Fos cooperation with PTEN loss elicits keratoacanthoma not carcinoma, owing to p53/p21^WAF-induced differentiation triggered by GSK3β inactivation and reduced AKT activity

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Summary

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 *Fos* (*HK1.Fos*). RU486-treated *HK1.Fos/Δ5Pten^flx* mice exhibited hyperplasia, hyperkeratosis and tumours that progressed to highly differentiated keratoacanthomas, rather than to carcinomas, owing to re-expression of high p53 and p21^WAF levels. Despite elevated MAP kinase activity, cyclin D1 and cyclin E2 overexpression, and increased AKT activity that produced areas of highly proliferative papillomatous keratinocytes, increasing levels of GSK3β inactivation induced a novel p53/p21^WAF expression profile, which subsequently halted proliferation and accelerated differentiation to give the hallmark keratosis of keratoacanthomas. A pivotal facet to this GSK3β-triggered mechanism centred on increasing p53 expression in basal layer keratinocytes. This increase in expression 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 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 that has attracted 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 individuals with Cowden Disease 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 that are 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), cooperates with PTEN loss in prostate carcinogenesis (Mulholland et al., 2006) when *p53* is also compromised (Chen et al., 2005), and bladder cancer models have identified compensatory roles for *p21^WAF* that counter initial *Pten^null*-mediated hyperplasia (Yoo et al., 2006).

Multistage skin carcinogenesis studies also implicate these molecules. 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). Furthermore, employing conditional PTEN knockouts, studies showed that DMBA-initiated *c-ras^Ha* (*Hras1*) activation achieved increased malignancy after TPA promotion (Suzuki et al., 2003). However, two-stage chemical carcinogenesis using 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 synergism of *c-ras^Ha* with *Pten* loss (Li et al., 2002) produced benign papillomas, but required TPA for malignant conversion, which involved a separate *Pten*-mediated mechanism of cell cycle deregulation that superseded initial *Pten*/*ras^Ha* synergism (Yao et al., 2006).

Given that the oncogene *Fos* is a major effecter of TPA promotion (Schlingemann et al., 2003) and cooperates with *c-ras^Ha* during papillomatogenesis and malignant conversion (Greenhalgh et al., 1990; Greenhalgh et al., 1993a; Greenhalgh et al., 1995; Saez et

al., 1995), this study investigated whether activated Fos would cooperate with PTEN loss in papillomatogenesis and drive this *c-ras^{Ha}*-independent Δ5Pten-mediated mechanism of malignant progression. Indirect links between Fos and PTEN deregulation already exist, as Δ5Pten could substitute for activated c-*ras^{Ha}* during TPA promotion (Yao et al., 2006), and Fos-mediated photo-carcinogenesis associates with both AKT activation and GSK3β inactivation (Gonzales and Bowden, 2002). Furthermore, UVB-mediated p53 mutation and subsequent PTEN loss induces AP1 expression (Wang et al., 2005), whereas, in reverse, PTEN specifically targets Fos expression via AKT signalling to downregulate AP1 activity (Koul et al., 2007).

Direct cooperation between activated *Fos* and inducible *Pten* loss in adult skin 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 cooperates 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, owing to the expression specificity of the K14 promoter (Yao et al., 2006). These mice were bred with *HK1.fos* mice that exclusively express epidermal FBJ/R *v-fos* in ~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 or 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 2.5 year 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 [over 12 months (Greenhalgh et al., 1993b)], whereas RU486-treated *K14.cre/Δ5Pten^{flx}* siblings exhibited epidermal hyperkeratosis without spontaneous papillomas (Yao et al., 2006). Transgene expression/ablation analysis (supplementary material Fig. S1) confirms permanent ablation of PTEN exon 5 following RU486 treatment, and demonstrates *HK1.fos* expression in normal appearing skin. *HK1.fos* expression was elevated in KAs, consistent with their increased differentiation and anomalous murine K1 expression in proliferative basal layers.

