated and translocated to the nucleus. The dimerization of c-Fos and pc-Jun results in the formation of the AP-1 complex, capable of regulating growth, apoptosis and differentiation via gene transcription (Eferl and Wagner, 2003). c-Jun and c-Fos are highly expressed in the granular layer, below the outermost cornified layer in the skin epidermis (Figure 2).

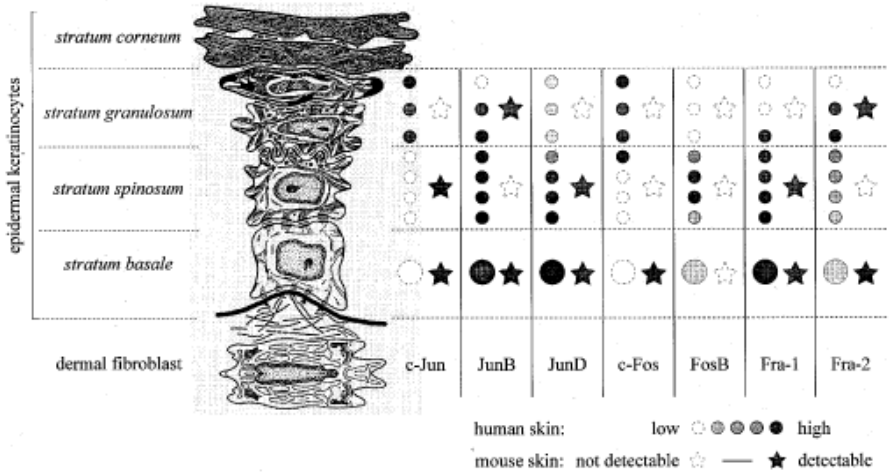

Figure 2- Expression pattern of AP-1 subunits in human skin. c-Jun and c-Fos are highly expressed at detectable levels in stratum granulosum in mouse skin (Angel et al., 2001). The differential expression of the different Fos and Jun members in the epidermal layers suggests a regulatory function of AP-1 in differentiation of keratinocytes (Angel et al., 2002).

Members of the ATF protein family can also heterodimerize with members of the Fos and Jun protein families (Angel et al., 2001). It must be noted that Jun proteins can homodimerize, while Fos proteins cannot (Hess et al., 2004). Instead, they form heterodimers with members from the Jun family and increase the DNA-binding activity of the transcription factors (Hess et al., 2004). These proteins contain docking sites for other kinases, such as c-Jun NH_2-terminal Kinase (JNK) and ERK (Eferl and Wagner, 2003). AP-1 activity is regulated through signaling pathways involving Protein Kinase C (PKC), Ras, and the p38 family of MAPK (Reviewed by Gerdes et al., 2006). AP-1 controls cell proliferation by the positive regulation of cyclin D1 and of JunB (Reviewed by Shaulian and Karin, 2001). c-Jun has a negative regulatory effect on p53, cyclin A, cyclin E, $p21^{Cip1}$, $p16^{Ink4a}$ and $p19^{ARF}$ in fibroblasts (Reviewed by Shaulian and Karin, 2002).

AP-1 activity has been implicated in skin tumorigenesis (Gerdes et al., 2006). Activation of AP-1 by JNK, PKC family members, TNF-α and constitutive activation of Ras promote tumorigenesis (Gerdes et al., 2006). Papillomas in murine skin did not differentiate to carcinomas in a c-Fos knockout model (as cited in Gerdes et al., 2006). Cot has been shown to enhance c-Jun expression (Chiariello et al., 2000). The lack

of a transactivation domain in a c-Jun containing AP-1 dominant negative did not produce a skin phenotype, however its deletion resulted in the development of smaller papillomas in a mouse knockout model (as cited in Gerdes et al., 2006).

