

CDK4 regulation by TNFR1 and JNK is required for NF- κ B-mediated epidermal growth control

Jennifer Y. Zhang,^{1,2} Shiyong Tao,^{1,2} Robin Kimmel,^{1,2} and Paul A. Khavari^{1,2}

¹Veterans Affairs Palo Alto Healthcare System, Palo Alto, CA 94304

²Program in Epithelial Biology, Stanford University School of Medicine, Stanford, CA 94305

Nuclear factor κ B (NF- κ B) mediates homeostatic growth inhibition in the epidermis, and a loss of NF- κ B function promotes proliferation and oncogenesis. To identify mechanisms responsible for these effects, we impaired NF- κ B action in the epidermis by three different genetic approaches, including conditional NF- κ B blockade. In each case, epidermal hyperplasia was accompanied by an increase in both protein levels and tissue

distribution of the G1 cell cycle kinase, CDK4. CDK4 up-regulation required intact TNFR1 and c-Jun NH₂-terminal kinase (JNK) function. *Cdk4* gene deletion concomitant with conditional NF- κ B blockade demonstrated that CDK4 is required for growth deregulation. Therefore, epidermal homeostasis depends on antagonist regulation of CDK4 expression by NF- κ B and TNFR1/JNK.

Introduction

Nuclear factor κ B (NF- κ B)/Rel transcription factors exert important effects in diverse tissues, including epithelia. NF- κ B subunits are inhibited by inhibitor of κ B (I κ B) proteins and are activated by an upstream cascade involving I κ B kinases, which is controlled by a number of cell surface receptors, such as TNFR1 (Baldwin, 2001; Dixit and Mak, 2002). In skin, one role for NF- κ B appears to be in the inhibition of epithelial growth. Epidermis lacking the RelA NF- κ B subunit displays hyperproliferation that is epithelial cell autonomous (Zhang et al., 2004). RelA inhibits epidermal growth by opposing the action of another group of TNFR1 effectors, namely the c-jun NH₂-terminal kinase (JNK) cascade (Zhang et al., 2004). NF- κ B epidermal effects are not confined to physiologic growth restraint, but have also been implicated recently in epidermal squamous cell carcinoma (SCC), which is the second most common cancer in the United States. A majority of human SCCs display evidence of NF- κ B hypofunction, and experimentally induced NF- κ B blockade with I κ B α promotes SCC in both murine and human epidermal tissue (van Hogerlinden et al., 1999; Dajee et al., 2003; Lind et al., 2004). Therefore, NF- κ B affects epithelial homeostasis as well as carcinogenesis, although the NF- κ B targets altering cellular growth in these settings are unknown.

The critical transition through the G1 phase of the cell cycle into DNA replication is regulated by CDKs, including

CDK4/6 and their D-type cyclin partners, which act in concert to remove the mid-G1 Rb block (Murray, 2004). Although redundancy in CDK-cyclin-mediated G1 progression is clear (for review see Su and Stumpff, 2004), the functional importance of G1 CDKs and cyclins is supported by the overlapping defects resulting from their ablation as well as by their frequent amplification in human cancers (Cheung et al., 2001; Yasmeen et al., 2003; for review see Su and Stumpff, 2004). Additionally, in human epidermal cells, CDK4 down-regulation has been identified as a safeguard against neoplastic transformation by oncogenic Ras (Lazarov et al., 2002). In this context, CDK4 protein degradation is triggered by Ras in a process that can be inhibited by I κ B α (Dajee et al., 2003), suggesting that NF- κ B opposes epidermal tumorigenesis by altering levels of a core cell cycle regulator. In agreement with this, NF- κ B caused selective CDK4 down-regulation, which led to G1 arrest (Dajee et al., 2003; Hinata et al., 2003). However, these experiments relied on overexpression of active NF- κ B subunits, and it is uncertain whether these findings point to a physiologic role for CDK4 regulation by NF- κ B in epidermal growth control.

Here, we demonstrate that interfering with NF- κ B function in epidermis by multiple distinct genetic approaches increases expression and tissue distribution of CDK4. This CDK4 up-regulation is dependent on both TNFR1 and JNK cascade function, and is consistent with a model in which TNFR1 originates antagonistic effects on the core cell cycle machinery through opposing actions by two of its major downstream effectors, NF- κ B and JNK. *Cdk4* ablation abolished epidermal growth impacts of conditional NF- κ B blockade, confirming the functional importance of CDK4 in this setting.

