Fos co-operation with PTEN loss elicits keratoacanthoma not carcinoma due to p53/p21^{WAF}-induced differentiation triggered by GSK3β inactivation and reduced AKT activity

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RUNNING TITLE: PTEN/fos synergism in skin cancer

ABBREVIATIONS:

HK1.fos; HK1.ras	transgenic mice expressing v-ras^{Ha} or FBJ/R v-fos from a modified human keratin K1 vector.
PTEN^{wt}; Δ5PTEN$^{wt/flx, flx}$	transgenic mice wild type, heterozygous or homozygous for lox-P flanked exon 5 PTEN.
K14.creP	transgenic mice expressing RU486 responsive cre recombinase from a keratin K14 promoter.
K14.cre/Δ5PTEN$^{wt, wt/flx, flx}$	progeny of mating
HK1.fos/Δ5PTEN$^{wt, wt/flx, flx}$	
SCC	Squamous cell carcinoma.
KA	Keratoacanthoma

ABSTRACT

To investigate gene synergism in multistage skin carcinogenesis, the RU486-inducible cre/lox system was employed to ablate *PTEN* function [*K14.cre/Δ5PTEN*^{flx}] in mouse epidermis expressing activated *v-fos* [*HK1.fos*]. RU486-treated *HK1.fos/Δ5PTEN*^{flx} mice exhibited hyperplasia, hyperkeratosis and tumours that progressed to highly differentiated keratoacanthomas rather than carcinomas, due to re-expression of high p53 and p21^{WAF} levels. Despite elevated MAP kinase activity, cyclin D1/E2 over expression and increased AKT activity forming areas of highly proliferative, papillomatous keratinocytes, increasing levels of GSK3β inactivation exceeded a threshold that induced p53/p21^{WAF} expression to halt proliferation and accelerate differentiation, giving the hallmark keratosis of keratoacanthomas. A pivotal facet to this GSK3β-triggered mechanism centred on increasing p53 expression in basal layer keratinocytes. This reduced activated AKT expression and released inhibition of p21^{WAF}, which accelerated keratinocyte differentiation, as indicated by unique basal layer expression of differentiation-specific keratin K1, alongside premature filaggrin and loricrin expression. Thus, *fos* synergism with *PTEN* loss elicited a benign tumour context where GSK3β-induced, p53/p21^{WAF} expression continually switched AKT-associated proliferation into one of differentiation, preventing further progression. This putative compensatory mechanism required the critical availability of normal p53 and/or p21^{WAF} otherwise deregulated *fos*, *Akt* and *GSK3β* associate with malignant progression.

INTRODUCTION

PTEN is a tumour suppresser gene attracting significant interest given its high mutation frequency in human cancers and its roles in apoptosis/proliferation via negative regulation of AKT/PKB activity (Downward, 2004; Parsons, 2004). Consistent with the direct protein-protein interactions that regulate p53 function (Freeman et al., 2003; Lei et al., 2006), *PTEN* mutation in Cowden Disease patients results in cancer predisposition (Liaw et al., 1997) associated with cutaneous hyperkeratosis (Fistarol et al., 2002), suggesting that roles in keratinocyte differentiation can be added to PTEN activities essential for normal development. In transgenic mice, *PTEN* hetrozygotes (Stambolic et al., 2000) or conditional knockouts (Li et al., 2002; Suzuki et al., 2003) exhibit neoplasia associated with increased anti-apoptotic AKT activities, cell migration/adhesion anomalies (Masahito et al., 1998; Subauste et al., 2005) and cell cycle control failure (Di Cristofano et al., 2001; Weng et al., 2001). In addition, recent models demonstrate that GSK3β, which integrates WNT and β-catenin signalling (Karim et al., 2004), co-operates with PTEN loss in prostate carcinogenesis (Mulholland et al., 2006) when *p53* is also compromised (Chen et al., 2005), whilst bladder models identified compensatory roles for $p21^{WAF}$ that countered initial $PTEN^{null}$-mediated hyperplasia (Yoo et al., 2006).

Multistage skin carcinogenesis studies implicate these molecules also; roles for p53 are well established (Brash, 2006), if sometimes paradoxical (Greenhalgh et al., 1996; Wahl, 2006), as are those for $p21^{WAF}$ (Topley et al., 1999; Devgan et al., 2006). In classic two-stage DMBA/TPA chemical carcinogenesis, AKT activation and GSK3β inactivation typically correlate with tumour progression (Leis et al., 2002; Segrelles et al., 2006) and employing conditional PTEN knockouts, DMBA-initiated c-ras^{Ha} activation achieved increased malignancy following TPA promotion (Suzuki et al., 2003). However, two-stage chemical carcinogenesis employing heterozygous *PTEN* knockouts identified a mutual exclusivity between *PTEN* loss and *c-*ras^{Ha} activation (Mao et al., 2004). This was partly resolved on finding that ras^{Ha} synergism with *PTEN* loss (Δ5PTEN, Li et al., 2002) gave benign papillomas, but required TPA for malignant conversion, which involved a separate *Δ5PTEN*-mediated mechanism of cell cycle deregulation that superseded initial Δ5PTEN/ras^{Ha} synergism (Yao et al., 2006).

Given that the oncogene *fos* is a major effecter of TPA promotion (Schlingemann et al., 2003) and co-operates with ras^{Ha} during papillomatogenesis and malignant conversion (Greenhalgh et al., 1990, 1993a, 1995; Seaz et al., 1995), this study investigated whether activated fos would cooperate with PTEN loss in papillomatogenesis and drive this ras^{Ha}-independent, Δ5PTEN-mediated mechanism of malignant progression. Indirect links between FOS and PTEN deregulation already exist, as Δ5PTEN could substitute for activated ras^{Ha} during TPA promotion (Yao et al., 2006) and c-fos-mediated photo-carcinogenesis associates with both AKT activation and GSK3β inactivation (Gonzales and Bowden, 2002). Further, UV-B-mediated p53 mutation and subsequent PTEN loss induces AP-1 expression (Wang et al., 2005) whilst in reverse, PTEN specifically targets c-fos expression via AKT signalling, to down regulate AP-1 activity (Koul et al., 2007).

Direct co-operation between activated *fos* [*HK1.fos*] and inducible *PTEN* loss in adult skin [*Δ5PTEN*] resulted in an unanticipated keratoacanthoma [KA] aetiology, rather than malignant progression to squamous cell carcinoma [SCC]. Analysis of the underlying mechanism demonstrated that compensatory p53 and $p21^{WAF}$ expression prevented progression via switching highly mitotic, papilloma keratinocytes into a programme of accelerated differentiation, manifest by unique, novel basal layer expression of early differentiation-specific keratin K1. This $p53/p21^{WAF}$ expression profile was apparently induced by progressively increasing levels of GSK3β inactivation. In addition pivotal roles for AKT were identified, where $p53/p21^{WAF}$-mediated reduction of AKT activity in basal layer keratinocytes of benign tumours appeared to be a key facet underlying the switch in progression to KA, not SSC.

RESULTS

PTEN loss co-operates with HK1.fos expression to elicit keratoacanthomas with atypical keratinocyte differentiation.

Investigation of PTEN loss and fos activation in skin carcinogenesis was achieved by employing topical RU486 application to activate *cre* recombinase (*K14.creP*, Berton et al., 2000) and ablate *loxP*-flanked PTEN exon 5 *(Δ5PTEN*; Li et al., 2002**)** in proliferative basal layer keratinocytes and hair follicles, due to the expression specificity of the K14 promoter (Yao et al., 2006). These mice were bred to HK1.fos mice that exclusively express epidermal v-fos in approx 25-30% transit amplifying cells and all suprabasal keratinocytes by virtue of a truncated, human keratin 1 based vector (*HK1.fos*, Greenhalgh et al., 1993b) but not hair follicles nor internal epithelia. All RU486-treated *HK1.fos/Δ5PTEN*^{flx} mice [Fig. 1], exhibited bilateral ear tumours by 6-7 weeks [n = 55; produced over a 2.5yr period], which rapidly progressed to form keratoacanthomas [KAs]. KA aetiology in treated *HK1.fos/Δ5PTEN*$^{flx/wt}$ heterozygotes [n = 35] was slower and required an ear-tag wound promotion stimulus. Treated *HK1.fos [PTEN*^{wt}*]* controls developed wound-dependent ear hyperplasia by 3-4 months and papillomas after long latency [> 12 mo (Greenhalgh et al., 1993b)], whilst RU486-treated *K14.cre/Δ5PTEN*^{flx} siblings exhibited epidermal hyperkeratosis without spontaneous papillomas (Yao et al., 2006). Transgene expression/ablation analysis [Supp. Data: Fig. 1S] confirms permanent ablation of PTEN exon 5 following RU486 treatment, and demonstrated *HK1.fos* expression in normal appearing skin, which appeared elevated in KAs, consistent with their increased differentiation and anomalous murine K1 expression in proliferative basal layers [below].

The KA outcome of fos cooperation with PTEN contrasts to induction of malignant conversion in co-operation with ras^{Ha} [Greenhalgh et al., 1990; 1995; Saez et al., 1995] or TPA-mediated, i.e. fos-associated (Schlingemann et al., 2003), conversion of *HK1.ras/Δ5PTEN* papillomas (Yao et al., 2006). This difference may centre on inherent abilities of an epidermis to cope with specific genetic insults as reflected by the histotypes produced. The histotype of *HK1.fos* skin [Fig. 1A] was indistinguishable

from normal despite HK1.fos expression [supp data, Fig. 1: lane 5] that gave an underlying doubling in mitotic index (below Fig. 3; Greenhalgh et al., 1993b). Hence, prior to wound promotion, as v-fos potentiates the functions of c-fos, *HK1.fos*-induced proliferation was counterbalanced via c-fos functions in regulation of keratinocyte turnover and/or differentiation (Angel et al., 2001; Mehic et al., 2005), which also involved down regulation of AKT activity [below]. Similarly, as observed in cancer prone Cowden disease patients (Stambolic et al., 2000; Fistarol et al., 2002**),** treated *K14.cre/Δ5PTEN*^{flx} epidermal histotypes exhibited a relatively mild hyperplasia dominated by hyperkeratosis [Fig. 1B], with blooms of "ghost" cells indicative of incomplete stratification. This suggests that a proliferative response due to PTEN loss was rapidly translated into hyperkeratosis to eliminate potentially neoplastic cells at an early stage and this also involved AKT activation in regulation of differentiation [below] (Calautti et al., 2005). Furthermore, Δ5PTEN expression would compromise PTEN-mediated functions in cell-cell adhesion and cell-matrix interactions (Masahito et al., 1998; Subauste et al., 2005) that threaten epidermal barrier function, hence this hyperkeratotic response may also conscript epidermal homeostasis mechanisms in order to maintain epidermal integrity.

At approx 5-6 mo, when *HK1.fos* mice displayed only wound-dependent hyperplasia/early papillomatogenesis [Fig 1C], *HK1.fos/Δ5PTEN*^{flx} mice possessed mature KAs [Fig. 1D]. These tumours comprised two distinct histotypes: one of significant differentiation, with "fronds" of keratinocytes interspaced within massive areas of keratosis; and a second papillomatous area comprising highly proliferative keratinocytes, similar to late stage, aggressive papillomas or possibly carcinoma *in situ*. Furthermore, while keratinocyte differentiation in *HK1.fos* phenotypes displayed an ordered nature with sequential expansion of each cellular compartment [Fig. 1C], keratotic, but not papillomatous, *HK1.fos/Δ5PTEN*^{flx} KA histotypes, displayed a distinctly disordered differentiation pattern [Fig. 1E-G]. Here cornified and granular cells co-existed alongside proliferative basal cells, culminating in the appearance of micro-cysts [Fig. 1E, G: arrows] and a prominent *stratum luciduim* [Fig. 1F: arrows] indicative of incorrect cornification. This confusion of differentiated and proliferative cell sub-types in

each epidermal compartment suggests that *HK1.fos-Δ5PTEN^{flx}* keratinocytes received abruptly conflicting proliferation and differentiation signals in this keratotic/differentiated histotype.

Premature differentiation marker expression in keratoacanthomas associates with reduced progression marker expression and decreased proliferation.

Tumours were analysed for expression of keratin K1, an early-stage differentiation marker, and late-stage differentiation markers filaggrin and loricrin, all proteins which typically become lost during carcinogenesis, and also for keratin K13, a simple epithelia keratin, employed as a marker of papilloma progression, which typically becomes uniform prior to malignant conversion (Greenhalgh et al., 1995). As observed previously, *HK1.fos* papillomas exhibit a delay in the onset of K1 expression due to expansion of the proliferative basal layer compartment [Fig. 2A, indicated by the K14 keratin counterstain]. This result was also observed in papillomatous *HK1.fos/Δ5PTEN^{flx}* KA histotypes [not shown], however highly differentiated *HK1.fos/Δ5PTEN^{flx}* KA histotypes exhibited novel, K1 expression in the proliferative basal layers. K1 expression was quite strong, given the lack of yellow colour from [red] K14 co-expression, although K14 expression itself remained unchanged [see Fig. 3]. Typically, AP1-regulated keratin K1 is expressed as differentiating keratinocytes commit to leave the basal layer (Rothnagel et al., 1993) and this result suggests that *HK1.fos/Δ5PTEN^{flx}* keratinocytes accelerated their commitment to differentiation in these keratotic areas.

