

# 14-3-3 $\sigma$ /Stratifin and p21 limit AKT-related malignant progression in skin carcinogenesis following MDM2-associated p53 loss

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## Abstract

To study mechanisms driving/inhibiting skin carcinogenesis, stage-specific expression of 14-3-3 $\sigma$  (Stratifin) was analyzed in skin carcinogenesis driven by activated *ras*<sup>Ha</sup>/*fos* expression (*HK1.ras/fos*) and ablation of *PTEN*-mediated AKT regulation (*K14.creP/Δ5PTEN*<sup>flx/flx</sup>). Consistent with 14-3-3 $\sigma$  roles in epidermal differentiation, *HK1.ras* hyperplasia and papillomas displayed elevated 14-3-3 $\sigma$  expression in supra-basal keratinocytes, paralleled by supra-basal p-MDM2<sup>166</sup> activation and sporadic p-AKT<sup>473</sup> expression. In bi-genic *HK1.fos/Δ5PTEN*<sup>flx/flx</sup> hyperplasia, basal-layer 14-3-3 $\sigma$  expression appeared, and alongside p53/p21, was associated with keratinocyte differentiation and keratoacanthoma etiology. Tri-genic *HK1.ras/fos-Δ5PTEN*<sup>flx/flx</sup> hyperplasia/papillomas initially displayed increased basal-layer 14-3-3 $\sigma$ , suggesting attempts to maintain supra-basal p-MDM2<sup>166</sup> and protect basal-layer p53. However, *HK1.ras/fos-Δ5PTEN*<sup>flx/flx</sup> papillomas exhibited increasing basal-layer p-MDM2<sup>166</sup> activation that reduced p53, which coincided with malignant conversion. Despite p53 loss, 14-3-3 $\sigma$  expression persisted in well-differentiated squamous cell carcinomas (wdSCCs) and alongside elevated p21, limited malignant progression via inhibiting p-AKT<sup>473</sup> expression; until 14-3-3 $\sigma$ /p21 loss facilitated progression to aggressive SCC exhibiting uniform p-AKT<sup>473</sup>. Analysis of TPA-promoted *HK1.ras-Δ5PTEN*<sup>flx/flx</sup> mouse skin, demonstrated early loss of 14-3-3 $\sigma$ /p53/p21 in hyperplasia and papillomas, with increased p-MDM2<sup>166</sup>/p-AKT<sup>473</sup> that resulted in rapid malignant conversion and progression to poorly differentiated SCC. In 2D/3D cultures, membranous 14-3-3 $\sigma$  expression observed in normal HaCaT and SP1<sup>ras61</sup> papilloma keratinocytes was unexpectedly detected in malignant T52<sup>ras61/v-fos</sup> SCC cells cultured in monolayers, but not invasive 3D-cells. Collectively, these data suggest 14-3-3 $\sigma$ /Stratifin exerts suppressive roles in papillomatogenesis via MDM2/p53-dependent

**Abbreviations:**  $\Delta 5PTEN$ <sup>flx/flx</sup>, transgenic mice homozygous for lox-P flanked exon 5 *PTEN*; *HK1.fos*, transgenic mice expressing *FBJ/R v-fos* from a truncated human keratin K1 promoter; *HK1.fos-Δ5PTEN*<sup>flx/flx</sup>, bi-genic progeny of mating *HK1.fos* and *K14.creP-Δ5PTEN* mice; *HK1.ras*, transgenic mice expressing *v-ras*<sup>Ha</sup> exclusively in the epidermis employing a truncated human keratin K1 promoter; *HK1.ras/fos-Δ5PTEN*<sup>flx/flx</sup>, tri-genic progeny of mating *HK1.ras*, *HK1.fos* and *K14.creP-Δ5PTEN* mice; *K14.creP*, transgenic mice expressing RU486 responsive cre recombinase from a keratin K14 promoter; *K14.cre-Δ5PTEN*<sup>flx/flx</sup>, bi-genic progeny of mating *K14.creP* and  $\Delta 5PTEN$  mice; KA, keratoacanthoma; SCC, squamous cell carcinoma.

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mechanisms; while persistent p53-independent expression in early wdSCC may involve p21-mediated AKT1 inhibition to limit malignant progression.