Correspondence to Paul A. Khavari: khavari@CMGM.stanford.edu

Abbreviations used in this paper: 4OHT, 4-hydroxytamoxifen; BMZ, basement membrane zone; ER, estrogen receptor; I κ B, inhibitor of κ B; JNK, c-Jun NH₂-terminal kinase; NF- κ B, nuclear factor κ B.

Antagonist regulation of CDK4 expression by NF- κ B and TNFR1/JNK therefore mediates homeostatic growth control in epidermis.

Results and discussion

To study the basis for the growth deregulation that occurs with NF- κ B hypofunction in the nontransformed epidermis, we examined the levels of cell cycle regulators. We focused initially on CDK4 because of prior findings in neoplasia overexpression studies (Dajee et al., 2003). To define effects on CDK4 in the context of NF- κ B subunit loss of function by genetic deletion, we began with RelA-deficient epidermis. This tissue, generated by an embryo-grafting approach to immunodeficient mice that circumvents the mid-gestational embryonic lethality of *RelA* knockout mice (Zhang et al., 2004), displayed a markedly more widespread distribution of CDK4 protein; in contrast, distribution of another G1 CDK, CDK2, was unaltered (Fig. 1 a). Although normally confined to the proliferative basal layer of epithelium that is adherent to the underlying basement membrane zone (BMZ), CDK4 protein was found many cell layers above the BMZ in RelA-deficient epidermis, which is consistent with the recently demonstrated expansion of the proliferative cell compartment that occurs in this context (Zhang et al., 2004). Quantitatively, CDK4 protein levels were more than fourfold above the normal level seen in epidermal extracts from RelA-null tissue (Fig. 1 b). Therefore, NF- κ B RelA deficiency leads to CDK4 up-regulation in nonneoplastic epidermis, indicating that RelA is required to maintain physiologic CDK4 expression levels in this setting.

TNFR1 activates NF- κ B as well as other effector pathways, including the JNK kinase cascade, which has emerged as a major oppositional force to NF- κ B action in a variety of cell types (De Smaele et al., 2001; Tang et al., 2001; Reuther-Madrid et al., 2002). In light of recent findings indicating that NF- κ B restrains epidermal proliferation by antagonizing TNFR1-JNK action (Zhang et al., 2004), we wished to determine if the CDK4 up-regulation that occurs with NF- κ B impairment requires intact TNFR1 and JNK function. To examine TNFR1, *Tnfr1*^{-/-}*RelA*^{-/-} double knockout mice were generated; these mice are viable through the postnatal period (Alcama et al., 2001). Epidermal extracts demonstrated no increase in CDK4 levels with RelA deficiency on a TNFR1-null background (Fig. 2 a), confirming that TNFR1 is required for the CDK4 up-regulation that occurs with NF- κ B loss of func-

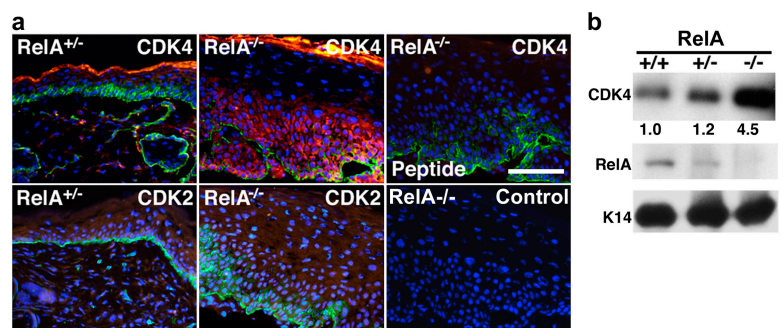
tion. TNF α , the major TNFR1 ligand, down-regulated CDK4 protein levels in primary human epidermal cells, indicating that CDK4 represents a downstream target of TNF action (Fig. 2 b). CDK4 down-regulation by TNF α was blocked by expression of an NF- κ B inhibitory I κ B α super-repressor mutant (Van Antwerp et al., 1996) as well as by active MKK7 (Fig. 2 b). Although neither I κ B α nor MKK7 altered levels of TNFR1 protein (Fig. 2 c), antibodies to TNFR1 blocked the CDK4 increases seen in epidermal cells expressing I κ B α (Fig. 2 d). JNK cascade inhibition, via both genetic and pharmacologic means, also blocked I κ B α -driven CDK4 up-regulation in human epidermal cells in vitro (Fig. 2 e). Consistent with prior studies (Dajee et al., 2003) using overexpression of NF- κ B subunits, altered NF- κ B function failed to change CDK4 mRNA levels significantly (Fig. 2 f), indicating that CDK4 regulation by NF- κ B occurs at the posttranscriptional level. Expression of I κ B α in genetically engineered human epidermal tissue regenerated on immunodeficient mice recapitulated the expansion of CDK4 expression seen in RelA null tissue; this expansion was largely reversed by either coexpression of dominant-negative JNK or topical application of a JNK inhibitor (Fig. 2 g). Epidermal CDK4 up-regulation caused by NF- κ B hypofunction, therefore, requires intact function of both TNFR1 and the JNK cascade.