THE ROLE OF AP-1 IN THE FORMATION OF THE SEBACEOUS ADENOMA PHENOTYPE

Inhibiting AP-1 and DNA binding has been shown to block carcinoma formation in papillomas and convert squamous tumors into sebaceous ones in a mouse multistage skin carcinogenesis model (Gerdes et al., 2006). This inhibition resulted in mild hair loss, sebaceous gland hyperplasia, especially on the eyelids and focal skin erosions in the murine epidermis (Gerdes et al., 2006). The hyperplasic sebaceous glands became independent of the adjacent hair follicles around them (Gerdes et al., 2006). These sebaceous adenomas were observed in a tumor initiating agent dependent manner. In addition, expression of the dominant negative in squamous papillomas induced 26% of the tumors to become mixed sebaceous/ squamous lesions and 14% to sebaceous adenomas (Gerdes et al., 2006). Almost half of the squamous tumors were either converted or trans-differentiating to sebaceous adenomas.

The lipid marker used for differentiating sebaceous cells was Oil-Red O (Gerdes et al., 2006). In addition to inducing the sebaceous phenotype, the dominant negative that inhibits AP-1 and DNA binding also up-regulated two antagonists of the *wnt* family and down-regulated *c-Myc*, a *wnt* target gene. The members of the *wnt* family are highly conserved signaling proteins that control cell-cell signaling. Phosphorylated c-Jun (pc-Jun), an AP-1 component, was bound to the promoters in the *wnt* and *frizzled* families (Gerdes et al., 2006).

Previous Research

Unpublished results in a *Tpl2-/-* mouse have been shown to induce a sebaceous phenotype in tumors induced by the two-stage skin chemical carcinogenesis model (Table 1). Thus, we hypothesized whether MAP3K8 regulated AP-1 expression results in the transdifferentiation of squamous carcinomas into sebaceous adenomas.

Genotype	n (Animals)	n (Tumors)	Squamous Papillomas	Squamous Carcinomas	Sebaceous Adenomas	Mixed Phenotype	Conversion to Sebaceous Phenotype
C57Bl/6	30	6	5	1[1]	0	0	0%
Tpl2-/-	31	113	95	8[2]	8	2	8.85%

Table 1. Unpublished data from DeCicco et al [in prep], showing numbers of mixed phenotype tumors and sebaceous adenomas observed in *C57Bl/6* and *Tpl2-/-* mice treated under the two-step chemical skin carcinogenesis model over 52 weeks.

[1] Contained areas of spindle cells.

[2] 4 tumors were defined as “well-differentiated” and 2 tumors were defined as “poorly differentiated”.

Materials and Methods

Tissue culture of Primary Keratinocytes. Primary keratinocytes were extracted from 8 *C57Bl/6* and 8 *Tpl2-/-* neonatal mice as per protocol on isolation of primary keratinocytes from newborn mouse epidermises (Lichti et al., 2008). Extracted primary keratinocytes were plated in LoCa medium on 20-mm dishes as per protocol on plating primary keratinocytes for culture (Lichti et al., 2008). After confluence was observed on day 3, 10µl of TPA was added to all tissue cultures except controls after an hour of incubation in fresh LoCa solution. Protein lysates were collected at untreated, 5mins, 15mins, 30mins, 1h, 3h, 6h and 18h time-points with M-PER reagent and Halt Phosphatase Inhibitor Cocktail at 10µl /ml (Pierce).

Immunoblot Analysis. Protein extracts were quantified using the BCA Assay as per manufacturer's protocol (Thermo-Scientific). Samples were resolved with sodium dodecyl sulfate and prepared at 20µg/well. The Invitrogen system for immunoblotting was followed with these modifications. The extracts were run on 12% Bis-Tris Criterion XT precast gels at 145V for 90 minutes, transferred onto nitrocellulose membranes at 145V at 0.6A per gel cassette. Membranes were blocked for 60 minutes in 10% milk in TBS-Tween20 and incubated overnight with shaking at 4°C in primary antibody (Cell Signaling) dilutions of 1:1000. Membranes were incubated in secondary antibody (Cell Signaling) dilutions of 1:2000 in 5% milk for 60 minutes. Blots were developed using SuperSignal West extended Dura substrate (Pierce) chemiluminescent reagent and individually wrapped single-coated Kodak BioMax MR films pellicula. Membranes were reprobed using Restore Stripping Buffer (Thermo-Scientific) for beta-actin protein at a primary antibody dilution of 1:4000 and a secondary antibody dilution of 1:2000 (Cell Signaling) to confirm equivalent loading.