The KA outcome of Fos cooperation with PTEN contrasts with induction of malignant conversion in cooperation with *c-ras^{Ha}*- (Greenhalgh et al., 1990; Greenhalgh et al., 1995; Saez et al., 1995) or TPA-mediated, i.e. Fos-associated (Schlingemann et al., 2003), conversion of Δ5Pten/*ras^{Ha}* 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. For instance, the histotype of *HK1.fos* skin (Fig. 1A) was indistinguishable from normal, despite the fact that HK1.fos expression (supplementary material Fig. S1, lane 5) doubled the mitotic index [below Fig. 3 (Greenhalgh et al., 1993b)]. Hence, prior to wound promotion, this *HK1.fos*-induced proliferation was possibly counterbalanced by an increase in keratinocyte turnover and/or differentiation, functions regulated in part by endogenous Fos (Angel et al., 2001; Mehic et al., 2005), and by downregulation of AKT activity. Similarly, as observed in cancer-prone individuals with Cowden Disease (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 proliferation in response to PTEN loss was rapidly translated into hyperkeratosis to eliminate potentially neoplastic cells at an early stage, an observation consistent with recent findings on roles for AKT activation during the initial stages of terminal differentiation (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 ~5-6 months, 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, whereas 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 lucidum (Fig. 1F, arrows) that is indicative of incorrect cornification. This confusion of differentiated and proliferative cell subtypes in each epidermal compartment suggests that *HK1.fos/Δ5Pten^{flx}* keratinocytes within the keratotic/differentiated histotype received abruptly conflicting proliferation and differentiation signals.

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), for the late-stage differentiation markers filaggrin and loricrin (both proteins that typically become lost during carcinogenesis), and also for keratin K13 [a simple epithelia keratin employed as a marker of papilloma progression, the expression of 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 owing 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
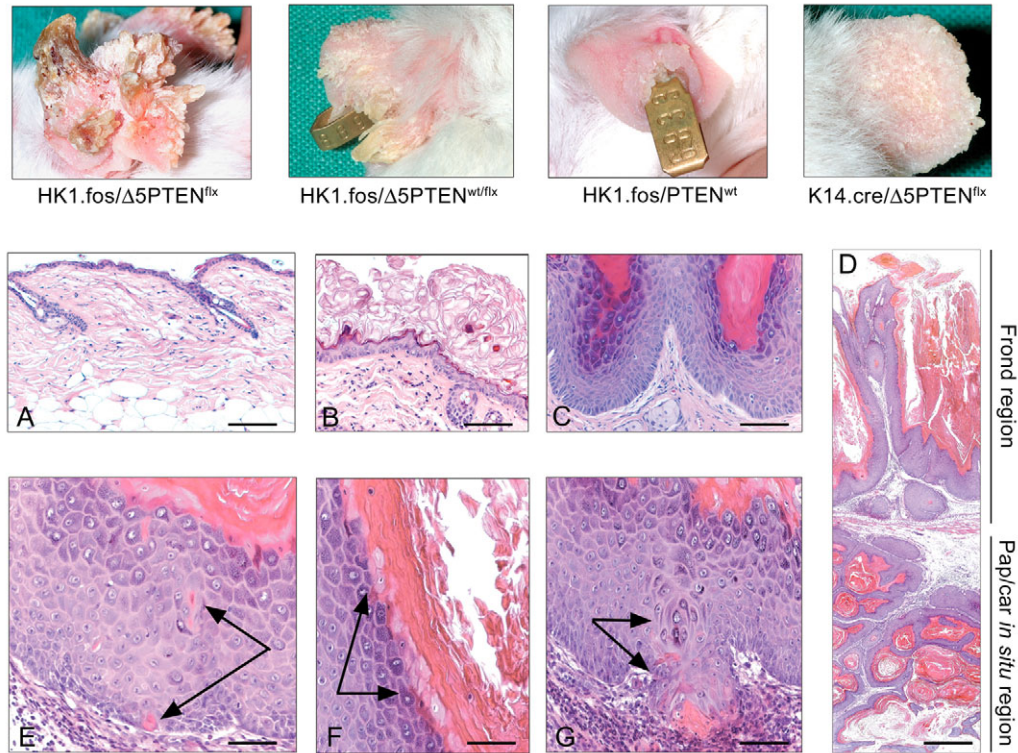
Fig. 1. Phenotype and histotype of *HK1.fos/Δ5Pten^flx^* mice. (Top row) RU486-treated homozygous and heterozygous *HK1.fos-Δ5Pten^flx^* mice exhibit ear keratoacanthomas (KAs). Control *HK1.fos* siblings possess small papillomas and RU486-treated *K14.cre/Δ5Pten^flx^* mice exhibit hyperkeratosis. Lower 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* papilloma 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 lucidum (arrows) and (G) premature differentiation gave rise to micro-cysts (arrows). Scale bars: 100 µm in A-C; 50 µm in E-G; ~175 µm in D.