CDK4 up-regulation in *RelA*^{-/-} epidermis could reflect either a requirement for CDK4 in the hyperproliferation that occurs with NF- κ B impairment or, in light of recent studies (for review see Su and Stumpff, 2004), it could represent a nonessential secondary effect. Distinguishing between these two possibilities requires the ability to examine the consequences of NF- κ B impairment in the absence of CDK4. Generation of *RelA*^{-/-}*Cdk4*^{-/-} double knockout mice, however, is a formidable challenge because of the death of *RelA*^{-/-} embryos by E14.5 (Beg et al., 1995) and the decreased viability and infertility of *Cdk4*^{-/-} mice (Rane et al., 1999). To circumvent this, we established a conditional approach to inhibit NF- κ B by fusing a 4-hydroxytamoxifen (4OHT)-responsive mutant estrogen receptor (ER) domain (Littlewood et al., 1995) to the COOH terminus of Δ SP, which is a p50 mutant that dominantly inhibits NF- κ B function (Logeat et al., 1991). Expression of Δ SP:ER in epidermal cells selectively blocked NF- κ B-directed reporter gene activity and down-regulated expression of the NF- κ B target, I κ B α , in a 4OHT-responsive manner (Fig. 3, a and b). Δ SP:ER thus mediates conditional NF- κ B inhibition.

To determine the effects of NF- κ B hypofunction in the setting of CDK4 deficiency in tissue, we first generated multi-

Figure 1. Increased CDK4 levels in *RelA*^{-/-} murine epidermis.

(a) Expression of CDK4 protein in *RelA*^{+/-} and *RelA*^{-/-} skin. CDK4, CDK2 (orange), the nidogen BMZ marker (green), and Hoechst 33342 (blue) are shown. Note the increase in CDK4 protein in *RelA*^{-/-} epidermis that extends many cell layers above the BMZ compared with the lack of altered CDK2 distribution. The addition of CDK4 peptide immunogen (peptide) abolished detection, and verified CDK4 antibody specificity. Control, secondary antibody alone. Bars, 30 μ m. (b) CDK4 immunoblots. E14.5 keratinocytes from *RelA*^{+/+}, *RelA*^{+/-}, and *RelA*^{-/-} mice embryos were immunoblotted for CDK4, RelA, and K14; optical densitometric quantitation for CDK4 protein is shown below each CDK4 band, normalized to keratin 14 [K14] loading control.