The premature expression profiles of loricrin and filaggrin [Fig. 2B, C] also indicated accelerated differentiation. In *HK1.fos* papillomas, AP-1-regulated loricrin expression, a major component of granular cells, remained restricted to the granular compartment, where c-fos is also highly expressed (Greenhalgh et al., 1993b; Mehic et al., 2005). Conversely, *HK1.fos/Δ5PTEN^{flx}* KAs exhibited premature elevated, suprabasal loricrin expression in areas of atypical differentiation, particularly highlighting the micro-cysts [Fig. 2B]. Similarly, filaggrin expression, another AP-1-regulated

component of cornification, with critical functions in barrier maintenance (Palmer et al., 2006), was reduced in *HK1.fos* papillomas, whereas *HK1.fos/Δ5PTEN* KAs expressed early, high filaggrin levels in suprabasal and occasional basal keratinocytes [Fig. 2C]. With respect to keratin K13, *HK1.fos* papillomas [Fig. 2D] exhibited the focal/patchy K13 expression profile typical of benign tumours (Greenhalgh et al., 1993b). However, while early *HK1.fos/Δ5PTEN*^{flx} tumours and the proliferative, papillomatous histotypes of KAs exhibited focal K13 expression, the differentiated regions lost K13 expression [Fig. 2D]. Thus, a hyperproliferative, K13-positive papillomatous keratinocyte population differentiated into a quiescent, K13-negative population and suggests that the temporal event(s) that switched progression to KA occurred at the overt, benign tumour stage and not in pre-neoplastic hyperplasia.

BrdU labelling data also support this idea. As shown in Fig. 3, acquisition of each additional mutation resulted in sequential increases in mitotic index [labelled nuclei/mm basement membrane], culminating in very high levels in HK1.fos/*Δ5PTEN*^{flx} papillomatous histotypes, until suddenly halted in the differentiated regions. Normal appearing *HK1.fos* epidermis possessed a mitotic index [10.1^{+}/- 2.1] approximately double that of non-transgenic adult epidermis [4.7^{+}/- 3.0], which in *K14.cre/Δ5PTEN*^{flx} genotypes [13.7^{+}/- 3.6] gave mild hyperplasia. Additional doubling of mitotic index occurred in *HK1.fos/Δ5PTEN*^{flx} skin [26.1 $^{+}$/- 5.7] to levels observed in *HK1.fos* papillomas [27.2 $^{+}$/- 7.1, Fig. 3B], whilst KA keratinocytes possessed a very high mitotic index [90.2 $^{+}$/- 17.6;] comparable to aggressive SCCs, with extensive supra-basal BrdU-labelling [Fig. 3B], whereas keratinocytes of differentiated histotypes possessed a significantly reduced mitotic index [36.6 $^{+}$/- 6.7; p = < 0.0001], although this remained higher than that of typical HK1.fos papillomas [p = 0.001 Students t test]. Thus, BrdU labelling indicated a potent counter to hyperproliferation arose after benign tumour formation, which inhibited further progression.

***HK1.fos/Δ5PTEN* KAs express high levels of normal p53 whereas control *HK1.fos* and *K14.cre/Δ5PTEN* phenotypes loose p53 expression.**

Given the close relationship between PTEN and p53 regulation (Freeman et al., 2003; Lei et al., 2006; Wang et al., 2005), p53 status during *HK1.fos/Δ5PTEN*^{flx} KA aetiology was determined by western analysis of normal epidermis, pre-neoplastic phenotypes and tumours taken from separate animals [Fig. 4]; or from the same animals [Fig. 5], to compare KAs with similar keratosis/papilloma ratios, and/or age-matched littermate control phenotypes. Hyperkeratotic *K14.cre/Δ5PTEN*^{flx} epidermis exhibited little detectable p53 expression [Figs 4, 5: HK lanes] compared to normal epidermis [Fig. 4: aN, N, NE lanes]. Similarly, hyperplastic *HK1.fos* epidermis and papillomas also lost p53 expression and p53 levels were undetectable in "normal" appearing *HK1.fos* skin [Figs 4, 5: N, PAP, HP lanes]. This latter result was consistent with the doubled mitotic index [above] but inconsistent with the normal histotype. On rare occasions low-level p53 expression was recorded in *HK1.fos* phenotypes due to inflammation or presence of anagen follicles [Fig. 4,: aN lane] where HK1.fos was not expressed.

Conversely, in both homozygous and heterozygous *Δ5PTEN* animals, significantly high levels of p53 expression were recorded in *HK1.fos/Δ5PTEN* KAs [Figs. 4, 5: KA lanes]. Expression levels varied amongst randomly selected KAs [Fig. 4] but were usually high, and p53 expression increased with KA maturity/size [Fig. 5], e.g. heterozygous *HK1.fos/Δ5PTEN*$^{flx/wt}$ KAs developed less rapidly and typically possessed lower increases in p53 expression when analysed alongside the faster growing, mature KAs of homozygotes [Fig. 4: #8898 vs. #9593 KAs]. A result also recorded on comparison of larger, wound-promoted ear-tagged vs. untagged ear KAs from the same animals [Fig. 5, lanes: KA^{T} vs. KA]. In addition, pre-neoplastic *HK1.fos/Δ5PTEN*^{flx} or -$^{wt/flx}$ epidermis expressed low-level p53 [Fig. 4, lanes: HK #8898, #9593; Fig. 5, HK lanes: 5,6,9] suggesting p53 expression was an early feedback response to *HK1.fos* synergism with *PTEN* loss. This high p53 expression in benign tumours

was consistent with the reduced BrdU labelling in keratotic, differentiated KA histotypes compared to high labelling indices of papillomatous regions, and together with decreased K13 tumour marker expression, elevated p53 would inhibit further tumour progression. This idea was further supported by sequence analysis of *p53* cDNAs from HK1.fos/*Δ5PTEN*^{flx} KAs [n = 5], which found full-length transcripts without detectable mutation or alternate splicing [not shown], hence normal p53 tumour suppressor functions appeared intact (Nister et al., 2005). *HK1.fos/Δ5PTEN* KAs also lacked spontaneous c-*ras*^{Ha} activation (Corominas et al., 1989; Greenhalgh et al., 1990, 1995; Lieu et al., 1991) [n = 5; not shown]. Thus, high expression of normal p53 in KA aetiology may be rendered impotent by *ras*^{Ha} activation leading to SCC, an idea currently under investigation in triple *HK1.ras/fos/Δ5PTEN*^{flx} mice.

Regulation of AKT activation is a pivotal target of tumour progression and epidermal homeostasis.

Consistent with loss of PTEN phosphatase function following ablation of exon 5 (Parsons, 2004), levels of activated AKT^{ser473} phosphorylation [p-AKT] rose in RU486-treated *K14.cre/Δ5PTEN*^{flx} epidermis [Fig. 4: HK lanes]. *HK1.fos/Δ5PTEN*^{flx} KAs also exhibited increased p-AKT expression [Figs 4,5], however levels were not as high as expected and, compared to total AKT expression levels, p-AKT expression varied significantly with the degree of keratosis vs. hyperproliferation [Fig. 4, KA*] or KA size/maturity [Fig. 4, lanes: $^{\#}$8898 and $^{\#}$9593]. Analysis of histology-matched KAs [Fig. 5] found only moderate increases in p-AKT expression compared to hyperplastic epidermis taken from the same animal. Moreover, p-AKT levels in pre-neoplastic *HK1.fos/Δ5PTEN*^{flx} epidermis were consistently lower than age-matched *K14.cre/Δ5PTEN*^{flx} littermate epidermis [Fig. 5, *K14.cre/Δ5PTEN*^{flx} HK lanes: 1, 2; vs. *HK1.fos/Δ5PTEN*^{flx} HK lanes: 5,6, 9]. This suggests that p-AKT inhibition was a target of the early, low–level p53 feedback response, consistent with *PTEN*^{null} prostate carcinogenesis, where NKX3.1 inhibits p-AKT to stabilise p53 expression (Lei et al., 2006).

The fact that the p-AKT expression increase in *HK1.fos/Δ5PTEN^{flx}* KAs was lower than that of comparable *ras^{Ha}/Δ5PTEN* synergism (Yao et al., 2006) was also consistent with inhibition of AKT by high p53 levels. However, this moderate p-AKT expression profile masked a significant expression level in *HK1.fos/Δ5PTEN^{flx}* papillomatous areas as detected by immunohistochemical analysis [below, Fig. 6; Supp data: Fig. 2s], suggesting that AKT played significant roles in papillomatogenesis and continuation of this activity was essential for further malignant progression (Segrelles et al., 2006; Yao et al., 2006).

An earlier role for AKT regulation was identified in *HK1.fos* "normal" appearing or hyperplastic epidermis, which exhibited little p-AKT expression compared to total AKT expression levels, and levels remained relatively low until overt papillomas appeared [Fig. 4 *HK1.fos* lanes: N, HP, P; Fig. 5, lanes N, P]. Thus, p-AKT down regulation maybe an element of epidermal resistance to early carcinogenesis. This observation may explain the delay in papilloma appearance and the longstanding puzzle that a p53-negative, *HK1.fos* epidermis exhibited a normal histotype, despite a mitotic index that gave hyperplasia/hyperkeratosis in *K14.cre/Δ5PTEN^{flx}* skin [Figs 1,3]. Given the direct links between fos and PTEN (this study; Koul et al., 2007; Wang et al., 2006), coupled to the intimate interactions between p53 and PTEN (Freeman et al., 2003), HK1.fos-mediated p53 loss maybe countered in part by a PTEN-mediated feedback involving p-AKT down regulation, which facilitates keratinocyte turnover and differentiation (Angel et al., 2001; Calautti et al., 2005) and this is under investigation. Hence *HK1.fos* phenotypes required a wound-promotion stimulus, eliciting high p-ERK 1/2 and increased cyclin D1/E2 expression [below], to antagonise/interdict such putative countermeasures and restore p-AKT expression in *HK1.fos* papillomas [Figs 4,5 lanes: PAP]. Adding further complexity to AKT oncogenicity, in p53-negative *K14.cre/Δ5PTEN^{flx}* epidermis, where AKT would be released from PTEN control, elevated p-AKT expression [Fig. 4, HK lanes; Fig. 5: HK lanes 1,2] was accompanied by a rapid translation of hyperplasia into hyperkeratosis [Fig. 1B], as observed in Cowdens Disease, but no papillomas [unless promoted by TPA (Yao et al., 2006)], demonstrating

that AKT regulation in keratinocyte differentiation can dictate differing outcomes depending on the context(s) of gene expression.

HK1.fos/Δ5PTEN keratoacanthoma aetiology identifies significant roles for GSK3β inactivation.

Key insights into *HK1.fos/Δ5PTEN* KA development derived from analysis of GSK3β expression (Karim et al., 2004), a gene functionally inactivated by several oncogenes including AKT, via phosphorylation at serine 9 [p-GSK3β] (Parsons, 2004). In being an AKT target, elevated levels of p-GSK3β were displayed by hyperkeratotic *K14.cre/Δ5PTEN*^{flx} epidermis [Fig. 4, HK lanes], and normal or early hyperplastic *HK1.fos* epidermis exhibited reduced p-GSK3β expression following p-AKT down regulation [Fig. 4, lanes: N, HP]. However, *HK1.fos* papillomas expressed moderate p-GSK3β levels higher than that attributable to p-AKT expression [Figs. 4, 5: PAP lanes], and increasing *HK1.fos* hyperplasia displayed low-level p-GSK3β when p-AKT remained undetectable [Supp data: Fig. 3S]. As *HK1.fos* papillomatogenesis required wound-promotion, this fos-associated p-GSK3β inactivation uncoupled from AKT activity, may derive from high levels of ERK1/2 expression or increased cyclins [below, Figs 4, 5: PAP lanes].

Inactivation of GSK3β was found to be instrumental to the eventual KA outcome, as increased p-GSK3β expression correlated to elevated p53 expression [Figs 4, 5]. This association was initially unclear due to differing keratosis/papilloma ratios [Fig. 4, lanes: KA vs. KA*], however analysis of KAs with similar keratosis/papilloma ratios consistently expressed high levels of inactivated p-GSK3β, concomitant with high p53, but not p-AKT expression, which remained only similar to that of *K14.cre/Δ5PTEN*^{flx} epidermis [Fig. 5: KA vs. HK lanes]. Hyperplastic *HK1.fos/Δ5PTEN*^{flx} epidermis also possessed moderately elevated p-GSK3β levels, associated with low-level p53 expression, again uncoupled from that of p-AKT, which was down regulated [Fig. 5 lanes: HK 5,6,9]. The moderate p-GSK3β expression associated with low-level p53 expression in *HK1.fos/Δ5PTEN*^{flx} epidermis,

coupled to the major increases in p-GSK3β expression alongside the burst of p53 expression in KAs, suggests that inactivation of GSK3β function triggered p53 re-expression (Ghosh and Altieri, 2005). Furthermore, the apparent burst of p53 and abrupt reduction in keratinocyte proliferation that prevented further progression, required a high threshold level of GSK3β inactivation and this may have been achieved from the moderate, AKT independent p-GSK3β expression *HK1.fos/Δ5PTEN^{flx}* observed in early preneoplastic hyperplasia [above] coupled to that derived from increasing p-AKT activity in papillomatous areas [Fig. 5: KA lanes].

HK1.fos/Δ5PTEN^{flx} KAs exhibit novel p21^{WAF} expression deregulated cell cycle control and elevated MAP kinase signalling.