#### KEYWORDS

fos, keratoacanthoma, organotypic culture, PTEN, ras, skin carcinogenesis, TPA promotion, transgenic mouse

## 1 | INTRODUCTION

The 14-3-3 family of phospho-proteins modulate an extensive and wide variety of pathways ranging from metabolism and autophagy; through cell cycle regulation, proliferation, and apoptosis; to cell motility, spatial awareness, and differentiation.<sup>1–5</sup> In carcinogenesis, deregulated 14-3-3 $\sigma$  is the most common isoform,<sup>6–8</sup> however, given the plethora of pathways and their complex interactions,<sup>1–5</sup> 14-3-3 $\sigma$  causality remains elusive; exhibiting both tumor suppressive and oncogenic roles dependent upon context. Loss of tumor suppressive functions, via promoter hypermethylation rather than mutation/deletion,<sup>6,9</sup> appear in diverse tumors including receptor-positive breast cancers,<sup>8</sup> liver,<sup>9</sup> small cell and neuroendocrine lung cancers<sup>10</sup> and correlate with poor prognosis. In contrast, promoter hypo-methylation giving 14-3-3 $\sigma$  overexpression appears in lung adenocarcinoma,<sup>11</sup> nonsmall cell lung cancer,<sup>12</sup> and colorectal cancers<sup>13</sup>; with overexpression of the secreted form appearing in liver cancer.<sup>14</sup> 14-3-3 $\sigma$  positivity was more common in triple-negative versus receptor-positive breast cancers and despite acting as an inhibitory MDM2 chaperone,<sup>3,4</sup> resultant p53 increase conferred poorer prognosis.<sup>8</sup> Gastrointestinal cancers further highlight contrasting p53 interactions, as some studies show independent 14-3-3 $\sigma$ /p53 expression,<sup>15</sup> others showed strong correlation with p53 mutations; for example, in colonic tumors; yet survival rates were associated with 14-3-3 $\sigma$  loss in tumors wild type for p53.<sup>16</sup>

In epidermis, 14-3-3 $\sigma$  is associated with multiple roles in differentiation, spatial awareness/cellular orientation, and motility of both interfollicular and follicular keratinocytes.<sup>5,17–19</sup> In human skin carcinogenesis, while 14-3-3 $\sigma$  was implicated in basal cell carcinoma via classic TSG loss, this study found increased 14-3-3 $\sigma$  expression in squamous cell carcinoma (SCC).<sup>20</sup> This suggests that initial, increased 14-3-3 $\sigma$  protected p53 expression<sup>3,4,21</sup> via MDM2 chaperone/degradation, thus maintaining nuclear p53 levels.<sup>3,4,22</sup> However, if p53 was mutated by UV-B, this mechanism would be counter-productive, resulting in an oncogenic loop of p53 loss-/gain-of-function mutant expression<sup>21</sup> again reflecting contrasting roles in specific tumor contexts.<sup>9–16</sup> Mouse models highlight tumor suppressive roles, as the *Er/Er<sup>+/-</sup>* repeated epilation stain possesses germline 14-3-3 $\sigma$  mutations that result in epidermal hyperplasia, failed follicular differentiation, and susceptibility to SCC.<sup>23,24</sup> Conditional 14-3-3 $\sigma$  knockout co-operated with ErbB2 in mammary carcinogenesis<sup>25</sup>; while in DMBA/TPA carcinogenesis, 14-3-3 $\sigma$  loss resulted in rapid papilloma formation, supporting a promoting role.<sup>26</sup>

To further investigate inhibitory/oncogenic functions, 14-3-3 $\sigma$  expression was investigated in skin carcinogenesis driven by *ras<sup>Ha</sup>/fos*

activation and conditional loss of *PTEN*-mediated AKT inhibition.<sup>27–29</sup> *HK1.ras/fos- $\Delta$ 5PTEN<sup>flx/flx</sup>* mice display papillomatogenesis associated with compensatory p53/p21 that delayed/inhibited malignant conversion or produced benign keratoacanthomas (KAs) (*HK1.fos- $\Delta$ 5PTEN<sup>flx/flx</sup>*).<sup>27–29</sup> Following p53 loss and malignant conversion, *HK1.ras/fos- $\Delta$ 5PTEN<sup>flx/flx</sup>* tumors retained p21 expression, which limited progression to well-differentiated SCC (wdSCC); until p21 loss<sup>29</sup> gave progression to poorly differentiated SCC (pdSCC). This provided an opportunity to investigate stage-specific 14-3-3 $\sigma$  interactions with MDM2 and p53 that inhibit/drive papilloma conversion; while loss of *PTEN*-mediated AKT inhibition investigated links between p21 and 14-3-3 $\sigma$  that may limit malignant progression via AKT inhibition.<sup>30–32</sup>