shown); however, highly differentiated *HK1.fos/Δ5Pten^flx^* KA histotypes exhibited novel, K1 expression in the proliferative basal layers. K1 expression was very 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, AP1-regulated loricrin expression, a major component of granular cells, remained restricted to the granular compartment, where 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 AP1-regulated component of cornification, with crucial 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, although 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. This 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) produced mild hyperplasia. Additional doubling of mitotic index occurred in *HK1.fos/Δ5Pten^flx^* skin (26.1 ± 5.7) to levels observed in overt *HK1.fos* papillomas (27.2 ± 7.1, Fig. 3B), whereas papillomatous KA keratinocytes possessed a very high mitotic index (90.2 ± 17.6), comparable with that of SCCs, and with extensive suprabasal BrdU-labelling (Fig. 3B). Keratinocytes of differentiated histotypes, however, possessed a significantly reduced mitotic index (36.6 ± 6.7; P=<0.0001), although this remained higher than
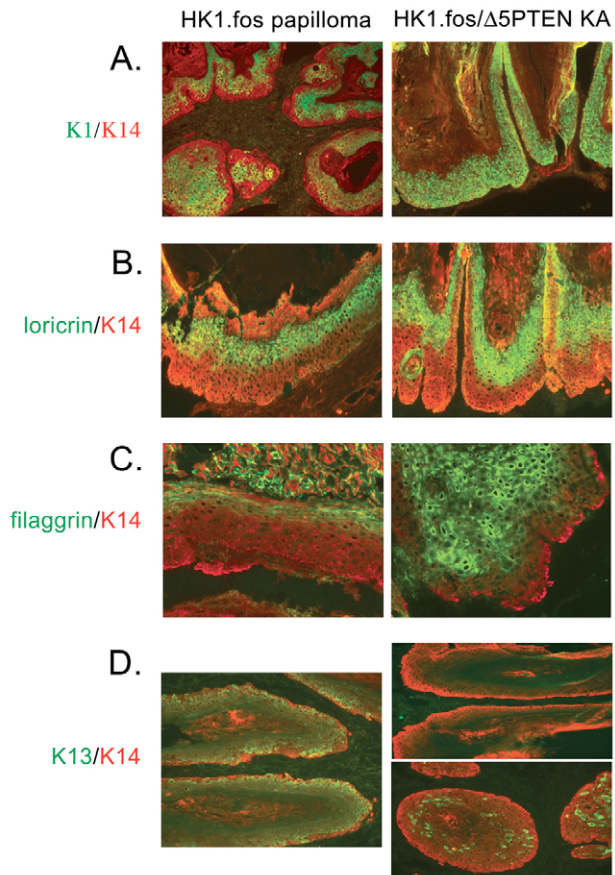
Fig. 2. Expression of differentiation markers in *HK1.fos/Δ5Pten* tumours. (A) *HK1.fos* papillomas exhibit a delay in onset of suprabasal keratin K1 expression (green), consistent with expansion of the proliferative basal cell compartment, counterstained with K14 (red). *HK1.fos/Δ5Pten*^{flx} KAs exhibit strong, atypical K1 expression in proliferative basal cells of differentiated regions (no separate K14/red is visible), suggesting an accelerated terminal differentiation. (B,C) Late-stage differentiation markers (B) loricrin and (C) filaggrin remained confined to the granular layer in *HK1.fos* papillomas. By 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 tumour progression marker keratin K13, which was focally expressed in papillomatous *HK1.fos/Δ5Pten*^{flx} areas, whereas K13 expression was lost in differentiated KA histotypes.

that of typical HK1.fos papillomas (*P*=0.001; Student's *t*-test). Thus, BrdU labelling indicated that a potent inhibition of hyperproliferation arose after benign tumour formation that 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 control phenotypes from their age-matched littermates. Hyperkeratotic *K14.cre/Δ5Pten*^{flx} epidermis exhibited little