The mechanism underlying KA aetiology was extended to investigate cell cycle deregulation via western analysis of p21^{WAF}, cyclin D1 and E2 expression together with MAP kinase signalling via analysis of ERK 1/2 activation. Analysis of p21^{WAF} was doubly attractive, since p21^{WAF} possesses roles in keratinocyte differentiation (Topley et al., 1999) separate to that of cell cycle regulation (Devgan et al., 2006) and can be an early response to PTEN loss (Yoo et al., 2006). All *HK1.fos/Δ5PTEN^{flx}* KAs exhibiting p-GSK3β hyper-inactivation and high p53 expression, also exhibited novel, high p21^{WAF} expression levels [Fig. 5: KA lanes]. However, unlike induction of low-level p53 expression by moderate p-GSK3β expression in *HK1.fos/Δ5PTEN^{flx}* hyperplasia [Figs 4, 5], below the high GSK3β inactivation threshold, p21^{WAF} expression was not induced [Fig. 5, lanes: HK, P, vs. KA]. Further, p53 negative *HK1.fos* papillomas and *K14.cre/Δ5PTEN^{flx}* phenotypes, with lower GSK3β inactivation levels were negative for p21^{WAF} expression [Fig. 5]. Thus p21^{WAF} expression was specific to mature KAs, and the data suggest that p21^{WAF} expression arose following induction of p53, possibly as a consequence of p53-mediated down regulation of AKT activity [below (Zhou et al., 2001)]. Moreover, this temporal p21^{WAF} expression indicated that the critical changes in progression

occurred at the overt benign tumour stage, a result consistent with the K13/BrdU labelling data and previous roles for $p21^{WAF}$ associated with inhibition of malignant conversion (Topley et al., 1999).

Analysis of cyclins D1, E2 and MAP Kinase signalling in *HK1.fos/Δ5PTEN^{flx}* KAs [Figs. 4, 5] was also consistent with the idea that persistent keratinocyte hyperproliferation was continually switched into differentiation. Increasing hyperplasia in *HK1.fos/Δ5PTEN^{flx}* epidermis was reflected by elevated cyclin expression [Fig. 5: HK lanes 5,6,9] alongside increased p-ERK 1/2 expression [Fig. 4: HK lanes], which were retained at moderate levels in mature KAs, despite the p53/$p21^{WAF}$ expression profile, due to the hyperproliferation observed in papillomatous areas [Fig. 5: KA lanes]. Thus, in *HK1.fos/Δ5PTEN^{flx}* tumour aetiology, induction of both p53 and $p21^{WAF}$ were able to halt excessive proliferation, unlike activated *ras^{Ha}* cooperation with PTEN loss, where p53 remained lost and strong cyclin D1/E2 over expression was associated with AKT-mediated progression to carcinoma (Yao et al., 2006).

Analysis of control phenotypes [Figs 4, 5] found that normal *HK1.fos* epidermis exhibited a small elevation in cyclin D1 but not E2, and slightly elevated p-ERK 1/2 expression, compared to total ERK 1/2 levels [Figs. 4, 5: N lanes] (Karin, 1995), consistent with its doubled mitotic index. *HK1.fos* papillomas [Fig. 5: PAP lane] exhibited increased cyclin D1 and E2 expression (Bamberger et al., 2001), together with very high levels of activated p-ERKs 1/2 expression [Fig. 4 lanes: P, HP, N], which suggested that MAP Kinase signalling during wound-promoted *HK1.fos* papillomatogenesis facilitated escape from AKT-linked countermeasures [above] to restore p-AKT activity (Segrelles et al., 2006). In *K14.cre/Δ5PTEN^{flx}* epidermis, similar small elevations in both cyclins D1 and E2 (Di Cristofano et al., 2001; Weng et al., 2001) were recorded, associated with promotion from ear tagging [Fig. 5, lanes: HK^{T} vs. HK], alongside increased p-ERK 1 and 2 levels [Fig. 4, end panel: HK lanes]; all consistent with p-AKT regulation of PI3 Kinase and interactions with MAPK signalling (Parsons, 2004; Downward, 2004).

Immunohistochemical analysis identified p-GSK3β-associated p53/p21^{WAF} expression and down regulation of p-AKT activity in basal layer keratinocytes.

To further clarify these molecular interactions, the *in situ* expression profiles of p53, p21^{WAF}, p-GSK3β and p-AKT were determined via immunohistochemical analysis of differentiated, transitional and papillomatous *HK1.fos/ΔPTEN^{flx}* KA histotypes [Fig. 6. See also Supp. data: Fig. 2S]. Analysis of *HK1.fos* and *K14.cre/ΔPTEN^{flx}* control phenotypes are given in Supp. data: Fig. 3S. In all differentiated KA histotypes, p53 was strongly expressed throughout each epidermal compartment, including proliferative basal layer keratinocytes [Fig. 6A]. In transitional areas, initially p53 expression was low and predominantly suprabasal, but expression became increasingly stronger and appeared in the basal layer [Fig. 6B; Supp data: Fig. 2S]. Conversely, papillomatous areas possessed little detectable p53 protein [Fig. 6C; Supp data: Fig. 2S]. However, low-level, suprabasal/granular p53 expression was observed in hyperplastic *HK1.fos/ΔPTEN^{flx}* epidermis and occasional papillomatous areas, both associated with elevated, suprabasal expression of p-GSK3β [not shown]. Differentiated KA histotypes exhibited strong p21^{WAF} expression in all layers [Fig. 6D; Supp. data: Fig. 2S], and again this began in transitional histotypes with a low-level suprabasal and cytoplasmic p21^{WAF} expression profile, until elevated expression appeared in the nuclei of basal cells associated with increased differentiation [Fig. 6E], prior to becoming strong and uniform in all compartments. This expression profile appeared to trail the wave of high p53 expression [Supp data: Fig. 2S], as all papillomatous KA histotypes always lacked detectable p21^{WAF} expression, even if p53 was detectable [Fig. 6F; Supp. data: Fig. 2S], and p21^{WAF} was undetectable in hyperplastic *HK1.fos/ΔPTEN^{flx}* epidermis [not shown] or *HK1.fos* and *K14.cre/ΔPTEN^{flx}* control phenotypes [Supp. data: Fig. 3S]. Given the roles for p21^{WAF} in epidermal differentiation (Topley et al., 1999), this basal layer expression of p21^{WAF} would be consistent with the premature commitment of *HK1.fos/ΔPTEN^{flx}* keratinocytes to terminal differentiation, as indicated by novel, basal layer K1 expression [above], whilst the confused, atypical nature of epidermal differentiation maybe due to continued, p21^{WAF}

expression in the suprabasal/granular layers when normally p21 expression shuts down (Devgan et al., 2006).

Expression of p-GSK3β in differentiated KA regions paralleled this p53/p21^{WAF} profile, with strong, expression in the basal layers and each epidermal compartment [Fig. 6G]. In transitional histotypes, uniform p-GSK3β expression preceded basal p53/p21^{WAF} expression [Fig. 6H; Supp data: Fig. 2S], as p-GSK3β already appeared earlier in the suprabasal layers of papillomatous KA histotypes [Fig. 6I; Supp. data: Fig. 2S] and hyperplastic *HK1.fos/ΔPTEN^{flx}* epidermis [not shown]. In HK1.fos/*ΔPTEN^{flx}* epidermis, moderate suprabasal p-GSK3β expression was associated with supra-basal p53 expression, prior to p53 loss in papillomatogenesis, and this could be observed in occasional papillomatous areas also, suggesting the beginnings of a counter to hyperproliferation, but this was insufficient to induce p21^{WAF}. Thus, increasingly high and basal layer expression of p-GSK3β in the transitional areas induced a corresponding increase in basal layer expression of first p53, to halt proliferation, and later p21^{WAF} to increase differentiation rate.

Analysis of p-AKT in *HK1.fos/ΔPTEN^{flx} KAs* [Fig. 6J-L] demonstrated a reverse of these expression profiles, as differentiated or transitional p53/p21^{WAF}-positive areas expressed decreasing levels of p-AKT [Fig. 6J, K]. Conversely, p53/p21^{WAF}-negative papillomatous histotypes exhibited high p-AKT expression levels [Fig. 6L], a result masked in western analysis, as p-AKT expression faded with increasing differentiation and KA maturity [Fig. 6J; Supp data: Fig. 2S]. Moreover, p-AKT expression consistently appeared in the basal layers of papillomatous areas [Fig. 6L], suggesting that AKT activity helped provide a continuous supply of hyperproliferative keratinocytes, hence the lack of KA regression; while in increasingly p53/p21^{WAF} positive transitional areas, p-AKT expression became suprabasal [Fig. 6K], following the appearance of high basal layer p53 expression [Fig. 6B], that culminated in reduced, suprabasal p-AKT expression in differentiated histotypes [Fig. 6J]. Analysis of consecutive sections found that co–expression of p21^{WAF} and p-AKT appeared particularly

antagonistic, with high p-AKT expression being almost mutually exclusive to that of $p21^{WAF}$ [Fig. 6E,K]; and in composite micrographs, uniform increasing $p21^{WAF}$ expression paralleled down regulation of p-AKT [Supp data: Fig. 2S]. Collectively, it may be that p53-mediated reduced p-AKT expression in basal keratinocytes was instrumental to releasing $p21^{WAF}$ activity (Zhou et al., 2001) and the commitment to premature differentiation (Topley et al., 1999).

Analysis of *HK1.fos* phenotypes reflected the western data, with little p-AKT, p53 or $p21^{WAF}$ expression in hyperplastic epidermis or papillomas, whilst *HK1.fos* papillomas/late-stage hyperplasia exhibited the low-level, suprabasal p-GSK3β expression profile observed in HK1.fos/$\Delta PTEN^{flx}$ epidermis [Supp. data: Fig. 3S]. RU486-treated *K14.cre/*$\Delta PTEN^{flx}$ epidermis also lacked p53 and $p21^{WAF}$, but consistent with the K14.creP expression profile and loss of phosphatase activity, displayed p-AKT expression together with p-GSK3β expression in basal layers and follicles [Supp. data: Fig. 3S].

DISCUSSION

HK1.fos/Δ5PTEN^{flx} mice demonstrated direct co-operation between inducible PTEN loss and activated FOS expression, which resulted in preneoplastic hyperplasia/hyperkeratosis and a rapid development of overt benign tumours that progressed to KA not SCC. Importantly, this study found that in the context of *HK1.fos/Δ5PTEN^{flx}* benign tumours, significant re-expression of p53 and p21^{WAF}, previously lost in control phenotypes, now inhibited further malignant progression. This compensatory p53/p21^{WAF} expression profile was triggered by increasing levels of GSK3β inactivation (Ghosh and Altieri, 2005), which inhibited p-AKT activity in basal layer keratinocytes (Lei et al., 2006) to reduce proliferation, as indicated by BrdU labelling, and initiate p21^{WAF}-mediated differentiation (Devgan et al., 2006; Topley et al., 1999; Zhou et al., 2001), associated with novel basal layer expression of keratin K1 and premature loricrin and filaggrin expression. This potential sentinel mechanism, deployed at the benign tumour stage and critically dependent on normal p53 and p21^{WAF} functions, was able to continually block malignant progression via switching keratinocyte hyperproliferation into differentiation, resulting in the hallmark keratosis of KA.

This outcome of KA rather than SCC, was in sharp contrast to the high frequency of TPA-promoted [i.e. fos-associated (Schlingemann et al., 2003)] carcinomas observed in *HK1.ras/Δ5PTEN^{flx}* mice (Yao et al., 2006) or *ras^{Ha}*-activated, DMBA/TPA carcinogenesis studies involving PTEN knockouts (Mao et al., 2004; Suzuki et al., 2003). Nonetheless, early *HK1.fos/Δ5PTEN^{flx}* synergism was consistent with promotion roles assigned to *fos* (Greenhalgh et al., 1993a, 1993b; Saez et al., 1995) and the fact that PTEN loss could act as a weak initiator for TPA promotion (Yao et al., 2006). Indeed, whilst rapid, papillomatogenesis presented few surprises, as *HK1.fos-Δ5PTEN^{flx}* apparently substituted for *ras^{Ha}* activation observed in previous rabbit ear models of KA (Corominas et al., 1989), exhibiting moderate elevation in MAP Kinase signalling (Parsons, 2004; Downward, 2004; Karin, 1995) and over expression of cyclin D1 (Bamberger et al., 2001; Burnworth et al., 2006) or cyclin E2 (Di Cristofano et al., 2001; Weng et al., 2001). Incremental increases in keratinocyte proliferation

culminated in very high BrdU labelling indices in papillomatous KA histotypes, with typical delays in expression of differentiation markers and the appearance of focal keratin K13 expression, an early marker of tumour progression. (Greenhalgh et al., 1995). However, the initial appearance of K13 and high BrdU labelling abruptly diminished in transitional and differentiated KA histotypes, indicating a potent inhibition of proliferation appeared at the benign tumour stage which accelerated terminal differentiation rather than apoptosis, given the premature expression of keratin K1, loricrin and filaggrin. The resulting disorder to keratinocyte differentiation, also observed in cyclin D1–transformed HaCaT keratoacanthomas (Burnworth et al., 2006), highlighted a clash between proliferative/oncogenic and compensatory/differentiation pathways. Here novel, basal layer expression of keratin K1 was perhaps a major contributor to the KA outcome, as it not only indicated a sudden, accelerated commitment to differentiation (Rothnagel et al., 1993) but also, basal layer K1 expression would itself significantly inhibit further tumour progression, as introduction of K1, or its partner K10, into carcinoma cells reverses the malignant phenotype via enforced differentiation (Kartasova et al., 1992; Santos et al., 2002).