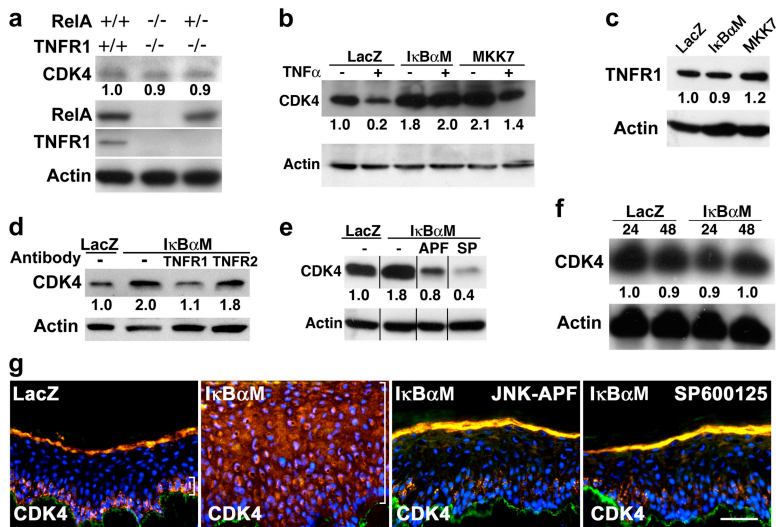


Figure 2. CDK4 up-regulation caused by NF- κ B hypofunction requires intact function of TNFR1 and JNK. (a) A lack of CDK4 up-regulation with RelA deficiency in the absence of TNFR1 in epidermal extract immunoblots from *RelA*^{+/+}, *RelA*^{-/-}, *Tnfr1*^{+/+}, *RelA*^{-/-}*Tnfr1*^{-/-}, and *RelA*^{+/-}*Tnfr1*^{-/-} mice. (b) TNF α decreases CDK4 protein levels. Primary human keratinocytes expressing I κ B α super-repressor mutant (I κ B α M), active MKK7, or LacZ control were treated with (+) or without (-) TNF α . (c) NF- κ B blockade fails to increase TNFR1 expression in keratinocytes that express I κ B α M as well as MKK7 and LacZ controls. (d) Blockade of I κ B α -induced CDK4 up-regulation by antibodies to TNFR1 in keratinocytes expressing I κ B α M or LacZ treated with blocking antibodies to TNFR1 or TNFR2. (e) JNK inhibition prevents CDK4 up-regulation because of NF- κ B blockade. Keratinocytes expressing either LacZ control or I κ B α M were either cotransduced with a retroviral expression vector for dominant-negative JNK1-APF (APF) or treated with the JNK inhibitor SP600125. (f) Northern blot of human keratinocytes harvested 24 and 48 h after expression of I κ B α M or LacZ control. (g) Interfering with JNK cascade function blocks CDK4 up-regulation in response to NF- κ B blockade in vivo. JNK function was impaired in human epidermis engineered to express I κ B α via either coexpression of

the dominant-negative JNK-APF mutant or topical application of SP600125. Representative micrographs from five independently generated animals per group are shown. Note the presence of the CDK4 protein (orange) confined to the basal layer in control tissue (small bracket) and its spread upward in epidermis expressing I κ B α M (large bracket) compared with the diminished CDK4 distribution seen in I κ B α -expressing epidermis subjected to JNK inhibition. Bar, 50 μ m.

ple lines of epidermis-targeted K14- Δ SP:ER transgenic mice; all three expressing lines demonstrated comparable findings (unpublished data). K14- Δ SP:ER mice displayed increased epidermal CDK4 protein levels in response to topical 4OHT (Fig. 4 a), confirming that NF- κ B inhibition in mature epidermis also produces CDK4 up-regulation. K14- Δ SP:ER mice were then bred with *Cdk4*^{-/-} mice to generate K14- Δ SP:ER *Cdk4*^{-/-} mice. Conditional NF- κ B blockade via 4OHT increased epidermal proliferation in *Cdk4*^{+/+} epidermis, resulting in marked hyperplasia in 5 d; in contrast, NF- κ B inhibition in CDK4-deficient tissue produced no changes in either epidermal mitotic index or morphology (Fig. 4, b-d). Therefore, CDK4 is required for the epidermal hyperproliferation that occurs with NF- κ B loss of function.

The present work indicates that suppressing CDK4 up-regulation and confining CDK4 within the epidermal basal layer represents a nonredundant role for NF- κ B RelA that serves as a physiologic restraint on epidermal growth. Moreover, these findings indicate that CDK4 is essential for the increased epidermal proliferation that accompanies NF- κ B im-