Key findings demonstrated 14-3-3 $\sigma$  expression increase in hyperplastic and papilloma basal-layers was paralleled by increased p53/p21 expression. Subsequently, reduced 14-3-3 $\sigma$  in papilloma basal-layers was associated with supra-basal-to-basal increases in p-MDM2<sup>166</sup> activation; with corresponding reduction in p53, which contributed to malignant conversion. Further, following p53 loss, persistent 14-3-3 $\sigma$  expression observed in wdSCCs, alongside elevated p21 may limit early-stage malignant progression via inhibition of p-AKT1<sup>473</sup> activation, as 14-3-3 $\sigma$ /p21 loss led to increased p-AKT1<sup>473</sup> and progression to aggressive SCC. Although an oncogenic role for persistent 14-3-3 $\sigma$  cannot be excluded, as also suggested by persistent 14-3-3 $\sigma$  expression in 3D cultures, TPA promotion of *HK1.ras- $\Delta$ 5PTEN<sup>flx</sup>* mice induced rapid transit to pdSCC, associated with early 14-3-3 $\sigma$ /p53/p21 loss and immediate, uniform p-AKT1<sup>473</sup> expression, which collectively support tumor suppressive roles for 14-3-3 $\sigma$  in this model.

## 2 | METHODS

### 2.1 | Transgenic genotypes and induction of tumors

Adult transgenic mice (5–16 weeks old) expressing activated *ras<sup>Ha</sup>* and/or *v-fos* from a human keratin K1-based vector, modified to express in basal- and supra-basal keratinocytes (*HK1.ras/fos*) have been described previously.<sup>33</sup> To explore malignant conversion, *HK1.ras/fos* mice were crossed to mice expressing a keratin K14-driven, RU486-inducible cre fusion protein (*K14.creP<sup>34</sup>*) and mice expressing a *lox-P*-flanked-*PTEN* exon 5 allele ( *$\Delta$ 5PTEN<sup>flx/flx</sup>*).<sup>27–29</sup> *HK1.ras/fos- $\Delta$ 5PTEN<sup>flx/flx</sup>* progeny were genotyped by PCR (see

Supporting Information: Table 1). PTEN function was inactivated (15 per cohort in repeat experiments<sup>27–29</sup>) following 3 topical treatments with 15  $\mu\text{L}$ , 2  $\mu\text{g}/\text{mL}$  RU486 (mifepristone; Sigma) in ethanol; controls (5 per cohort) received ethanol alone; giving an archive of up to 60 RU-486 treated ear tumor and 30 dorsal skin biopsy samples per cohort. Tumors were raised in adult RU486-treated, bi-genic *HK1.ras*- $\Delta 5\text{PTEN}^{\text{flx/flx}}$  mice ( $n = 10$ ) via 2 weekly treatments with 2.5  $\mu\text{g}/50 \mu\text{L}$  acetone TPA (50  $\mu\text{L}$  of  $1.6 \times 10^{-4}$  M TPA; Sigma) with controls ( $n = 5$ ) receiving RU486 and ethanol alone.

As observed previously, individuals from each transgenic cohort produced similar, if not identical phenotypes; for instance, all *HK1.ras* mice exhibit papillomas by 8–10 weeks<sup>27,33</sup> and while *HK1.fos* provides a promotion stimulus,<sup>28</sup> all bi-genic *HK1.ras/fos* mice lack spontaneous malignant conversion.<sup>33</sup> Similarly, bi-genic *HK1.ras/\Delta 5\text{PTEN}^{\text{flx/flx}}* papillomas required TPA promotion to achieve malignancy<sup>27</sup>; while bi-genic *HK1.fos-\Delta 5\text{PTEN}^{\text{flx/flx}}* progeny developed benign KAs, not wdSCC, associated with compensatory p53/p21 expression.<sup>28</sup> Additional  $\text{ras}^{\text{Ha}}$  expression in tri-genic *HK1.ras/fos-\Delta 5\text{PTEN}^{\text{flx/flx}}* progeny led to malignant conversion associated with spontaneous p53 loss, whereas p21 retention limited progression to wdSCC<sup>29</sup> (see Supporting Information: Figures S2 and S7). All experiments adhered to UK Experimental Regulations governing animal experimentation (Licence: P82170325 to DAG). In addition to this licence, Animal Care and Use was approved by Glasgow University Advisory Committee on Genetic Manipulation: Animal Use Committee (GM Center number: 318).