Human KA aetiology is also typified by an initial rapid growth phase, followed by arrest and regression. In several respects murine *HK1.fos/Δ5PTEN*^{flx} KA aetiology mimics that of humans, producing a tumour with a highly proliferative papillomatous/carcinoma in *situ* histotype, underlying areas of massive keratosis. However, whether *fos/PTEN*^{null} synergism drives human KA aetiology remains to be confirmed, although fos roles in hyperproliferative disease and keratinocyte differentiation/turnover (Angel et al., 2001; Mehic et al., 2005) and the hyperkeratosis following PTEN loss (Fistarol et al., 2002; Stambolic et al., 2000; Yao et al., 2006) would be consistent with the increased differentiation in KAs. In addition, most human KAs are devoid of p53 mutations and exhibit increased p21^{WAF} expression (Ahmed et al., 1997; Perez et al., 1997; Ren et al., 1996). These data add fuel to the debate on whether KA represents a differentiated extreme of SCC or a class of benign tumour in their own right, with a separate molecular aetiology. Given the contrasting results for activated fos or ras^{Ha} synergism with PTEN in KA vs. previous SCC aetiology (Yao et al 2006), and the

relative lack of typical initiating *ras*^{Ha} or *p53* mutations (Ahmed et al., 1997; Lieu et al., 1991; Perez et al., 1997; Ren et al., 1996), these murine data suggest a separate molecular aetiology. However, again this idea awaits analysis of whether additional/appropriate mutations of *ras*^{Ha} or *p53* interdict a murine KA aetiology mediated by fos, PTEN and the p53/p21^{WAF} switch.

Initially, p53 status had been assessed in *HK1.ras/Δ5PTEN*^{flx} KA aetiology given its well-characterised roles in skin tumourigenesis (Brash, 2006) and close links with PTEN function where PTEN loss invokes p53 loss (Freeman et al., 2003; Wang et al., 2005; Chen et al., 2005), unless compensatory mechanisms stabilised p53 (Lei et al., 2006). Hence control *K14.cre/Δ5PTEN*^{flx} lost p53 expression, however resultant hyperplasia was rapidly translated into hyperkeratosis [below]. Similarly, hyperplastic *HK1.fos* epidermis or papillomas were negative for p53 expression, but again surveillance systems sensitive to fos-mediated p53 loss were invoked, which maintained a degree of normality until promoted (Greenhalgh et al., 1993b), consistent with earlier *HK1.fos* cooperation studies with *p53* knockout mice, where *HK1.fos/p53*^{null} epidermis paradoxically failed to exhibit benign tumours (Greenhalgh et al., 1996). These observations reflect in human photo-carcinogenesis, as p53 mutations frequently initiate keratinocytes (Brash, 2006), but tumour aetiology requires additional events over time, including UV-induced c-fos-mediated tumour promotion (Gonzales and Bowden, 2002; Wang et al., 2005).

Against this background, high levels of p53 expression in basal layers of differentiated, KA histotypes was unexpected and identified p53 re-expression to be a key facet underlying a *HK1.fos/Δ5PTEN*^{flx} KA aetiology. Earlier *HK1.fos/Δ5PTEN*^{flx} hyperplasia had exhibited a low-level p53 expression response, associated with moderate GSK3β inactivation, which was subsequently lost giving rise to the hyperproliferative, p53-negative papillomatous KA histotype with elevated MAP Kinase/cyclin D1/E2 activities. Hence when re-expressed in the proliferative basal layers of transitional areas, increasing p53 expression abruptly reduced BrdU labelling and K13 expression, and demonstrated

that inhibition of tumour progression depended upon both intensity and locality of gene expression (Wahl, 2006). In human KAs, p53 expression also becomes increased (Perez et al., 1997) and is seldom mutated (Ren et al., 1996), consistent with the lack of p53 or alternate splicing observed in *HK1.fos/Δ5PTEN*^{flx} KAs, which suggested that normal p53 functions were intact (Nister et al., 2005). As with compensatory p53 expression in *PTEN*-mediated prostate carcinogenesis (Chen et al., 2005; Lei et al., 2006), these data predict that a KA aetiology requires fully functional p53 pathways. Hence loss of p53 in *ras*^{Ha}*/PTEN* cooperation or chemical carcinogenesis results in SCC (Suzuki et al., 2003; Mao et al., 2004; Yao et al., 2006). Indeed, the relative rarity of human KA compared to SCC may reflect the high frequency of UV-B-induced p53 mutations (Brash, 2006) that would interdict this putative compensatory mechanism.

High p21^{WAF} expression levels were observed in mature *HK1.fos/Δ5PTEN*^{flx} KAs but post the appearance of overt benign tumours, as pre-neoplastic or papillomatous histotypes displayed little detectable p21^{WAF} and these data suggest that p21^{WAF} inhibited malignant conversion (Topley et al., 1999). Consistent with this idea, western analysis of p21^{WAF} expression in KAs trailed that of p53 and this maybe a consequence of activated AKT expression in papillomatous histotypes [below], as p-AKT inhibits expression, nuclear localisation and function of p21^{WAF} (Zhou et al., 2001). Logically therefore, induction of high p53 re-expression would reduce p-AKT expression (Miyauchi et al., 2004) and facilitate p21^{WAF} escape from p-AKT inhibition. The resultant basal layer expression of p21^{WAF} would reduce proliferation, however p21^{WAF} roles in differentiation, separate to that of cell cycle control (Devgan et al., 2006), maybe of greater significance. In normal epidermal differentiation, p21^{WAF} expression increases when post-mitotic keratinocytes commit to differentiate (Topley et al., 1999; Devgan et al., 2006), echoing the normal keratin K1 expression profile (Rothnagel et al., 1993) and suggesting that p21^{WAF} functions in early decisions to commit to terminal differentiation. Therefore, high basal layer p21^{WAF} expression would accelerate this commitment to differentiate, indicated by basal layer K1 expression, and establish a mechanism that continually inhibited progression via terminal differentiation (Kartasova et al., 1992; Topley et al., 1999; Santos et

al., 2002). In addition, since *p21*^{WAF} has both positive and negative roles in keratinocyte differentiation, and actually inhibits the latter stages, when p21^{WAF} is normally down regulated (Devgan et al., 2006), intense p21^{WAF} expression in each epidermal compartment may explain the general disorder to keratinocyte differentiation in *HK1.fos/Δ5PTEN*^{flx} KA histotypes, manifest by premature loricrin/filaggrin expression and the appearance of micro-cysts, a problem further compounded by a increasing lack of p-AKT (Calautti et al., 2005), which would add to the failure to down regulate p21^{WAF} function (Devgan et al., 2006; Zhou et al., 2001).

Human KAs also exhibit elevated p21^{WAF} in two distinct patterns; one associated with reduced proliferation, one with increased differentiation (Ahmed et al., 1997). In a tissue continually exposed to environmental carcinogens, the ability to exert resistance to tumour progression at each stage is logical and may involve common components. In classic *ras/myc* co-operation, p21^{WAF} induction inhibited ras^{Ha}-activated skin carcinogenesis in *c-myc* null cells, until p21^{WAF} was itself compromised by re-introduction of oncogenic *myc* (Oskarsson et al., 2006). Similar compensatory effects of p21^{WAF} were observed in *Δ5PTEN*-mediated bladder carcinogenesis, where initial hyperplasia was countered by p21^{WAF} expression (Yoo et al., 2006). However, p21^{WAF} expression was not induced in *Δ5PTEN*-mediated prostate carcinogenesis (Mulholland et al., 2006), which relied on p53 interactions (Chen et al., 2005), whilst the reduced numbers of DMBA/TPA skin tumours in AKT knockout mice was independent of p53 (Skeen et al., 2006), highlighting multi-layered redundancies in these systems (Wahl, 2006). Perhaps in epithelia concerned with barrier functions, where terminal differentiation to eliminate pre-malignant cells is preferable to widespread apoptosis/senescence, induction of p21^{WAF} mediated differentiation (Topley et al., 1999; Devgan et al., 2006; Yoo et al., 2006) provides a necessary adjunct to p53-mediated apoptosis.

Regulation of AKT activity was also critical to early phenotypes and the KA tumour outcome. It is well accepted that loss of PTEN phosphatase results in elevated AKT activity (Parsons, 2004; Downward, 2004) and reduced p53 stability (Freeman et al., 2003; Lei et al., 2006), and AKT oncogenicity drives tumour progression in numerous mechanisms (Chen et al 2005; Lei et al 2006;

Skeen et al., 2006; Segrelles et al., 2006; Yao et al., 2006). Thus to counterbalance this neoplastic potential, early *HK1.fos/Δ5PTEN*^{flx} and control phenotypes either down-regulated AKT activity (Skeen et al., 2006) or exploited an emerging, anti-apoptotic role in epidermal differentiation that was associated with conversion of hyperplasia into hyperkeratosis (Calautti et al., 2005). In p53-negative *HK1.fos* epidermis, p-AKT down regulation helped maintain the differentiation/proliferation balance resulting in an overtly normal epidermis. Given recent glioma studies where PTEN^{wt} inhibits AP1 activity via reduced AKT signalling (Koul et al., 2007) and close links between PTEN/p53 loss and AP1 status (Wang et al., 2005), PTEN^{wt} may act to limit fos-mediated/p53-negative keratinocyte proliferation. Hence the lack of p-AKT in hyperplastic HK1.fos epidermis delayed papilloma formation, which required TPA/wound promotion (Greenhalgh et al., 1993b, 1995) to induced high p-ERK 1/2 expression (Karin, 1995; Schlingemann et al., 2003), increase cyclins D1/E2 (Bamberger et al., 2001) and restore p-AKT levels (Gonzales and Bowden, 2002).

Alternately in *K14.cre/Δ5PTEN* epidermis, elevated p-AKT expression increased differentiation to give hyperkeratosis (Fistarol et al., 2002; Stambolic et al, 2000), consistent with negative roles in reduction of endothelial cell lifespan (Miyauchi et al., 2004). In normal epidermis p-AKT expression is mainly suprabasal and *in vitro* its activities prevent p53-mediated apoptosis. This may provide a protected interval for keratinocytes to fully commit to terminal differentiation (Calautti et al., 2005). In pre-neoplastic *K14.cre/Δ5PTEN*^{flx} epidermis, elevated basal cell p-AKT expression disrupted this balance and increased proliferation due to concurrent loss of p53/p21^{WAF} cell-cycle regulation. However, instead of papillomatogenesis, resultant p-AKT-mediated hyperplasia was rapidly translated into hyperkeratosis, suggesting that basal expression of normally suprabasal p-AKT roles induced an early differentiation response. If correct, this elegant mechanism thus serves the dual purpose of rapidly eliminating potentially highly cancerous cells when PTEN tumour suppressor regulation and compensatory p53/p21^{WAF}-mediated apoptosis are interdicted (Brash, 2006), whilst maintaining epidermal tissue integrity and barrier functions under pathological conditions such as Cowden disease, where *PTEN* functions in adhesion signalling (Masahito et al., 1998; Subauste et al., 2005) are

potentially compromised and cutaneous keratinocytes lack normal $p21^{WAF}$ functions to initiate differentiation.

In *HK1.fos/Δ5PTEN^{flx}* KA aetiology, initial pre-neoplastic *HK1.fos/Δ5PTEN^{flx}* hyperplasia exhibited reduced p-AKT expression, alongside low-level p53-feedback, consistent with PTEN loss in prostate cancer where compensatory NKX3.1 inhibited p-AKT expression to stabilise p53 (Lei et al., 2006). With time increased MAP Kinase signalling and cyclin D1/E2 expression interdicted this early p53 countermeasure, resulting in high p-AKT expression in p53/$p21^{WAF}$–negative papillomatous histotypes. As outlined above subsequently high p53 co-expression fedback to reduce p-AKT activity in basal layers (Lei et al., 2006), inducing increasingly suprabasal p-AKT expression, which in turn facilitated basal layer expression of $p21^{WAF}$ (Zhou et al., 2001) and accelerate differentiation. This reduction in proliferative basal layer p-AKT expression appeared critical to inhibition of benign tumour progression i.e. unless significant p53/$p21^{WAF}$ co-expression induced a basal/suprabasal p-AKT expression switch to prevent sustained basal layer p-AKT activities, hyperproliferative benign tumour keratinocytes would be at risk for conversion, as demonstrated malignant transformation of DMBA-initiated, papilloma keratinocytes by introduction of constitutively active AKT (Segrelles et al., 2006), possibly via corruption of the anti-apoptotic AKT roles observed in normal differentiation (Calautti et al., 2005).

HK1.fos/Δ5PTEN^{flx} KA aetiology also indicated that a molecular trigger was required to induce basal layer p53/$p21^{WAF}$ expression and counter p-*AKT/v-fos/PTEN^{null}* oncogenicity. A prime candidate for this role emerged from analysis of GSK3β status, an unusual serine/threonine kinase where the unphosphorylated form is active and complexes with APC to target β-catenin for ubiquitin degradation (Karim et al., 2004). This tumour suppression role is inactivated by p-AKT phosphorylation, hence p-GSK3β co-operation with PTEN phosphatase loss in prostate carcinogenesis (Mulholland et al., 2006), and high p-GSK3β inactivation levels observed in DMBA/TPA carcinomas

(Leis et al., 2002) expressing elevated p-AKT activation (Segrelles et al., 2006). However, GSK3β status influences carcinogenesis in pathways separate to AKT, as pools of activated/inactivated GSK3β are interchangeable between PI3K/AKT and WNT/β-catenin pathways (Karim et al., 2004; Mulholland et al., 2006). Accordingly, while p-GSK3β inactivation paralleled p-AKT expression in p53-negative *K14.cre/Δ5PTEN*^{flx} and early *HK1.fos* hyperplasia, moderate p-GSK3β inactivation levels, uncoupled from p-AKT expression were observed in pre-neoplastic *HK1.fos/Δ5PTEN*^{flx} hyperplasia [and *HK1.fos* papillomas]. This moderate p-GSK3β expression appeared alongside low-level p53 expression, consistent with induction of p53 following GSK3β inactivation in colon carcinogenesis (Ghosh and Altieri, 2005). However, low-level p-GSK3β expression induced neither high p53 nor p21^{WAF} expression, therefore *HK1.fos/Δ5PTEN*^{flx} hyperplasia was susceptible to MAP kinase/cyclin D1/E2-associated promotion, resulting in restored, elevated basal layer p-AKT expression in the p53/p21^{WAF}-negative papillomatous histotypes [above], somewhat akin to the mechanism of p-AKT activation/p-GSK3β inactivation observed in *fos*-mediated [p53-null] HaCaT photo-carcinogenesis (Gonzales and Bowden, 2002).