Real Time-PCR. TRIzol RNA extraction was carried out on TPA-treated *Tpl2-/-* and *C57Bl/6* cultured keratinocytes as per the manufacturer's protocol (Invitrogen Life Technologies). For analysis, the expression level of c-Fos, c-Jun and GAPDH (control) cDNAs was determined using a BioRad iQ iCycler and Gene Expression Macro (version 2.0) from Bio-Rad (Hercules, CA). For cDNA synthesis, 1 µg of total RNA was reverse transcribed using iScript cDNA synthesis kit (Biorad). The primer sequences used were: GAPDH; CCTGCACCACCAACTGCTTA and TCATGAGCCCTTACAATG; c-Fos, GTAGAGCAGCTATCTCCTGAAGAGG and CGCTTGGAGTGTATCTGTCAGC; c-Jun, CAGAGAGGAAGCGCATGAGG and GTTGGCACCCACTGTTAACGT. Relative standard curves were generated from log input (serial dilutions of pooled cDNA) versus the threshold cycle (C_t), with GAPDH serving as an internal control. The slope of the standard curve was used to determine the efficiency of target amplification (*E*) using the equation $E = 100(10^{-1/\text{slope}}-1)$.

Densitometry Analysis. The area of each individual band film was measured with ImageJ software from a scanned high-resolution. The ratio between the area of each individual band and the respective band for β-actin was calculated. These ratios were normalized to the respective "untreated" or 0 time point for each genotype and graphed in Microsoft Excel.

Results and Discussion

Sebaceous transformation is induced in the *Tpl2-/-* genotype. Previously bred mice of *C57Bl/6* and *Tpl2-/-* genotypes demonstrated tumorigenesis following TPA skin painting. Out of the 113 observed tumors in the wild-type genotype, none were of a sebaceous phenotype as determined by histology (Table 1). Sebaceous adenomas are identified by the presence of mature sebaceous elements surrounded by basoloid periphery cells. This was confirmed qualitatively with Oil-Red O staining, a marker for lipid droplets in growing sebocytes (Data not shown here) (Doran et al., 1991). Although the conversion to a sebaceous phenotype is relatively low (8.85%), their presence was previously unobserved in a *Tpl2-/-* skin model.

Components of AP-1 protein expression change in the *Tpl2-/-* genotype. The knockout genotype has a higher c-Fos induction than the wild-type after 30 minutes of TPA treatment. Both genotypes show similar patterns of c-Fos expression; the highest expression is observed at 3h post TPA treatment (Fig. 3A). This expression is consistent across the other trials carried out (Figures not shown). Total endogenous c-Jun levels are induced at a faster rate in the *C57Bl/6* genotype than in the *Tpl2-/-* genotype (Fig. 3B). However, these values of fold induction are inconsistent due to sources of error, such as the difference in graininess of scanned images from different trials.

Lower phosphorylated c-Jun (pc-Jun) levels were observed in the *Tpl2-/-* genotype. pc-Jun expression follows the pattern of total c-Jun expression in the wild-type genotype, peaking at 15 minutes. Phosphorylated protein levels in the *Tpl2-/-* genotype do not reach the same levels of expression (Fig. 3A). Possible explanations include a slower rate of c-Jun phosphorylation in the *Tpl2-/-* genotype, or faster degradation of phosphorylated protein. These are not mutually exclusive as JNK phosphorylation of c-Jun on ser 73 protects c-Jun from ubiquitination (Fuchs et al., 1996). Hence, it is suggested that a lack of phosphorylation at Ser 63 prevents this protective effect in the *Tpl2-/-* genotype. To truly validate this statement, JNK activity in relation to the TPA time points must be monitored.