## 2.2 | Histology, immunofluorescence, and immunochemical analysis

Skin biopsies and organotypic rafts were fixed in buffered formalin (24 h at 4°C), embedded in paraffin and stained with haematoxylin and eosin. Differentiation status was assessed via double-label immunofluorescence. A minimum of 15, randomly selected ear tumor/dorsal skin biopsies were analyzed per genotype; and wherever possible, for direct comparison, the data shown in each figure employed serial sections from previously published archival tumor blocks.<sup>27–29</sup> Following antigen retrieval (boil 5 min/10 mM sodium citrate), sections were incubated overnight (4°C) with: rabbit anti-mK1 (BioLegend Cat#905601, RRID: AB2565051; 1:100) employing guinea-pig anti-K14 to delineate epidermis/tumor (Progen Cat#GP-CK14, RRID: AB2920669; 1:200). Expression was visualized with FITC-labeled anti-rabbit IgG (Jackson Labs Cat#711-096-152, RRID: AB2340597; 1:100) or biotinylated-goat anti-guinea pig (Vector Laboratories Cat#BA-7000, RRID: AB2336132; 1:100) and Streptavidin-Texas Red (Vector Laboratories Cat#SA-5006, RRID: AB2336754; 1:400) at room temperature for 60 min. 14-3-3 $\sigma$ /Stratifin analysis employed rabbit anti-14-3-3 $\sigma$ /Stratifin (Thermo-Fisher Cat#PA5-23507, RRID: AB2544623; 1:100) and p-AKT1<sup>s473</sup> activation employed rabbit anti-p-AKT1<sup>s473</sup> (Abcam Cat# ab81283, RRID: AB2224551; 1:100).

For immunohistochemical analysis, following antigen retrieval, sections were incubated overnight (4°C) with rabbit anti-14-3-3 $\sigma$ /Stratifin (1:100); and rabbit anti p-MDM2<sup>166</sup> (Abcam anti-MDM2 phospho-S166; Cat#ab131355, RRID: AB11157309; 1:400). p53 analysis employed Abcam Cat# ab31333, RRID: AB\_776980 (diluted 1:50) and p21 employed Proteintech Cat# #10355-1-AP, RRID: AB\_2077682 (diluted 1:200) which replaced Santa Cruz p53 (sc#393) and p21 (Cat# sc-397) (see Supporting Information: Figure S1). For analysis of 2D chamber cultures, cells were fixed in 10% formalin, washed and incubated overnight (4°C) with rabbit anti-14-3-3 $\sigma$ /Stratifin (1:100). Expression visualized via HRP-conjugated goat anti-rabbit antibodies (Vector Laboratories Cat#PAB21463HRP-1000, RRID: AB2916034; 1:100) at room temp for 60 min followed by DAB+ staining (Dako; Amersham Biosciences).

Photomicrographs employed Axiovision image software (Axiovision Imaging System, RRID: SCR002677; Zeiss Microscopes). Image J was employed to quantitate expression data via double-blind analysis. Typically, 20 areas were measured per inverted, 8-bit tif image (see Supporting Information: Figure S4) and optical density (OD; corrected minus background) results averaged per image. Up to 12 separate tumor images representative of each treated cohort were analyzed (see Supporting Information: Figures S3–S5) and significance determined via two-way ANOVA analysis.

## 2.3 | Cell culture and organotypic tumor invasion modeling

Immortalized human HaCaT keratinocytes (36; RRID: CVCL0038); were provided at passage 35 and used at passages 45–53. DMBA-initiated/TPA promoted,  $\text{ras}^{\text{Ha}}$ -transformed SP1 papilloma cells (RRID: CVCL5785; passage 47–53) and  $\text{ras}^{\text{Ha}}$ /fos transformed T52 carcinoma cells (37; DAG originated; passage 30–36) were cultured in DMEM (w/o  $\text{Ca}^{2+}$ ), supplemented with 10% (chelated) fetal calf serum (FCS) and 0.05 mM calcium (Low  $\text{Ca}^{2+}$ ).<sup>35</sup> C8161 melanoma cells (RRID: CVCL6813; passage number unknown) were cultured in DMEM/10% FCS.

For IHC or IF analysis, cells were plated at 1000 cells per chamber (Millicell EZ SLIDE; Millipore) in Low  $\text{Ca}^{2+}$  media, and 2 days later induced to differentiated by culture in 0.12 mM  $\text{Ca}^{2+}$  media for 48 h<sup>35</sup> and fixed in 10% formalin (Sigma).