As levels of p-GSK3β expression increased, possibly from a combination of moderate p-AKT-independent expression, observed in *HK1.fos/Δ5PTEN*^{flx} hyperplasia and that derived from increasing p-AKT expression during papillomatogenesis; it achieved a threshold of GSK3β inactivation that triggered the high, sustained p53/p21^{WAF} response. Again a key component centred on the switch of moderate, suprabasal p-GSK3β expression in papillomatous histotypes to one of high basal expression in transitional areas that induced p53, reduced p-AKT and initiated p21^{WAF}-mediated differentiation [above]. This attractive scenario thus explains why induction of high p53, and p21^{WAF} in particular, abruptly appeared in benign tumours, as the mechanism required substantial increases in p-GSK3β expression. Temporal GSK3β inactivation thus provided the sensory component of the mechanism geared to induce compensatory p53/p21^{WAF} responses, that actually required/exploited *HK1.fos/p-AKT* synergism in papillomatogenesis, yet continually blocked further progression. As this GSK3β-

associated mechanism of compensatory p53/p21^{WAF} may also induce apoptosis in alternate tumours (Ghosh and Altieri, 2005; Miyauchi et al., 2004; Yoo et al., 2006), it makes GSK3β inhibitors attractive for therapeutic intervention (Smalley et al., 2007; Tan et al., 2005). However, this should be approached with caution, given that GSK3β-inactivated inhibition of skin tumour progression directly contrasts with GSK3β-inactivated co-operation with PTEN loss that accelerates prostate carcinogenesis (Mulholland et al., 2006). Hence, potential efficacy may require tumor aetiologies where intact p53/p21^{WAF} response pathways (Nister et al., 2005; Wahl, 2006) can be induced (Smalley et al., 2007; Tan et al., 2005), as chemical carcinogenesis (Leis et al., 2002) and alternate models of AKT activation (Segrelles et al., 2006) show that should p53 and/or p21^{WAF} pathways become compromised, GSK3β inhibition could prove to be a double-edged sword.

In summary, this *HK1.fos/Δ5PTEN^{flx}* model links PTEN/PI3K/AKT signalling, ras/MAPK/fos pathways and the GSK3β/β-catenin/WNT axis and demonstrates that when deregulated by *fos* activation and/or *PTEN* loss, benign tumour progression can be inhibited by induction of p53 and/or p21^{WAF} pathways that limit oncogenic AKT activities. Collectively, these findings highlight the worth of inducible, transgenic models that allow mice to develop normally and thus yield valuable insights into the molecular relationships regulating normal tissue homeostasis. This carcinogenesis study also stressed the importance of context to the biological outcome of temporal, stage-specific gene expression, where common molecular expression profiles combined to give an unanticipated outcome that provides new insights into the capacity of the epidermis to cope with specific oncogenic insults.

METHODS

Genotypes, transgene expression and RU486 treatment.

HK1.fos (Greenhalgh et al., 1993b), RU486-inducible *K14.creP* regulator (Berton et al., 2000) and $\Delta 5PTEN^{flx}$ (Li et al., 2002) transgenic mice have been characterized previously. Breeding strategies maintained *HK1.fos* and *K14.creP* transgenes as heterozygotes in wild type [$PTEN^{wt}$], heterozygous [$\Delta 5PTEN^{wt/flx}$] or homozygous [$\Delta 5PTEN^{flx}$] *PTEN* backgrounds respectively. *PTEN* exon 5 ablation was achieved via activation of *cre* recombinase in dorsal skin treated topically with 2 ug RU486 in 50ul ethanol/week [mefipristone, Sigma] for 4 weeks, with controls receiving ethanol alone [*UK License:* 60/2929 to DAG]. *HK1.fos, K14.creP* and *Δ5PTEN* mice were genotyped by PCR and expression confirmed via rt/PCR (Greenhalgh et al., 1993a; Yao et al., 2006). For detection of *p53* or $c\text{-}ras^{Ha}$ mutations, tumour DNA was isolated as described (Yao et al., 2006), amplified with intron-specific oligonucleotides and sequenced.

Histology, Immunefluorescence and Bromo-deoxyuridine Labelling Analysis

Skin and tumour biopsies were fixed [10% formalin, 4^oC] and stained with haematoxylin and eosin, or frozen in OCT [Miles] and stored at -70°C. For immunefluorescence, frozen sections (5-7μm) were incubated overnight with: rabbit anti-K13 (Prof. D. Roop, Houston), anti-K1, anti-loricrin, anti-filaggrin [diluted 1:500] (Cambridge Bioscience) or guinea pig anti-K14 antibodies [dil 1:2000] (Research Diagnostics) and visualized by biotinylated goat anti-guinea pig/Streptavidin-Texas Red [diluted 1:100] (Vector Labs) or FITC-labelled anti-rabbit IgG [diluted 1:100; Jackson Labs]. For BrdU labelling, mice were injected IP with 125 mg/kg 5-bromo-4-deoxyuridine [Sigma] 2 hours prior to biopsy. Paraffin sections were subjected to antigen retrieval [10 mins boil/10mM sodium citrate] and BrdU labelling performed by overnight incubation at 4^0C with FITC-conjugated anti-BrdU dil 1:50 [Becton Dickinson], counterstained for K14 (above). For immunohistochemical analysis, sections were incubated with phospho-GSK3$\beta^{(ser9)}$ #9936 and phospho-$AKT^{(273)}$ #9271 [Cell Signaling Technology]; p53 (PAB 240) [CRUK Antibodies]; and $p21^{WAF}$ [#sc397, Santa Cruz] overnight [dil 1-

100/biotin-anti-goat 1:50) [Santa Cruz]; and visualized via HRP-conjugated strepavidin, incubated for 5 mins at room temp.

Western Analysis

Proteins were extracted from biopsy tissue as described (Yao et al., 2006). Proteins were subjected to western analysis employing antibodies to: total AKT #9272; phospho-AKT #9271, phospho-ERK #9101, total ERK p42/44 #9102, cyclin D1 #2922, cyclin E2 #4132, GSK3β #9315 and phospho GSK3$\beta^{(ser9)}$ #9936 [Cell Signaling Technology]; p53 (PAB 240) [CRUK Antibodies]; $p21^{WAF}$ #sc397 and β-actin #sc1616 [Santa Cruz]. Signals were detected with HRP-conjugated secondary antibodies (Dako) and ECL detection (Amersham Biosciences).

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LEGENDS

Figure 1. Phenotype and histotype of *HK1.fos/Δ5PTEN^{flx}* mice.

Upper panel: RU486-treated homozygous and heterozygous *HK1.fos-Δ5PTEN^{flx}* mice exhibit ear keratoacanthomas [KAs]. Control *HK1.fos* siblings possess small papillomas and RU486-treated *K14.cre/Δ5PTEN^{flx}* mice exhibit hyperkeratosis. *Lower panel:* [A] RU486-treated *HK1.fos* skin histology was indistinguishable from normal. [B] *K14.cre/Δ5PTEN^{flx}* epidermis exhibits mild hyperplasia, significant hyperkeratosis and ghost cells indicative of incorrect cornification. [C] *HK1.fos* papillomas histology displays expanded epidermal compartments but an overall ordered keratinocyte differentiation pattern. [D] A composite *HK1.fos/Δ5PTEN^{flx}* KA micrograph displays two distinct histotypes: an upper, differentiated area of massive keratosis interspaced with fronds of keratinocytes and a lower hyperproliferative, papilloma-like region. [E] *HK1.fos/Δ5PTEN^{flx}* KA keratinocytes of differentiated regions display a distinct disorder to the programme of differentiation, with cornified and granular cells co-existing alongside basal layer keratinocytes [arrows]. [F] Such regions also exhibited a prominent *stratum lucidium* [arrows] and [G] premature differentiation gave rise to micro-cysts. [Bars: A-C 100 μm; E-G 50 μm; and D approx. 175 μm].

Figure 2: Expression of differentiation markers in *HK1.fos/Δ5PTEN* tumours.

[A] *HK1.fos* papillomas exhibit a delay in onset of suprabasal keratin K1 expression [green] consistent with expansion of the proliferative basal cell compartment, counterstained with K14 [red]. *HK1.fos/Δ5PTEN^{flx}* KAs exhibit strong, atypical K1 expression in proliferative basal cells of differentiated regions [no separate K14/red is visible], suggesting an accelerated terminal differentiation. [B] Late-stage differentiation markers loricrin and [C] filaggrin remained confined to the granular layer in *HK1.fos* papillomas. In contrast, *HK1.fos/Δ5PTEN^{flx}* KAs display premature, elevated loricrin and filaggrin expression indicative of an accelerated disordered nature to differentiation e.g. loricrin in micro-cysts. [D] *HK1.fos* papillomas expressed tumor progression

marker keratin K13, which was focally expressed in papillomatous *HK1.fos/Δ5PTEN*^{flx} areas, whereas K13 expression was lost in differentiated KA histotypes

Figure 3: *HK1.fos/Δ5PTEN* synergism increases mitotic index in papillomatogenesis until KA is achieved.

Upper panel: Tabulated mitotic index from *HK1.fos/Δ5PTEN* epidermis and tumors. Despite a normal histotype, *HK1.fos* epidermis possessed a 2-fold increase in mitotic index over normal epidermis, similar to that of hyperkeratotic [HK] *K14.cre/Δ5PTEN*^{flx} epidermis. This index further doubled in *HK1.fos/Δ5PTEN* hyperplastic/hyperkeratotic [HPK] epidermis to levels observed in overt *HK1.fos* papillomas. The lower average mitotic labelling in *HK1.fos/Δ5PTEN* KAs comprised a low mitotic index in differentiated vs. a very high index in papillomatous histotypes. *Lower panel*: Double label BrdU immunefluorescence analysis of mitotic activity. Differentiated *HK1.fos/Δ5PTEN*^{flx} KA histotypes show low BrdU labelling [yellow] similar to that found in *HK1.fos* papillomas, conversely, papillomatous areas exhibit very high BrdU labelling.

Figure 4. Expression of p53, p-AKT, p-GSK3β and p-ERK 1/2 in *HK1.fos/Δ5PTEN* phenotypes.

Skin biopsies of keratoacanthomas [KA], papillomas [PAP], hyperplastic [HP] or hyperkeratotic [HK] epidermis, together with normal dorsal [N], anagen [aN] or ear skin [NE] were subject to western analysis. All *HK1.fos/Δ5PTEN* KAs expressed high p53 levels and phenotypic epidermis possessed low-level expression [mid panel] similar to normal controls [end panel]. Conversely, p53 expression was undetectable in *HK1.fos* phenotypes *[*lanes: PAP, HP, N] or hyperkeratotic *K14.cre/Δ5PTEN*^{flx} epidermis [end panel]. *HK1.fos/Δ5PTEN* KAs expressed high but variable p-GSK3β levels, depending on tumour maturity [*Δ5PTEN* heterozygous KA #8898 vs. homozygous KA # 9593]. However, KAs exhibited lower increases in p-AKT expression, which varied extensively with the degree of keratosis [Ka*]. Compared to total protein levels, *HK1.fos* epidermis exhibited low p-AKT and p-GSK3β

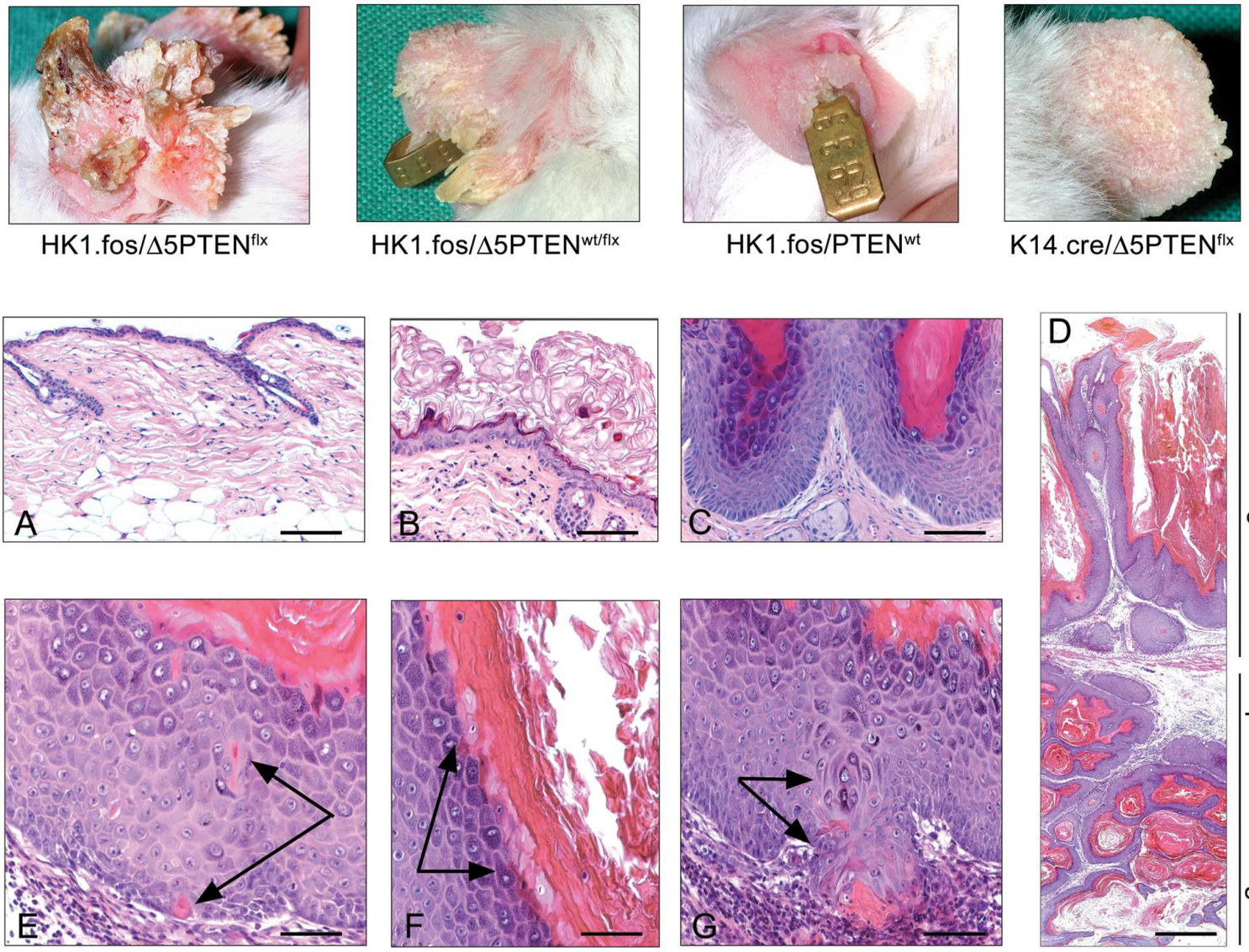
expression [first panel]; whilst p-GSK3β expression, but not p-AKT, increased in *HK1.fos* papillomas. Control *K14.cre/Δ5PTEN*^{flx} epidermis possessed elevated p-GSK3β and p-AKT expression [end panel]. All hyperplastic phenotypes expressed elevated p-ERKs 1 and 2, including "normal" *HK1.fos* epidermis and *HK1.fos* papillomas in particular, which remained steady, if slightly reduced, in all KAs. β-actin expression served as a loading control.