pairment. This does not reflect a global deficit in proliferation in CDK4-deficient epidermis, as shown recently by normal hyperproliferation of *Cdk4*^{-/-} mice in response to other stimuli, including phorbol ester and wounding (Rodriguez-Puebla et al., 2002). Although recent neoplasia-focused work suggests that active NF- κ B subunits regulate CDK4 expression at the posttranscriptional level in a proteasome inhibitor-sensitive manner (Dajee et al., 2003), further studies are needed to determine the mechanistic basis for CDK4 responsiveness to altered NF- κ B function. The current data are consistent with recent studies directed at epidermal neoplasia, where CDK4 levels were established to be the critical determinant of whether an epidermal cell undergoes permanent growth arrest or malignant transformation (Lazarov et al., 2002; Dajee et al., 2003). This appears to contradict the orthodox view that regulation of cyclin levels is the vital protein expression control mechanism of cellular proliferation (Murray, 2004) and points to CDK4 protein as a limiting factor in core cell cycle machinery in normal and neoplastic epidermis. In agreement with this possibility, epidermal overexpression of CDK4 causes greater hyperproliferation and malignant transformation than does expression of cyclin D1 from the same promoter (Robles et al., 1996; Miliani de Marval et al., 2004). The well-characterized positive regulation of D-type cyclins by NF- κ B (Guttridge et al., 1999) may thus be balanced by active suppression of CDK4, indicating that additional signals, such as JNK induction, are necessary to overcome CDK4 repression by NF- κ B to support G1 progression in epidermis.

We observed that intact TNFR1 and JNK function was required for CDK4 up-regulation caused by NF- κ B impairment. While NF- κ B-JNK antagonism is increasingly recognized as a potent regulator of signaling outcomes in a variety of settings, this work identifies the first core cell cycle target regulated in an opposing fashion by these pathways. TNFR1 activates both NF- κ B as well as JNK (Aggarwal, 2000), and our

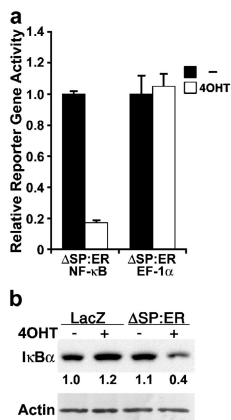


Figure 3. Generation of conditional NF- κ B loss of function. (a) Δ SP:ER inducibly inhibits NF- κ B reporter gene expression in response to 4OHT. Reporter gene activity \pm SD from triplicate independent experiments is shown in cells transfected with luciferase reporter plasmids for NF- κ B as well as EF-1 α specificity control after 48 h with or without 4OHT (50 nM). (b) Inducible suppression of an endogenous NF- κ B target. Extracts from primary human keratinocytes transfected with retrovectors encoding either LacZ or Δ SP:ER and grown for 48 h in the absence (-) or in the presence (+) of 4OHT were immunoblotted with I κ B α antibodies.