detectable p53 expression (Figs 4, 5: HK lanes) compared with 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 but inconsistent with the normal histotype. On rare occasions, low-level p53 expression was recorded in *HK1.fos* phenotypes owing 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 among 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 compare 8898 with 9593 KAs). A similar result was also recorded on comparison of larger wound-promoted ear-tagged with untagged ear KAs from the same animals (Fig. 5: lanes KA^{T} versus KA). In addition, pre-neoplastic *HK1.fos/Δ5Pten*^{flx} or *HK1.fos/Δ5Pten*$^{wt/flx}$ epidermis expressed low-level p53 (Fig. 4, lanes HK #8898 and #9593; Fig. 5, HK lanes 5, 6 and 9), suggesting that 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 with high labelling indices of papillomatous regions. As indicated by decreased K13 tumour marker expression, elevated p53 would inhibit further tumour progression. This idea was 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; Greenhalgh et al., 1995; Lieu et al., 1991) (*n*=5; not shown). Thus, high expression of normal p53 in KA aetiology may be rendered impotent by c-*ras*^{Ha} activation, which leads 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 with total AKT expression levels, *P*-AKT expression varied significantly with the degree of keratosis and hyperproliferation (Fig. 4, KA*) or with 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 with hyperplastic epidermis taken from the same animal. Moreover, *P*-AKT levels in pre-neoplastic *HK1.fos/Δ5Pten*^{flx} epidermis were consistently lower than in age-matched *K14.cre/Δ5Pten*^{flx} littermate epidermis (Fig. 5, *K14.cre/Δ5Pten*^{flx} HK lanes 1, 2; versus *HK1.fos/Δ5Pten*^{flx} HK lanes 5, 6 and 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
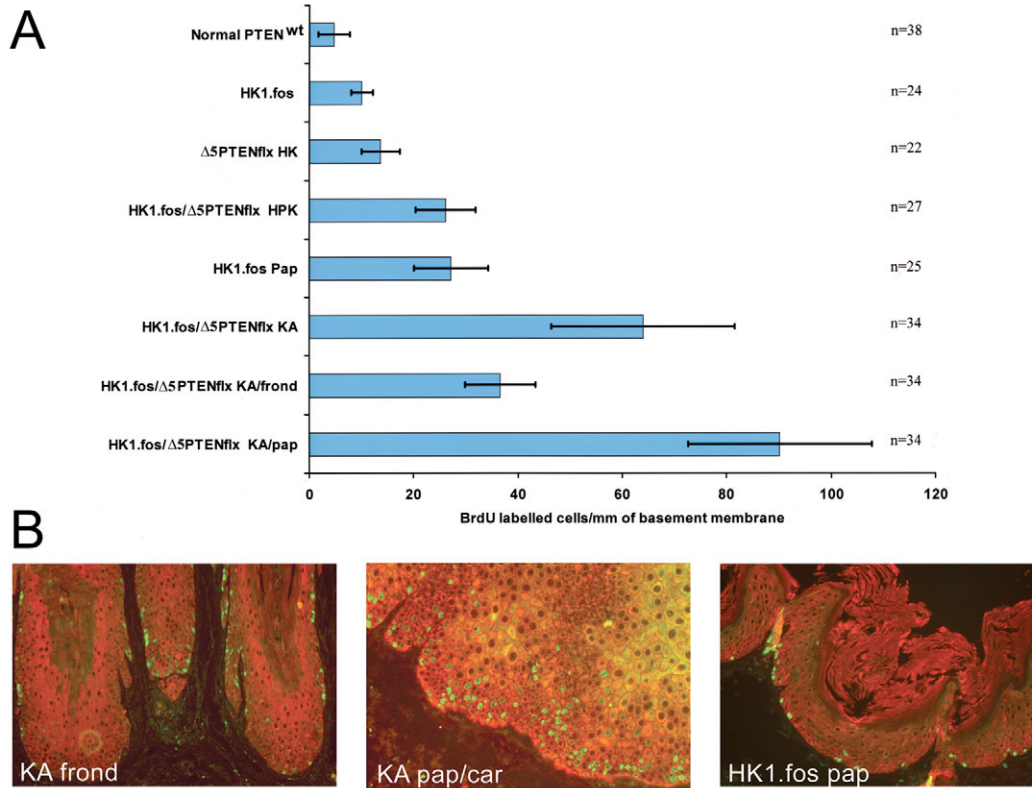
Fig. 3. *HK1.fos/Δ5Pten* synergism increases mitotic index in papillomatogenesis until KA is achieved. (A) Tabulated mitotic index from *HK1.fos/Δ5Pten* epidermis and tumours. Despite a normal histotype, *HK1.fos* epidermis possessed a twofold increase in mitotic index over normal epidermis, similar 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 versus a very high index in papillomatous histotypes. (B) Double-labelled BrdU immunofluorescence 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.

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 *c-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; supplementary material Fig. S2), 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 with 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 downregulation may be an element of the epidermal resistance to early carcinogenesis. This observation could explain the delay in papilloma appearance and the longstanding puzzle that a p53-negative *HK1.fos* epidermis exhibits a normal histotype, despite a mitotic index that produces 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., 2005), coupled to the intimate interactions between p53 and PTEN (Freeman et al., 2003), HK1.fos-mediated p53 loss may be countered, in part, by a PTEN-mediated feedback involving *P*-AKT