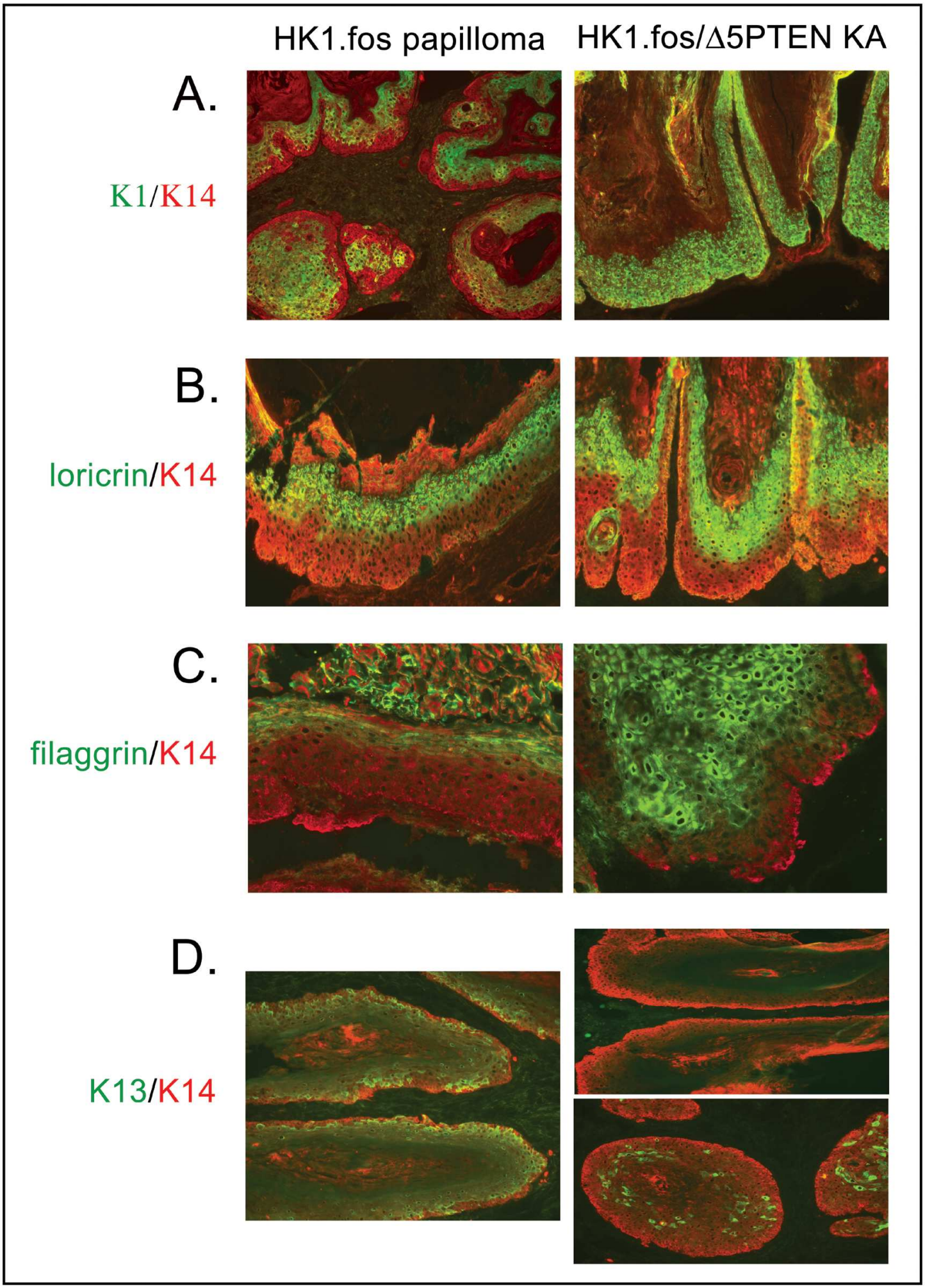
Figure 5: *HK1.fos/Δ5PTEN* KAs exhibit high p53 and novel p21^{WAF} expression associated with a threshold level of GSK3β inactivation.

Western analysis of p53, p-GSK3β and p-AKT was compared to p21^{WAF}, cyclin D1 and E2 expression in pathology matched KAs [similar keratosis/papilloma ratios] and age matched preneoplastic phenotypes. Hyperkeratotic [HK] *K14.cre/Δ5PTEN*^{flx} ear epidermis displayed little detectable p21^{WAF} or p53 and slightly increased expression of p-AKT, cyclins D1 and E2, with p-GSK3β being higher in the tagged [T], wound-promoted biopsy. Normal [N] appearing *HK1.fos* epidermis was negative for p21^{WAF} and p53 expression, with low p-GSK3β and decreased p-AKT levels, alongside slightly elevated cyclin D1. *HK1.fos* papillomas [PAP] expressed little p21^{WAF} and p53, but displayed increased p-GSK3β expression compared to p-AKT, together with elevated cyclins. *HK1.fos/Δ5PTEN*^{flx} epidermis [HK] expressed barely detectable p21^{WAF}, limited p53 and moderate p-GSK3β expression, whilst p-AKT expression was less than *K14.cre/Δ5PTEN*^{flx} controls. All *HK1.fos/Δ5PTEN*^{flx} KAs expressed high levels of p21^{WAF} and p53 that mirrored significant increases in p-GSK3β inactivation. However, p-AKT expression remained similar to *K14.cre/Δ5PTEN*^{flx} epidermis. All KAs exhibited elevated cyclin D1 and E2 expression, particularly in ear-tagged samples [KA^{T}]. β-actin served as a loading control.

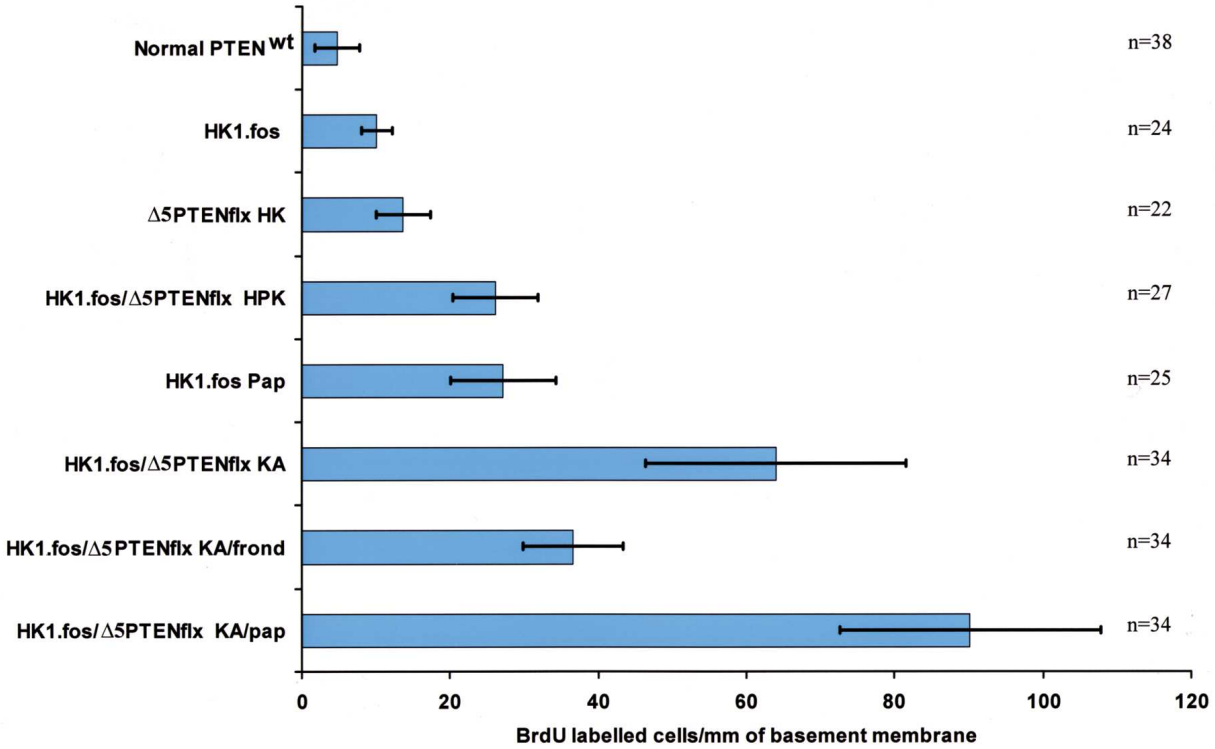
Figure 6: Immunohistochemical analysis of p53, $p21^{WAF}$, p-GSK3β and p-AKT expression in differentiated and proliferative *HK1.fos/Δ5PTEN* KA histotypes.

[A-C] p53 expression in differentiated [A], transitional [B] and papillomatous [C] KA histotypes. Note high, basal layer p53 expression in [A], was increasing in [B] from suprabasal to basal and absent in [C] papillomatous areas [Composite micrographs and immunohistochemical analysis of control *HK1.fos* and *K14.cre/Δ5PTEN*^{flx} phenotypes are shown in Supp data Figs. 2 and 3]. [D-F] Similarly strong basal $p21^{WAF}$ expression was observed in differentiated KA histotypes [D] which had increased and become nuclear in transitional areas [E], but was absent in papillomatous histotypes [F]. [G-I] Strong, basal layer p-GSK3β expression in differentiated KA areas [G] preceded that of p53/p21 in transitional areas [H] and was observed in papillomatous areas [I], where lower expression was confined to suprabasal layers. [J-L] Conversely, in differentiated KA areas p-AKT expression was reduced, cytoplasmic and undetectable in basal layers [J]; a process that began in transitional areas where expression became increasingly suprabasal and faded [K], unlike strong expression observed in papillomatous histotypes [L]. [Magnification bars: A and J: 25 μm; D and G: 50 μm; remainder: 100μm].