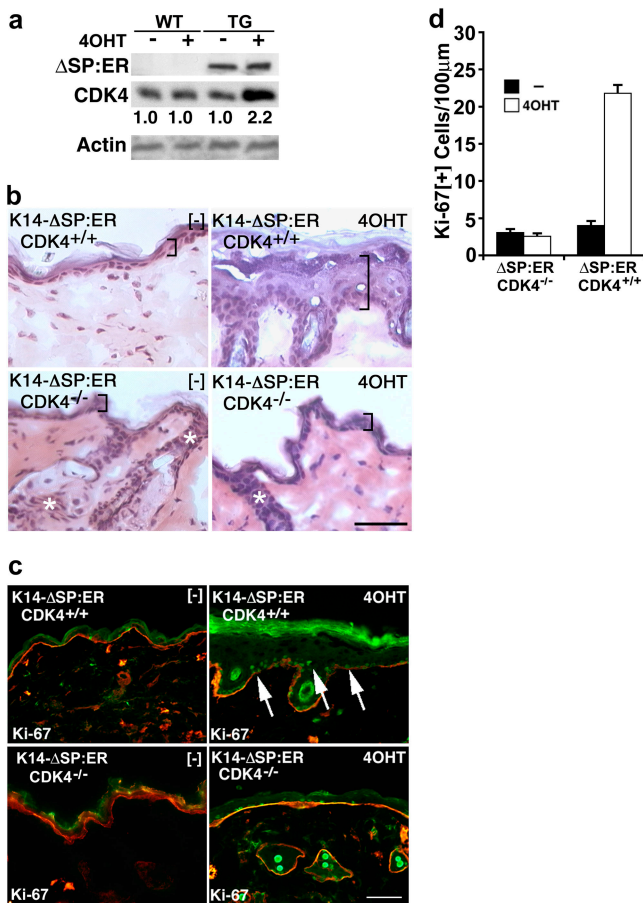


Figure 4. Epidermal hyperproliferation caused by NF-κB hypofunction requires CDK4. (a) Epidermal CDK4 protein levels increase upon inducible inhibition of NF-κB function. Epidermal extracts from wild-type (WT) and K14-ΔSP:ER transgenic mice (TG) treated with 4OHT or vehicle control for 5 d were immunoblotted for ΔSP:ER, CDK4, and actin. (b) Epidermal hyperplasia occurs with inducible NF-κB blockade and is CDK4 dependent. Histology of K14-ΔSP:ER/CDK4^{+/+} and K14-ΔSP:ER/CDK4^{-/-} murine skin treated with either 4OHT or vehicle alone for 1 wk. Note 4OHT-induced hyperplasia in K14-ΔSP:ER/CDK4^{+/+} epidermis and its complete absence in K14-ΔSP:ER/CDK4^{-/-} skin. Representative micrographs are shown for five independent mice per group. Bars, 50 μm. Asterisks denote normal hair follicles, and brackets delineate thickness of intrafollicular epidermis. (c) CDK4 is required for the increased proliferation seen with NF-κB inhibition. Note the increase in green Ki-67(+) cells (arrows) that occurs with NF-κB blockade only in the presence of CDK4. Bar, 50 μm. Ki-67 (green) and nidogen BMZ marker (orange) are shown. (d) Mitotic index quantitation of Ki-67(+) epidermal cells/100 μm skin of K14-ΔSP:ER/CDK4^{-/-} and K14-ΔSP:ER/CDK4^{+/+} murine skin in response to 4OHT. Error bars represent means ± SD from five independent mice for each group.

findings suggest that CDK4 levels, and their resultant effects on epidermal growth control, may depend on the relative balance between these two competing pathways. Although pro-growth functions by the JNK cascade, aside from CDK4 effects noted here, have not been systematically assessed in normal epidermis, the data suggest that JNK and its AP1 targets facilitate epidermal neoplasia. Inhibition of JNK-AP1 function, via deletion of either *Jnk2* (Chen et al., 2001) or *c-Jun* (Zenz et al., 2003) as well as by overexpression of dominant-negative c-Jun (Thompson et al., 2002) increases resistance to topical chemical carcinogenesis, but oncogenic AP1 subunits promote epi-

dermal tumor formation (Wang et al., 1995). Although further studies are needed to define the role of JNK cascade action in epidermal growth control, these data are consistent with a potential role for this NF-κB-opposed pathway in promoting epidermal proliferation.

Our findings suggest that the CDK4-dependent epidermal growth deregulation that occurs with NF-κB inhibition is intrinsic to epidermal cells and does not require significant inflammation. Pure cultures of both murine and human keratinocytes with impaired NF-κB function caused by RelA deficiency and IκBα overexpression, respectively, display both CDK4 up-regulation and hyperproliferation, underscoring the cell-autonomous nature of this process. In tissue, we detected neither an influx of inflammatory cells nor an up-regulation of inflammatory cytokines associated with the CDK4 induction that occurs with conditional NF-κB blockade (Fig. 4 b and not depicted). This lack of inflammation is consistent with prior findings with constitutively RelA-deficient epidermis (Zhang et al., 2004). Although inflammatory cell infiltration is readily excluded in these experiments by immunohistochemical analysis, such analyses do not exclude action by basal levels of local proinflammatory cytokines, such as TNFα, which are constitutively expressed by epidermal cells. TNFα represents a likely ligand for TNFR1 in the epidermal growth deregulation caused by NF-κB loss of function, as demonstrated recently by *RelA^{-/-}c-Rel^{-/-}Tnfα^{-/-}* mice, which display epidermal hypoplasia (Gugasyan et al., 2004). Moreover, the blockade of CDK4 up-regulation by antibodies to TNFR1 in pure cultures of keratinocytes observed here suggests that TNFR1 operates in an epithelial cell-intrinsic manner rather than by a more indirect route, such as via inflammatory cells. This premise is supported by the recent demonstration that *Tnfr1* deletion abolishes hyperplasia and tumorigenesis in epidermis overexpressing IκBα in a manner that cannot be restored by administration of *Tnfr1* wild-type hematopoietic cells (Lind et al., 2004). However, Lind et al. (2004) demonstrated that significant CDK4 up-regulation was observed only in tumorigenesis, and further study of differences between the K5-IκBα mice and the three distinct approaches pursued here is merited. Together, these data support a working model in which locally produced TNFα activates epidermal cell TNFR1, which then alters CDK4 protein levels in a NF-κB and JNK-regulated manner to control cell cycle progression in epidermis.