downregulation, which facilitates keratinocyte turnover and differentiation (Angel et al., 2001; Calautti et al., 2005). This is currently under investigation. Hence, *HK1.fos* phenotypes required a wound-promotion stimulus, eliciting high *P*-ERK1/2 and increased cyclin D1 and cyclin E2 expression (below), to antagonise/interdict such putative countermeasures and restore *P*-AKT expression in *HK1.fos* papillomas (Figs 4, 5, lane 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 and 2) was accompanied by a rapid translation of hyperplasia into hyperkeratosis (Fig. 1B), as observed in Cowden 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
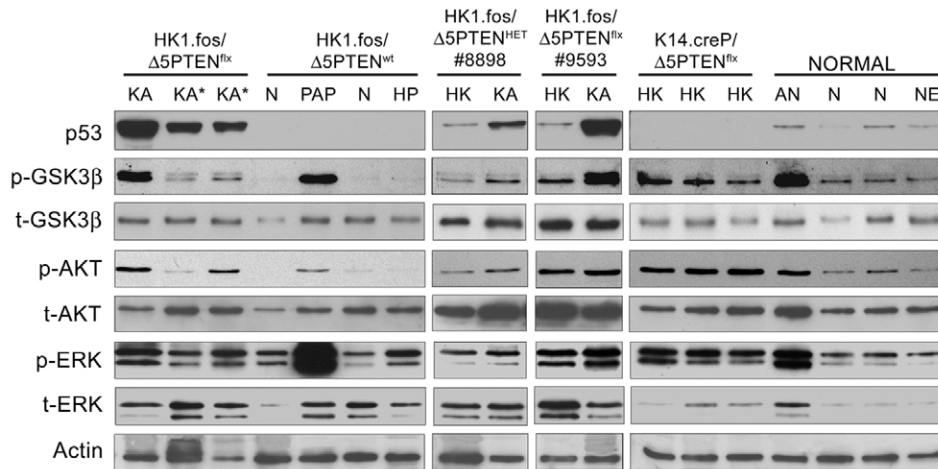
Fig. 4. Expression of p53, *P*-AKT, *P*-GSK3β and *P*-ERK1/2 in *HK1.fos/Δ5Pten* phenotypes. Skin biopsies of keratoacanthomas (KA), papillomas (PAP), hyperplastic (HP) or hyperkeratotic (HK) epidermis, together with normal dorsal (N), anagen (aN) or ear skin (NE) were subject to western analysis. All *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 and 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 versus homozygous KA 9593). However, KAs exhibited lower increases in *P*-AKT expression, which varied extensively with the degree of keratosis (Ka*). Compared with total (t-) protein levels, *HK1.fos* epidermis exhibited low *P*-AKT and *P*-GSK3β expression (first panel), whereas *P*-GSK3β expression but not that of *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*-ERK1 and *P*-ERK2, including 'normal' *HK1.fos* epidermis and *HK1.fos* papillomas in particular, which remained steady, if slightly reduced, in all KAs. β-Actin served as a loading control.

P-GSK3β expression following *P*-AKT downregulation (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 (supplementary material Fig. S3). 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 cyclin levels (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, owing to differing keratosis/papilloma ratios (Fig. 4, lanes: KA vs KA*); however, analysis of KAs with similar keratosis/ papilloma ratios consistently demonstrated high levels of inactivated *P*-GSK3β, concomitant with high p53, but not *P*-AKT, expression, which remained similar to that in *K14.cre/Δ5Pten^flx* epidermis (Fig. 5, KA versus 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 downregulated (Fig. 5, lanes HK 5, 6 and 9). The moderate *P*-GSK3β expression associated with low-level p53 expression in *HK1.fos/Δ5Pten^flx* epidermis, coupled with 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 burst of p53 and abrupt reduction in keratinocyte proliferation that prevented further progression required a high threshold level of GSK3β inactivation; this may have been achieved from the moderate AKT-independent *P*-GSK3β expression in *HK1.fos/ Δ5Pten^flx* observed in early preneoplastic hyperplasia (above), coupled with 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 cyclin E2 expression, together with MAP kinase signalling via analysis of ERK1/2 activation. Analysis of p21^WAF was doubly attractive, as 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 *HK1.fos/Δ5Pten^flx* hyperplasia where moderate levels of GSK3β inactivation induced a low level of p53 expression (Figs 4, 5), a high level of GSK3β inactivation was required to induce p21^WAF expression (Fig. 5, lanes HK and P versus KA) and below this threshold level of GSK3β phosphorylation, p21 was not expressed. Furthermore, p53-negative *HK1.fos* papillomas and *K14.cre/Δ5Pten^flx* phenotypes, which have lower GSK3β inactivation levels, were also 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 downregulation of AKT activity (Zhou et al., 2001). Moreover, this temporal p21^WAF expression indicated that the crucial changes in progression occurred at the overt benign tumour stage, a result consistent with the K13/BrdU labelling data and with previous roles for p21^WAF that are associated with inhibition of malignant conversion (Topley et al., 1999).