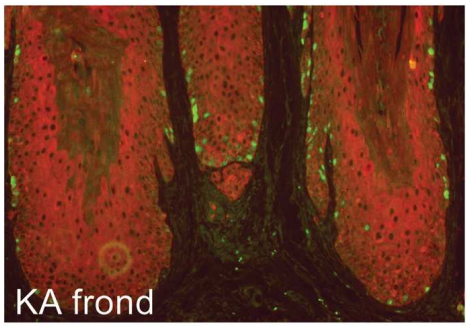

HK1.fos/Δ5PTEN^{flx}
HK1.fos/Δ5PTEN$^{wt/flx}$
HK1.fos/PTEN^{wt}
K14.cre/Δ5PTEN^{flx}
A
B
C
D
E
F
G
Frond region
Pap/car *in situ* region

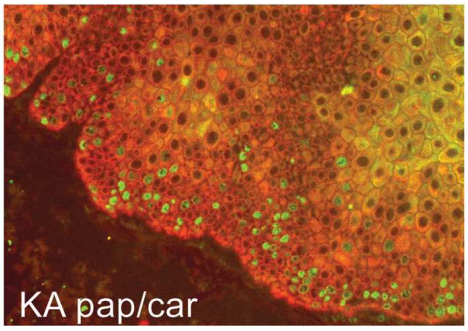

HK1.fos papilloma
HK1.fos/Δ5PTEN KA
A.
K1/K14
B.
loricrin/K14
C.
filaggrin/K14
D.
K13/K14

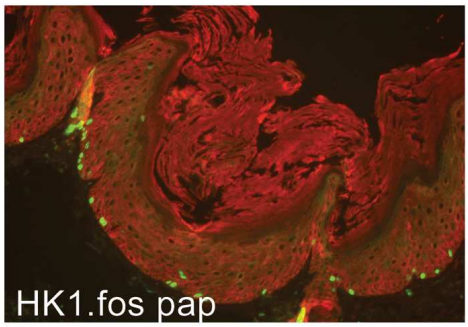
A

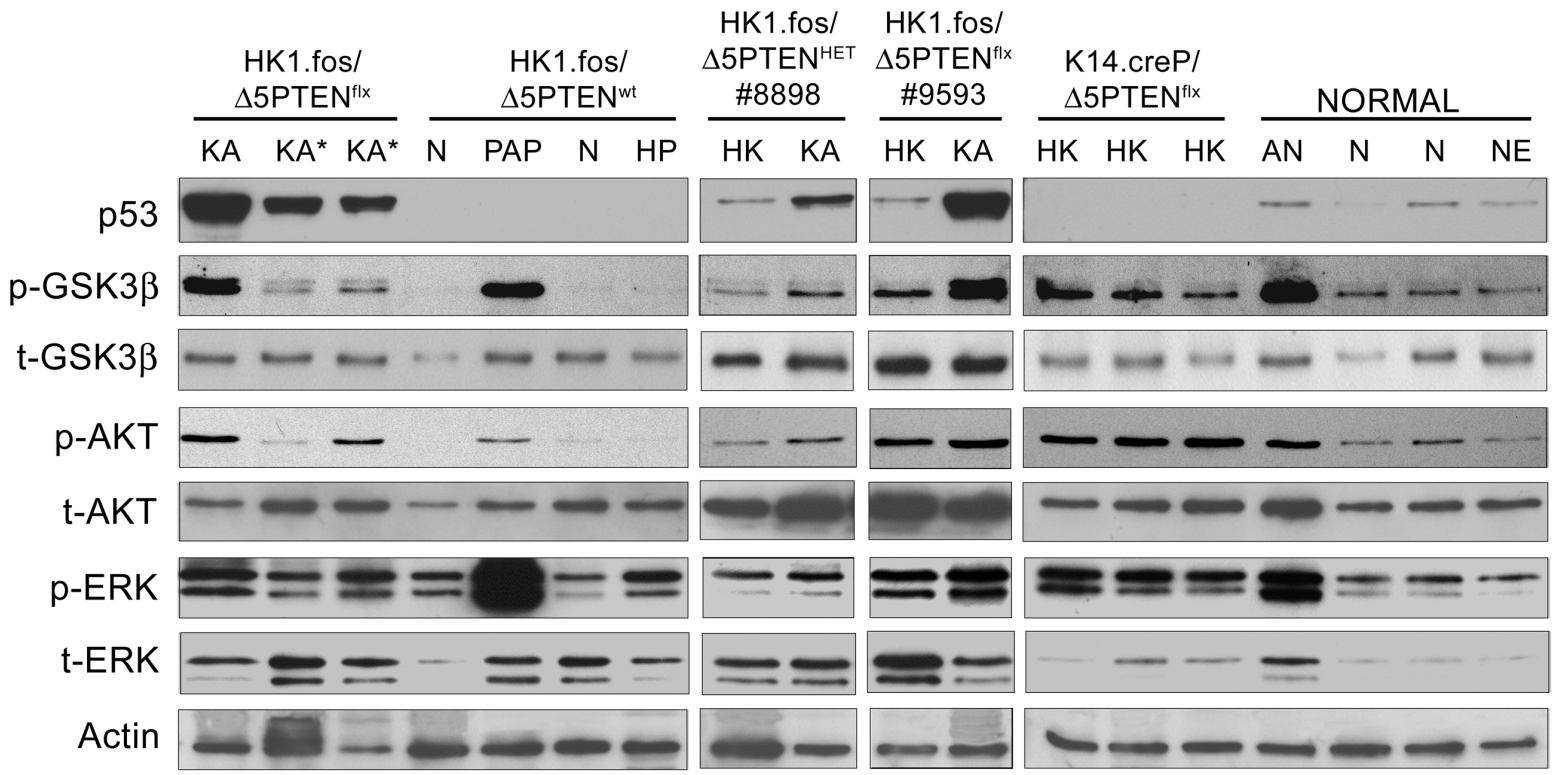

Normal PTEN wt
HK1.fos
Δ5PTENflx HK
HK1.fos/Δ5PTENflx HPK
HK1.fos Pap
HK1.fos/Δ5PTENflx KA
HK1.fos/Δ5PTENflx KA/frond
HK1.fos/Δ5PTENflx KA/pap
n=38
n=24
n=22
n=27
n=25
n=34
n=34
n=34
0
20
40
60
80
100
120
BrdU labelled cells/mm of basement membrane

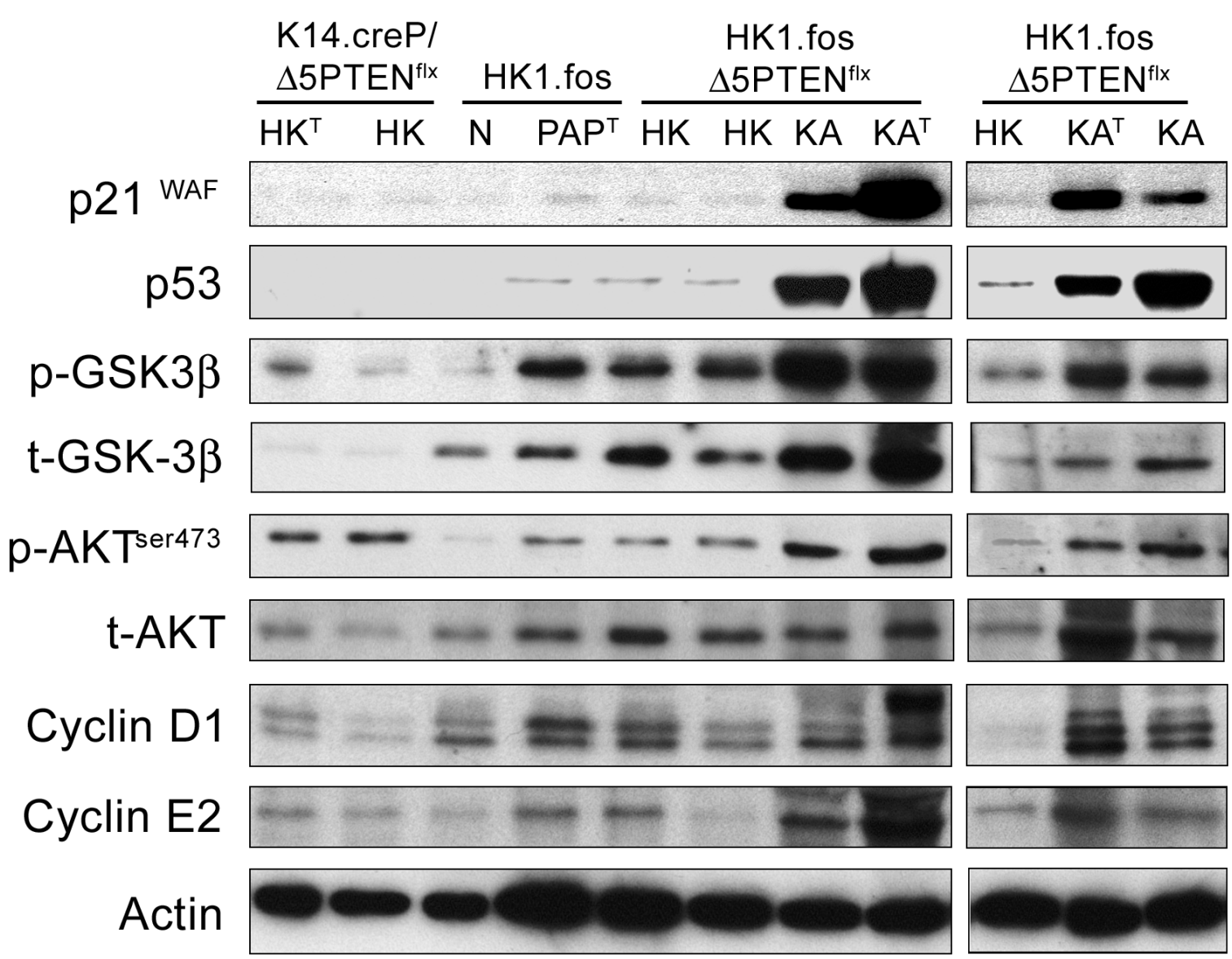
B

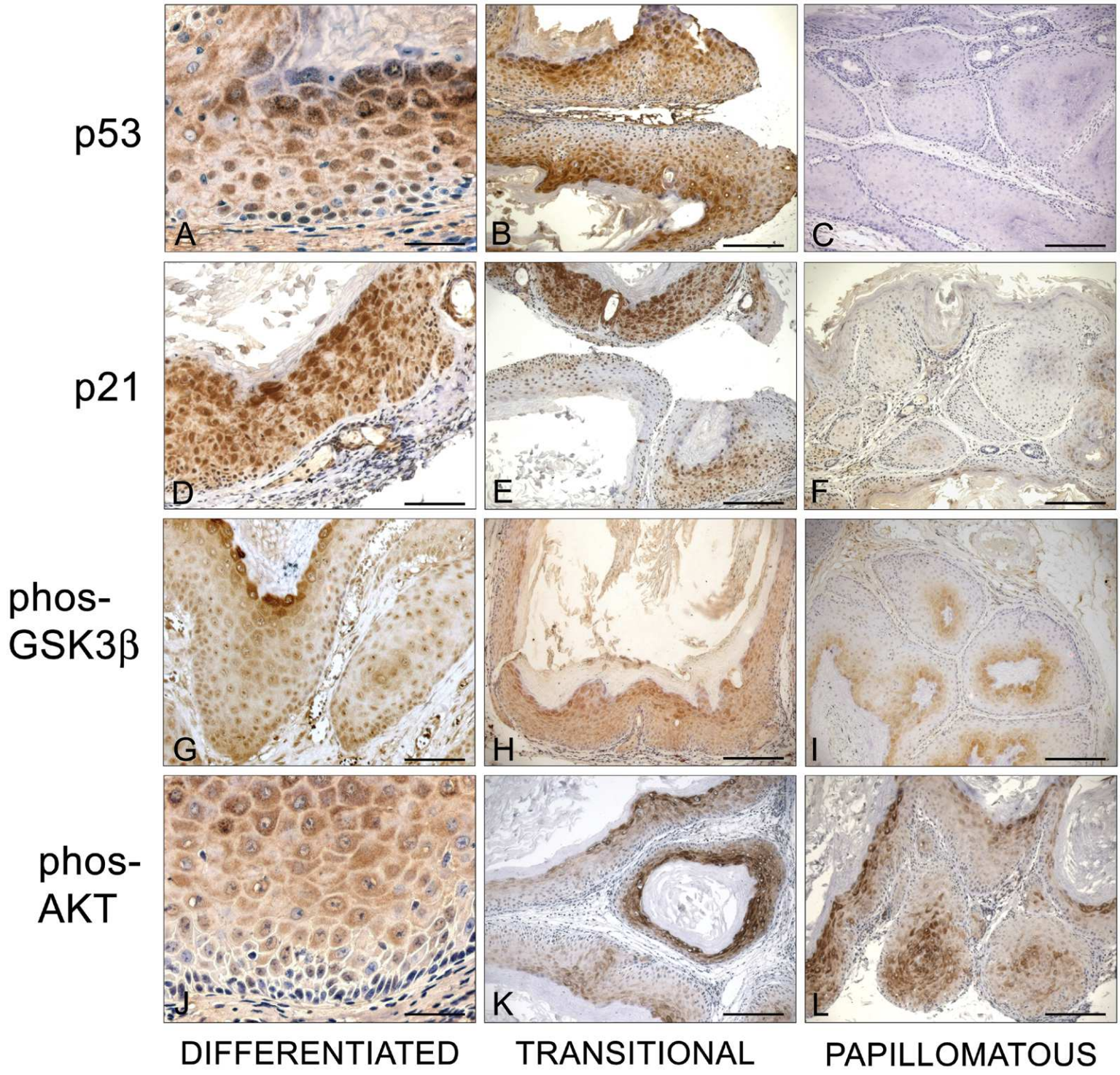

KA frond


KA pap/car


HK1.fos pap


HK1.fos/ Δ5PTEN^flx
HK1.fos/ Δ5PTEN^wt
HK1.fos/ Δ5PTEN^HET #8898
HK1.fos/ Δ5PTEN^flx #9593
K14.creP/ Δ5PTEN^flx
NORMAL
KA KA* KA* N PAP N HP HK KA HK KA HK HK HK AN N N NE
p53
p-GSK3β
t-GSK3β
p-AKT
t-AKT
p-ERK
t-ERK
Actin