Materials and methods

Cell culture and gene transfer

Primary human keratinocytes were grown as described previously (Robbins et al., 2001). We grew murine keratinocytes from *RelA^{+/+}*, *RelA^{-/-}*, and *RelA^{-/-}E14.5*-old embryos for 3 d before performing protein extraction. For gene transfer, 4×10^4 keratinocytes were plated on 35-mm wells 1 d before infection with retroviruses. No drug selection was used at any point, and >99% efficiency gene transfer was verified by immunofluorescence staining (Robbins et al., 2001). Retroviruses were generated encoding LacZ, IκBαM (Seitz et al., 1998), and dominant-negative JNK1-APF (Derijard et al., 1994) in the pLZRS backbone. Keratinocytes infected with retroviruses encoding LacZ, IκBαM, or MKK7 were treated with either neutralizing antibodies against TNFR1 (50 μg/ml; mAb 225; R&D Systems), TNFR2 control (50 μg/ml; mAb 226; R&D Systems), the JNK inhibitor SP600125 (10 μM; BIOMOL Research Laboratories, Inc.), or TNFα (15 ng/ml; Sigma-Aldrich) for 48 h before immunoblotting.

Animal studies

RelA^{+/+}, *RelA*^{+/-}, and *RelA*^{-/-} skin tissues were generated through embryonic skin grafting on immunodeficient CB.17 *scid/scid* mice (Oro et al., 1997) and analyzed at 6 wk. Inducible NF- κ B blockade was achieved through in-frame COOH-terminal fusion of the p50 mutant (Δ SP) dominant negative for NF- κ B function (provided by A. Israel, CNRS URA 2582, Paris, France; Logeat et al., 1991) with the 4OHT-responsive mutant ER (Littlewood et al., 1995). Δ SP:ER fusion was subcloned into the pENTR1A plasmid (Invitrogen) and transferred to pLZRS retrovector and pBSKII keratin 14 promoter plasmids modified to contain GATEWAY destination sequences (GIBCO BRL). We confirmed expression of Δ SP:ER protein in three independent lines of K14- Δ SP:ER transgenic mice by immunoblotting epidermal extracts. K14- Δ SP:ER mice were crossbred with *Cdk4*^{-/-} mice (provided by E. Reddy and M. Barbacid, Temple University School of Medicine, Philadelphia, PA; Rane et al., 1999) to generate K14 Δ SP:ER;*Cdk4*^{-/-} mice. To induce NF- κ B blockade, we applied 1 mg/d 4OHT (in 95% ethanol) for 5 d on back skin of 4-wk-old mice; five mice/group. For human skin studies, primary human keratinocytes were engineered to express LacZ control, I κ B α , or I κ B α + dominant-negative JNK1 (JNK-APF) and grafted on immunodeficient CB.17 *scid/scid* mice ($n = 5$ independent grafts per analyzed group). For JNK inhibition, we applied 1 mg/d SP600125 topically to human skin grafts for 5 d before analysis.

Protein expression analysis and microscopy

For immunostaining, 5- μ m cryosections were incubated either with primary rabbit antibodies against CDK4 or CDK2 (Santa Cruz Biotechnology, Inc.) along with rat anti-mouse nidogen (CHEMICON), or rat anti-Ki-67 (Dako-Cytomation) along with rabbit anti-mouse laminin 5 (gift of M.P. Marinkovich, Stanford University, Stanford, CA) followed by Cy2 or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Regenerated human skin cryosections were incubated with primary mouse antibodies against CDK4 (LabVision) along with rabbit anti-collagen VII (Calbiochem) followed by Cy2 or Cy3-conjugated secondary antibodies. Tissue sections were counterstained with Hoechst 33342 DNA for 15 s to visualize nuclei. Images were captured on a Zeiss Axiovert 100M.

Reporter gene assays

For reporter gene assays, cells were transfected with plasmids encoding Δ SP:ER and either NF- κ B or EFl α luciferase reporter plasmids along with CMV-driven renilla-luciferase internal controls for transfection efficiency using FuGENE reagents (Roche). 8 h after transfection, cells were treated with ethanol solvent or 50 nM 4OHT for 48 h before analysis with dual luciferase reagents (Promega).

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