Analysis of cyclin D1, cyclin 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*-ERK1/2 expression (Fig. 4: HK lanes), which were retained at moderate levels in mature KAs, despite the p53/p21^{WAF} expression profile, owing 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} was able to halt excessive proliferation, unlike activated *c-ras^{Ha}* cooperation with PTEN loss, where p53 remained lost and strong cyclin D1 and cyclin E2 overexpression 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 in cyclin E2, and slightly elevated *P*-ERK1/2 expression, compared with total ERK1/2 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 cyclin E2 expression (Bamberger et al., 2001), together with very high levels of activated *P*-ERKs 1/2 expression (Fig. 4, lanes P, HP and N), which suggested that MAP kinase signalling during wound-promoted *HK1.fos* papillomatogenesis facilitated escape from AKT-linked countermeasures (above) and restored *P*-AKT activity (Segrelles et al., 2006). In *K14.cre/Δ5Pten^{flx}* epidermis, similar small elevations in both cyclin D1 and cyclin E2 (Di Cristofano et al., 2001; Weng et al., 2001) were recorded [which are associated with promotion from ear tagging (Fig. 5, lanes: HK^T vs HK)], alongside increased *P*-ERK1 and 2 levels (Fig. 4, end panel: HK lanes). These results are 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 downregulation 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 supplementary material Fig. S2). Analysis of *HK1.fos* and *K14.cre/ΔPten^{flx}* control phenotypes are given in supplementary material Fig. S3. 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; supplementary material Fig. S2). Conversely, papillomatous areas possessed little detectable p53 protein (Fig. 6C; supplementary material Fig. S2). 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; supplementary material Fig. S2). 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 (supplementary material Fig. S2), as all papillomatous KA histotypes always lacked detectable p21^{WAF} expression, even if low levels of p53 were detectable (Fig. 6F; supplementary material Fig. S2), and
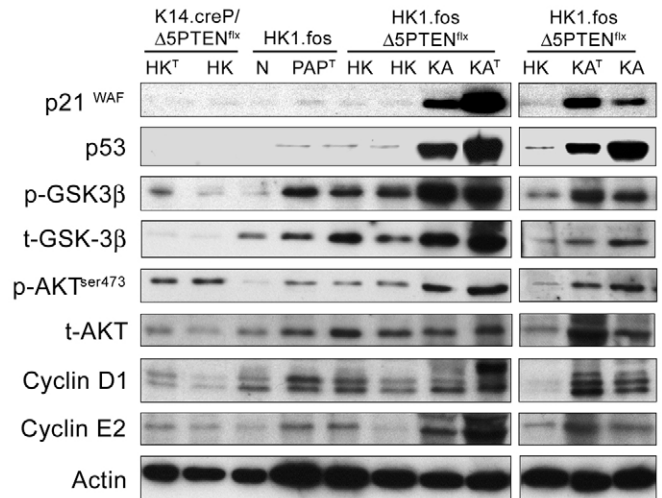

Fig. 5. *HK1.fos/Δ5Pten* KAs exhibit high p53 and novel p21^{WAF} expression associated with a threshold level of GSK3β inactivation. Western analysis of p53, total (t-) and phosphorylated (p-) GSK3β and AKT were compared with p21^{WAF}, cyclin D1 and cyclin E2 expression in pathology-matched KAs (similar 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, cyclin D1 and cyclin 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 expression, alongside slightly elevated cyclin D1. *HK1.fos* papillomas (PAP) expressed little p21^{WAF} and p53, but displayed increased *P*-GSK3β expression compared with *P*-AKT, together with elevated cyclins. *HK1.fos/Δ5Pten^{flx}* epidermis (HK) expressed barely detectable p21^{WAF}, limited p53 and moderate *P*-GSK3β expression, whereas *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 cyclin E2 expression, particularly in ear-tagged samples (KA^T). β-Actin served as a loading control.