K14.creP/
Δ5PTEN^flx
HK1.fos
HK1.fos
Δ5PTEN^flx
HK1.fos
Δ5PTEN^flx
HK^T
HK
N
PAP^T
HK
HK
KA
KA^T
HK
KA^T
KA
p21 WAF
p53
p-GSK3β
t-GSK-3β
p-AKT^ser473
t-AKT
Cyclin D1
Cyclin E2
Actin


p53
p21
phos-
GSK3β
phos-
AKT
A
B
C
D
E
F
G
H
I
J
K
L
DIFFERENTIATED
TRANSITIONAL
PAPILLOMATOUS

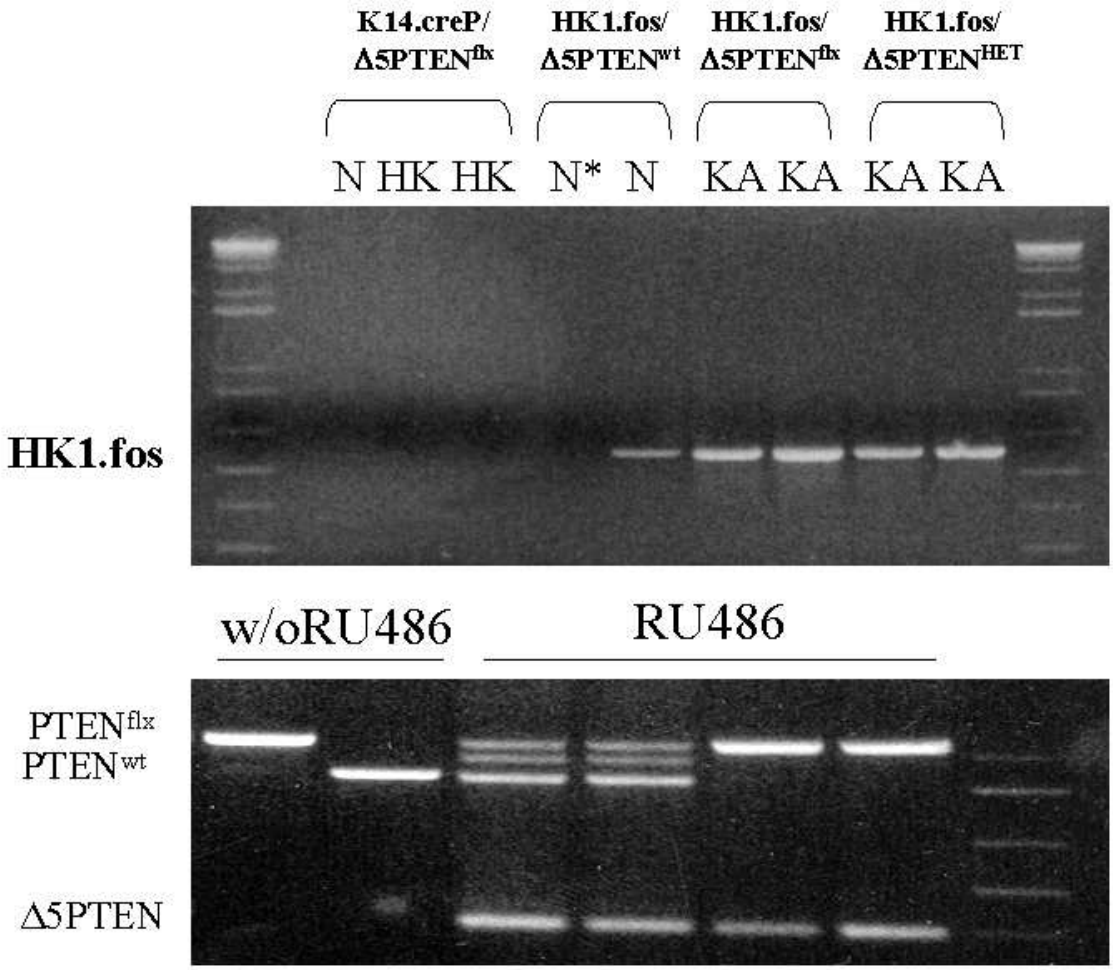


Figure 1S: Upper panel: reverse transcriptase/PCR analysis of *HK1.fos* expression identifies an approx 700bp *v-fos*–specific band in: non-phenotypic normal *HK1.fos* adult skin [lane N; N*: w/o reverse transcriptase control for DNA contamination]; and RU486-treated *HK1.fos/Δ5PTEN* ^{flx} or *HK1.fos/Δ5PTEN* ^{het} KAs.

Lower panel: Δ5PTEN expression in: Untreated skin [lanes 1 and 2] and RU486-treated skin from *HK1.fos/Δ5PTEN* ^{Het} [lanes 3,4] or *HK1.fos/Δ5PTEN* ^{flx} [lanes 5,6] identify a Δ5PTEN floxed allele-specific band [approx 1100bp], the wt allele [900bp] and the truncated Δ5PTEN floxed allele-specific band following exon 5 ablation in treated keratinocytes [400bp].

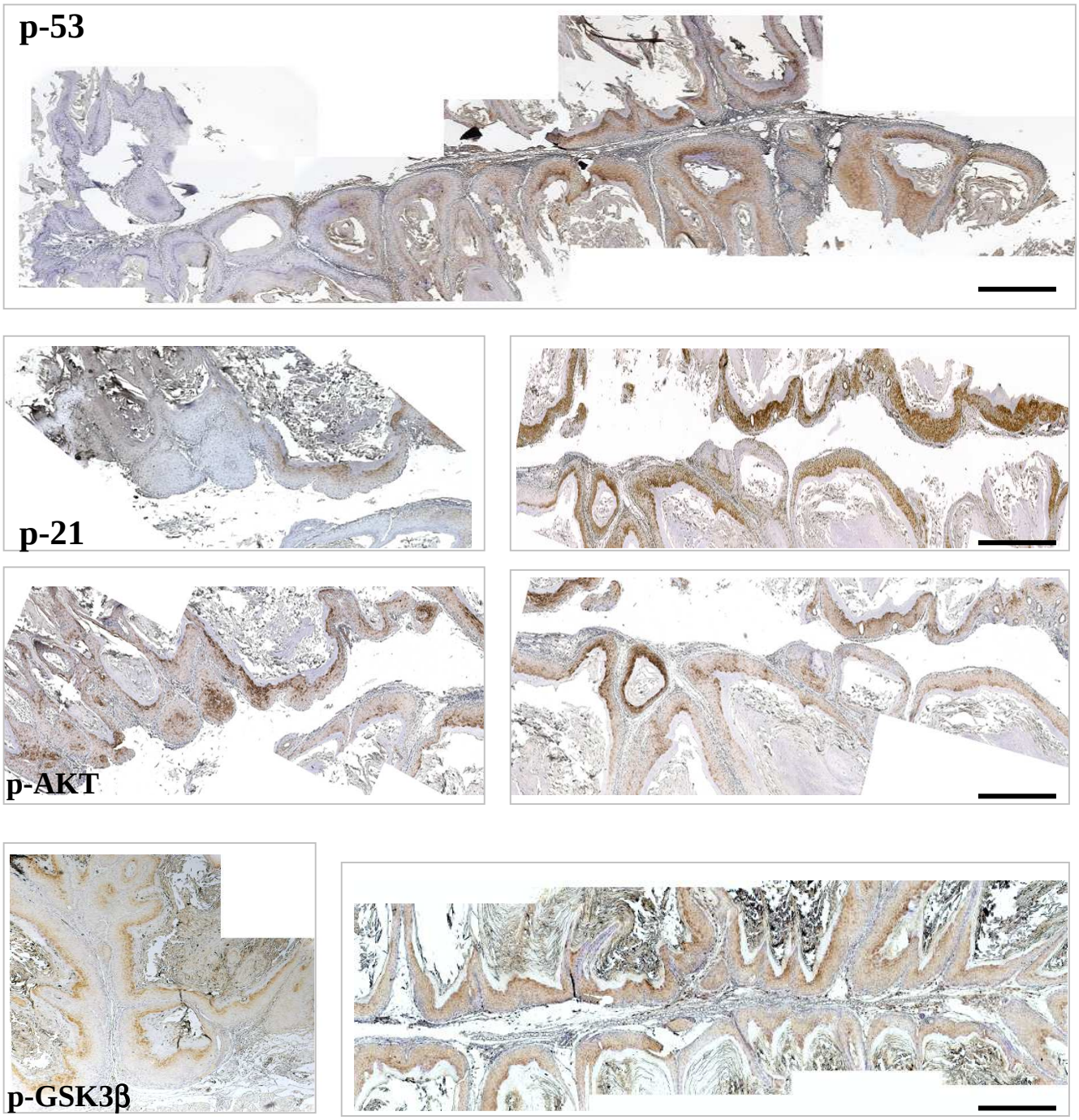


Figure 2S: Composite micrographs of protein expression in *HK1.fos/Δ5PTEN^{flx}* keratoacanthomas.

Adjacent sections are those shown in Fig. 6 and in each composite the left panel/area shows the proliferative, papillomatous histotype while the right displays differentiated, frond regions. Both p53 and p21^{WAF} exhibit little expression in the papillomatous areas, however strong, increasingly basal layer expression appeared in differentiated/keratotic areas, with p21^{WAF} expression appearing in both cytoplasm and nucleus. p-GSK3β expression increased in papillomatous histotypes prior to the p53/p21 expression burst, but this expression remained essentially suprabasal; whereas in differentiated histotypes, a further increase and basal layer p-GSK3β expression was observed. Conversely, an inverse relationship was recorded for p-AKT expression. Strong expression in papillomatous regions faded in the corresponding p53/p21^{WAF}-expressing differentiated histotypes. A more subtle finding in transitional areas demonstrated that strong p-AKT co-expression produced a less intense and suprabasal p53/p21^{WAF} expression profile and vice versa, increasing p53/p21^{WAF} expression produced less intense and suprabasal p-AKT co-expression, until p- AKT expression faded [Mag. Bar: 150 um].

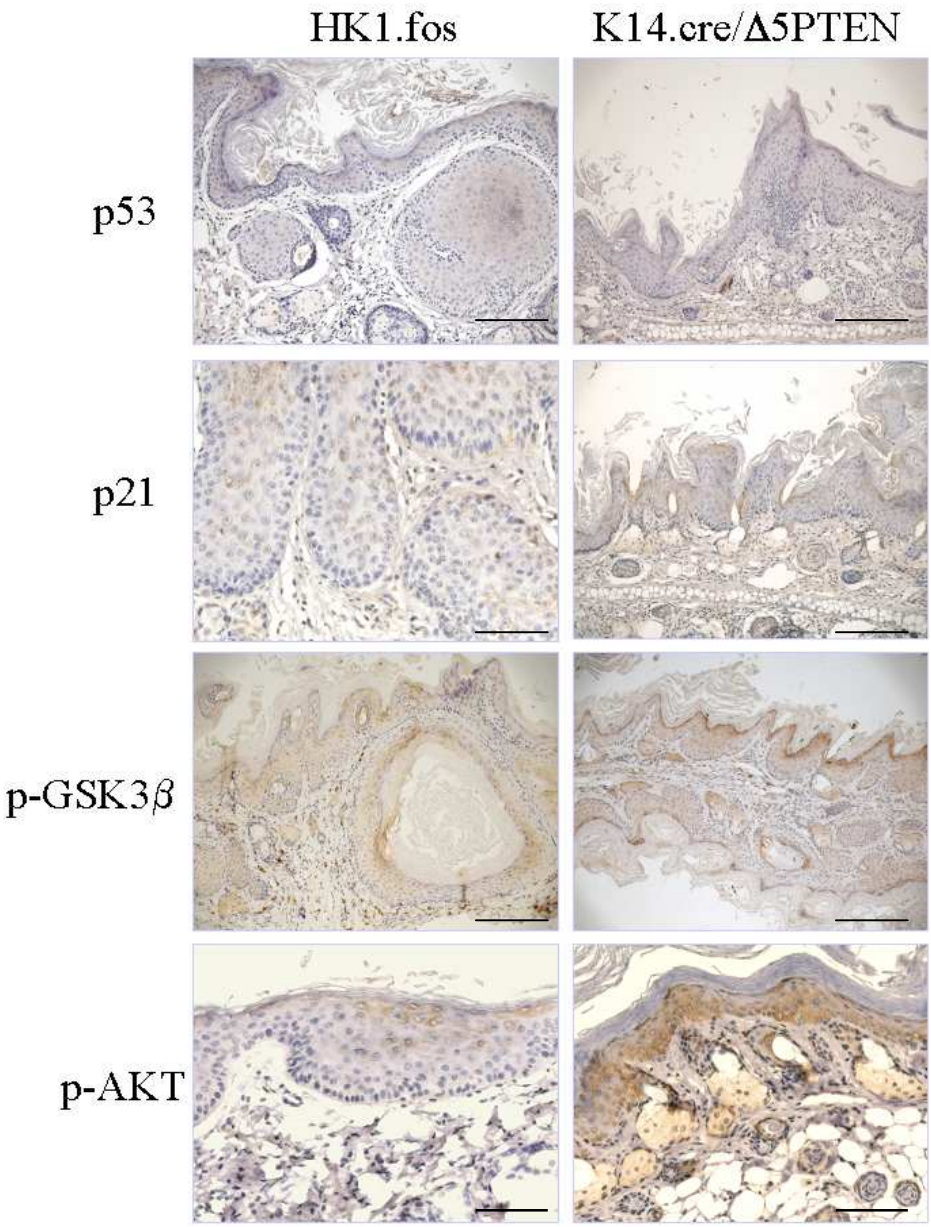


Figure 3S: Immunohistochemical analysis of p53, p21^{WAF}, p-GSK3β and p-AKT expression in *HK1.fos* and *K14.cre/Δ5PTEN* histotypes.

HK1.fos and *K14.cre/Δ5PTEN* sections are from control siblings age matched to those shown in Fig. 6 and Supp. Fig 2S. In both *HK1.fos* papillomas and *K14.cre/Δ5PTEN* epidermal hyperplasia, p53 and p21^{WAF} expression were consistently negative, as were normal and hyperplastic *HK1.fos* epidermis [not shown]. In normal or early hyperplastic *HK1.fos* epidermis, p-GSK3β levels were low [not shown], whereas in later hyperplasias/early papillomas, p-GSK3β expression increased to detectable levels, which remained suprabasal and similar but below to papillomatous *HK1.fos/Δ5PTEN* histotypes [Fig. 6I]. Conversely, hyperplastic *HK1.fos* epidermis possessed very little p-AKT expression, which remained low in papillomas [not shown]. Hyperplastic *K14.cre/Δ5PTEN* epidermis displayed strong p-AKT expression in each epidermal compartment including basal layers and follicles, yet no papillomas appeared and hyperkeratosis was the predominant histotype. This was mirrored by p-GSK3β in being a downstream target of AKT, but p-GSK3β expression was mainly suprabasal and below that observed in *HK1.fos/Δ5PTEN* KAs [Fig. 6] [mag. Bars 50 or 100 um].