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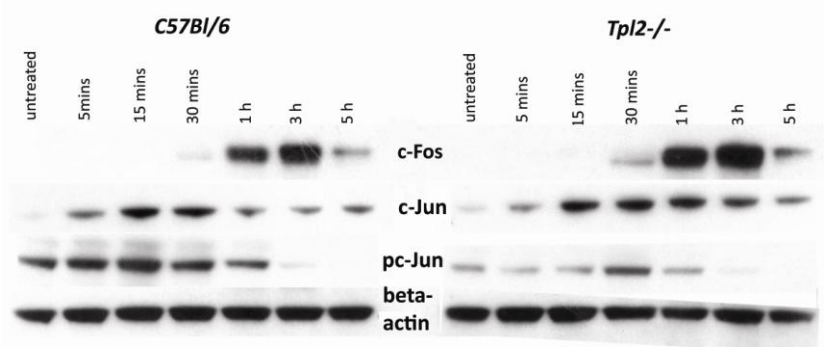

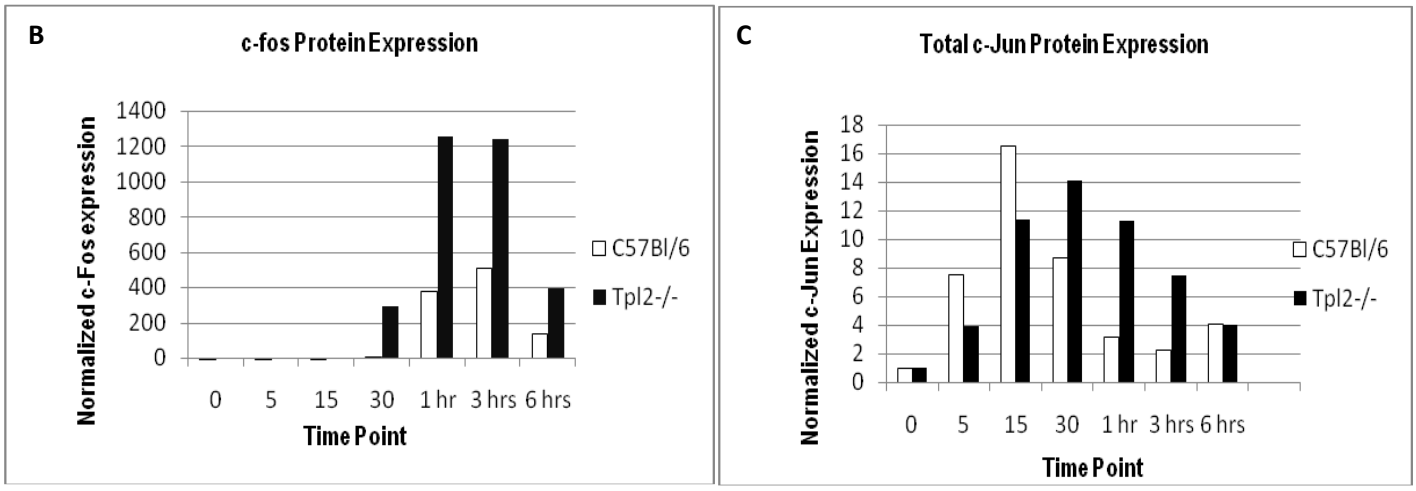

Figure 3. *A,* Compiled immunoblot image of endogenous levels of c-Fos, total c-Jun, pc-Jun proteins in cultured *C57Bl/6* and *Tpl2-/-* primary keratinocytes treated under different TPA time-points. The c-Fos antibody does not detect FosB, Fra 1 and Fra 2. The c-Jun antibody detects c-Jun regardless of phosphorylation state. The pc-Jun antibody detects endogenous levels of c-Jun only when phosphorylated at ser 63. *B, C,* Densitometric analysis of c-Fos and total c-Jun proteins, after normalizing to respective beta-actin levels.