p21^{WAF} was undetectable in hyperplastic *HK1.fos/ΔPten^{flx}* epidermis (not shown) or *HK1.fos* and *K14.cre/ΔPten^{flx}* control phenotypes (supplementary material Fig. S3). 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). The confused atypical nature of epidermal differentiation, however, may be 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; supplementary material Fig. S2), as *P*-GSK3β already appeared earlier in the suprabasal layers of papillomatous KA histotypes (Fig. 6I; supplementary material Fig. S2) and hyperplastic *HK1.fos/ΔPten^{flx}* epidermis (not shown). In HK1.fos/ΔPten^{flx} epidermis, moderate suprabasal *P*-GSK3β expression was associated with suprabasal p53 expression, prior to p53 loss in papillomatogenesis. This could also be observed in occasional papillomatous areas, suggesting the start of inhibition
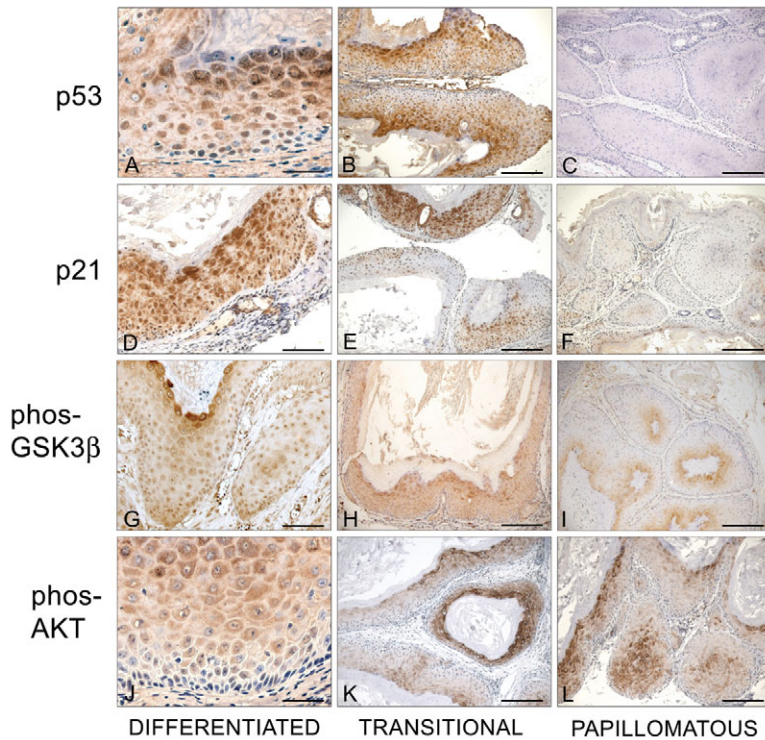
Fig. 6. Immunohistochemical analysis of p53, p21^WAF, *P*-GSK3β and *P*-AKT expression in differentiated and proliferative *HK1.fos/Δ5Pten* KA histotypes. (A-C) p53 expression in differentiated (A), transitional (B) and papillomatous (C) KA histotypes. High basal layer p53 expression (A) was increasing from suprabasal to basal (B), and was absent in papillomatous areas (C). (Composite micrographs and immunohistochemical analysis of control *HK1.fos* and *K14.cre/Δ5Pten^flx* phenotypes are shown in supplementary material Figs S2, S3.) (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). Scale bars: 25 μm in A,J; 50 μm in D,G; 100 μm in B,C,E,F,H,I,K,L.

of hyperproliferation; however, 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, which halts proliferation, and, later, p21^WAF, which increases 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; supplementary material Fig. S2). 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. In increasingly p53/p21^WAF-positive transitional areas, however, *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). In composite micrographs, a uniform increase in p21^WAF expression paralleled downregulation of *P*-AKT (supplementary material Fig. S2). Collectively, it is possible that p53-mediated reduced *P*-AKT expression in basal keratinocytes is instrumental to releasing p21^WAF activity (Zhou et al., 2001) and to 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, whereas *HK1.fos* papillomas/late-stage hyperplasia exhibited the low-level suprabasal *P*-GSK3β expression profile observed in HK1.fos/ΔPten^flx epidermis (supplementary material Fig. S3). RU486-treated *K14.cre/ΔPten^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 (supplementary material Fig. S3).

Discussion

HK1.fos/Δ5Pten^flx mice demonstrated direct cooperation 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) that is associated with novel basal layer expression of keratin K1, and with premature loricrin and filaggrin expression. This potential sentinel mechanism, which is deployed at the benign tumour stage and crucially dependent on normal p53 and p21^WAF functions, was able to block malignant progression by continually 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 *Δ5Pten^flx/c-ras^Ha* mice (Yao et al., 2006) or in *c-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; Greenhalgh et al., 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, although rapid, papillomatogenesis presented few surprises, as *HK1.fos-Δ5Pten^flx* apparently substituted

for *c-ras^Ha* activation observed in earlier KA studies (Corominas et al., 1989), exhibiting moderate elevation in MAP kinase signalling (Parsons, 2004; Downward, 2004; Karin, 1995) and overexpression 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 that accelerated terminal differentiation rather than apoptosis, given the premature expression of keratin K1, loricrin and filaggrin. The resulting disorder in keratinocyte differentiation, which was 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 indicated a sudden accelerated commitment to differentiation (Rothnagel et al., 1993) and 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 roles for Fos 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 fuel 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 *c-ras^Ha* synergism with PTEN in KA versus previous SCC aetiology (Yao et al., 2006), and the relative lack of typical initiating *c-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, this idea, again, awaits analysis of whether additional/appropriate mutations of *c-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.fos/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, p53 expression was lost in control *K14.cre/Δ5Pten^flx*; 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 human photo-carcinogenesis, as p53 mutations frequently initiate keratinocytes (Brash, 2006), but tumour aetiology requires additional events over time, including UV-induced 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 as being 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, permanent loss of p53 in *ΔPten/c-ras^Ha* 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 with 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 may be 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, the roles of p21^WAF in differentiation, separate to that of cell cycle control (Devgan et al., 2006), may be 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, as *p21^WAF* has both positive