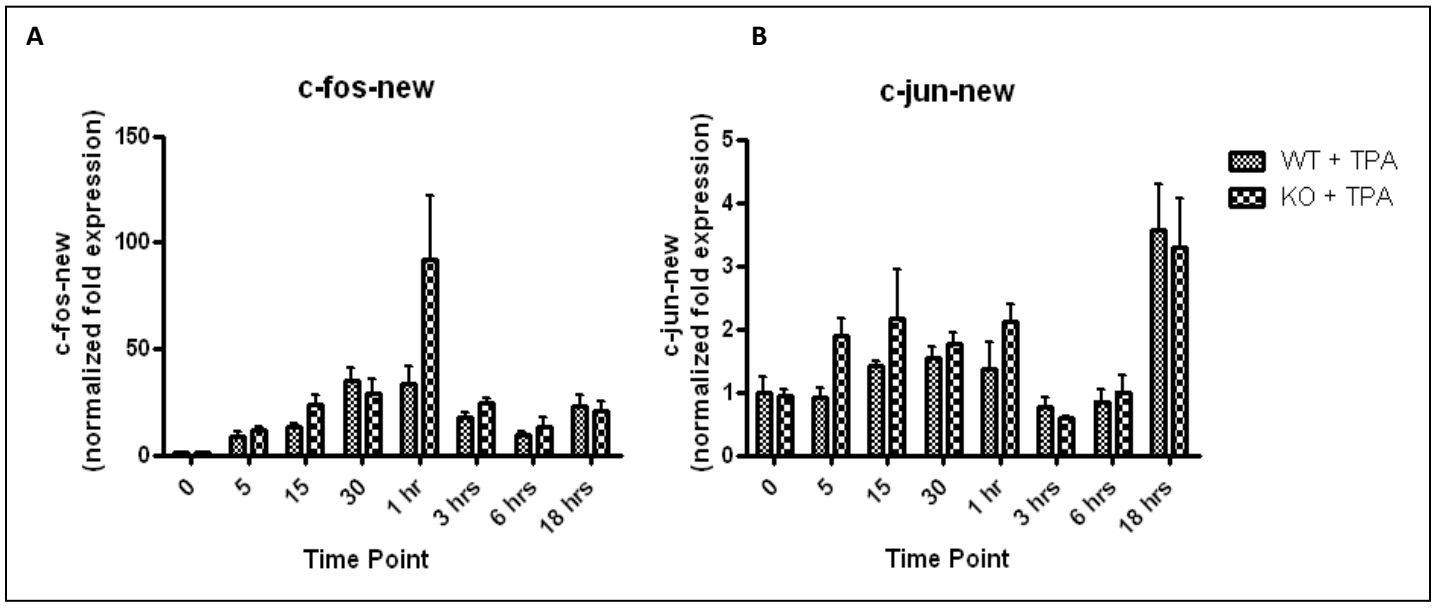

Figure 4. To examine *A, c*-Fos and *B*, c-Jun transcriptional expression, RNA was isolated from cultured *C57Bl/6* and *Tpl2-/-* primary keratinocytes treated at the same time-points shown in the immunoblot analysis. This mRNA expression was analyzed by reverse transcription- PCR.

mRNA expression reflects the protein expression of AP-1 components. Eukaryotic mRNA induction does not necessarily correlate with protein expression, due to alternative splicing, mRNA decay and other transcriptional processing. However, c-Fos protein expression correlates well with the high c-Fos mRNA induction at the 1h time point (Fig. 3B, 4A). This pattern of c-Fos mRNA induction at the 1h peak followed by reduction to background levels 3h later reflects c-Fos induction in epidermal wound healing in mice embryos (Martin and Nobes, 1992). Wound healing is a form of re-epithelization. This suggests a possible explanation for the higher proliferation observed in the *Tpl2-/-* genotype, as demonstrated by a larger number of hyperplasic squamous papillomas. Total c-Jun protein expression does not correlate well with the mRNA induction.

Conclusions

We demonstrated higher c-Fos expression in the *Tpl2-/-* system in comparison to the wild-type genotype in a two-stage cutaneous chemical carcinogenesis model. This protein expression is supported by a similar pattern of mRNA expression, suggesting higher AP-1 expression in the *Tpl2-/-* genotype as c-Fos is an AP-1 constituent. However, this does not directly signify higher AP-1 activation as the transcriptional activation of the other AP-1 component, c-Jun, is dependent on its phosphorylation at Ser-63 and Ser-73 (Potapova et al., 2001). Hence, the lower expression of c-Jun phosphorylated at Ser-63 in the *Tpl2-/-* suggests lowered AP-1 activation. AP-1 is implicated in cell cycle progression and differentiation, thus suggesting a possible *in vitro* explanation why the *Tpl2-/-* genotype demonstrates higher tumorigenesis and transdifferentiation to a sebaceous phenotype in the two-stage chemical carcinogenesis model *in vivo.*

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