and negative roles in keratinocyte differentiation, and actually inhibits the latter stages, when p21^{WAF} is normally downregulated (Devgan et al., 2006). Thus, 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 microcysts, a problem further compounded by an increasing lack of *P*-AKT (Calautti et al., 2005), which would add to the failure to downregulate 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 and 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* cooperation, p21^{WAF} induction inhibited c-*ras^{Ha}*-activated skin carcinogenesis in *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), whereas 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 crucial 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 by 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 downregulated 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 downregulation helped maintain the differentiation/proliferation balance, resulting in an overtly normal histology. Given recent glioma studies where PTEN^{wt} inhibits AP1 activity via reduced AKT signalling (Koul et al., 2007) and closes the 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*-ERK1/2 expression (Karin, 1995; Schlingemann et al., 2003), increase cyclin D1 and cyclin 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 produce 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 owing 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 activity 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). At the same time, it maintains 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 and cyclin 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 fed back to reduce *P*-AKT activity in basal layers (Lei et al., 2006), inducing increasingly suprabasal *P*-AKT expression that, in turn, facilitated basal layer expression of p21^{WAF} (Zhou et al., 2001) and accelerated differentiation. This reduction in proliferative basal layer *P*-AKT expression appeared crucial to inhibition of benign tumour progression, i.e. unless significant p53/p21^{WAF} co-expression induced a basal-to-suprabasal *P*-AKT expression switch to prevent sustained basal layer *P*-AKT activities, hyperproliferative benign tumour keratinocytes would be at risk for conversion. This is demonstrated by the ability of constitutively active AKT to indict the malignant transformation of DMBA-initiated papilloma keratinocytes (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/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 GSK3β cooperates with PTEN phosphatase loss in prostate carcinogenesis (Mulholland et al., 2006), and high *P*-GSK3β inactivation levels are observed in DMBA/TPA carcinomas (Leis et al., 2002) expressing elevated levels of activated *P*-AKT (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, whereas *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–cyclin 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 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 of 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, and actually required/exploited *HK1.fos/P-AKT* synergism in papillomatogenesis to increase *P*-GSK3β levels, which 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 interpreted 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 tumour 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 that regulate 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.

Materials and Methods
Genotypes, transgene expression and RU486 treatment
HK1.fos (Greenhalgh et al., 1993b), RU486-inducible *K14.creP* regulator (Berton et al., 2000) and *Δ5Pten*[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 (*Δ5Pten*[wt/flx]) or homozygous (*Δ5Pten*[flx]) *Pten* backgrounds. *Pten* exon 5 ablation was achieved via activation of *cre* recombinase in dorsal skin treated topically with 2 μg RU486 in 50 μl ethanol/week (mefipristone, Sigma) for 4 weeks, with controls receiving ethanol alone (UK License: 60/2929 to D.A.G.). *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-ras*[Ha] mutations, tumour DNA was isolated as described (Yao et al., 2006), amplified with intron-specific oligonucleotides and sequenced.

Histology, immunofluorescence and bromodeoxyuridine labelling analysis
Skin and tumour biopsies were fixed (10% formalin, 4°C) and stained with Haematoxylin and Eosin, or frozen in OCT (Miles) and stored at –70°C. For immunofluorescence, frozen sections (5-7 μm) 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 (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 intraperitoneally with 125 mg/kg 5-bromo-4-deoxyuridine (Sigma) 2 hours prior to biopsy. Paraffin sections were subjected to antigen retrieval (10 minute boil/10 mM sodium citrate) and BrdU labelling performed by overnight incubation at 4°C with FITC-conjugated anti-BrdU 1:50 (Becton Dickinson), counterstained for K14 (above). For immunohistochemical analysis, sections were incubated with phospho-GSK3β[(ser9)] 9936 and phospho-AKT[(273)] 9271 (Cell Signaling Technology), p53 (PAB 240) (CRUK Antibodies), and p21[WAF] (sc397, Santa Cruz) overnight (1:100/biotin-anti-goat 1:50) (Santa Cruz), and visualized via HRP-conjugated strepavidin, incubated for 5 minutes at room temp.

Western analysis
Proteins were extracted from biopsy tissue as described previously (Yao et al., 2006). Proteins were subjected to western analysis using 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 GSK3β[(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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