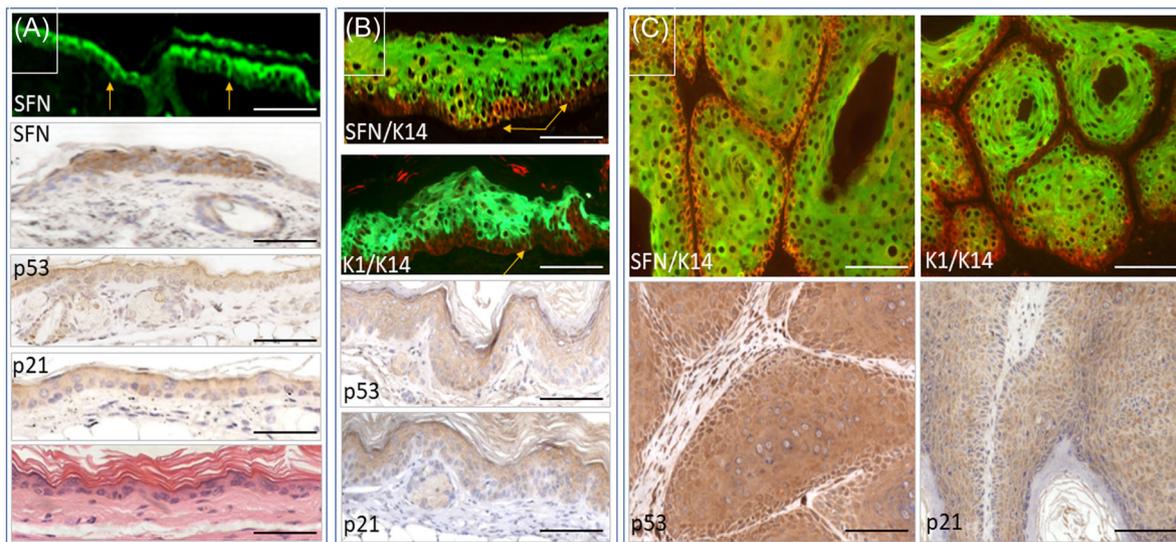
Organotypic invasion models<sup>36</sup> were prepared by extracting collagen from rat tail tendons with 0.5 M acetic acid. Typically, 3 mL 10X DMEM/30% FCS was added to 25 mL rat tail collagen (2 mg/mL; 4°C), neutralized with 0.22 M sodium hydroxide (pH 7.2) and dermal fibroblasts ( $7 \times 10^4$  cells/mL) seeded in 2.5 mL neutralized collagen and plated into 35 mm dishes. Collagen rafts formed within 7 days (~1.5 cm diameter) and were placed into 24-well dishes containing 1 mL media and seeded with  $10^4$  cells; employing C8161 melanoma cells (RRID: CVCL6813) as a positive control. Each cell-matrix was cultured for 3 days, transferred to 60 mm dishes containing a submerged grid and 3 days later, cells were raised to the air liquid interface for a further 8–10 days.<sup>36</sup>

### 3 | RESULTS

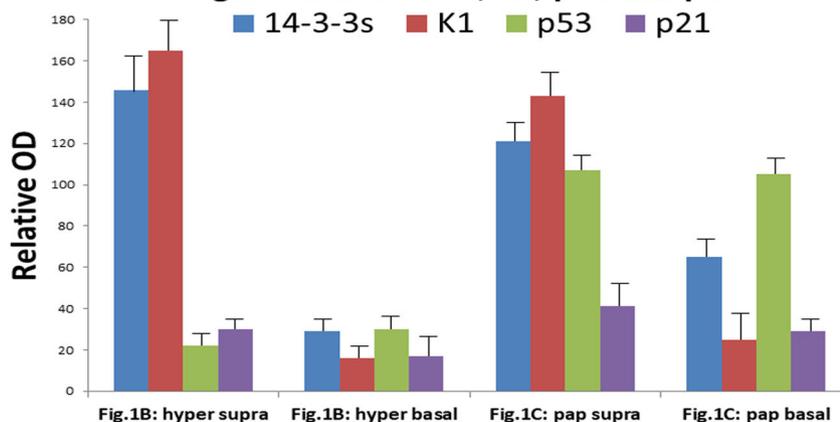
#### 3.1 | 14-3-3 $\sigma$ expression in differentiating supra-basal keratinocytes becomes elevated in proliferative, basal-layer papillomas

To investigate normal function and roles in papillomatogenesis, 14-3-3 $\sigma$  expression was compared to p53 and p21 status in normal skin and *HK1.ras* hyperplasia/papillomas (Figure 1A–C). In normal epidermis 14-3-3 $\sigma$  was expressed in supra-basal layers,

with occasional expression in proliferative, basal-layer keratinocytes (Figure 1A), paralleled by supra-basal keratin K1 expression, an early marker of differentiation, also expressed in occasional basal-layer keratinocytes on commitment to differentiate (Figure 1A); while p53/p21 were expressed in sporadic cycling keratinocytes (see also Supporting Information: Figure S7). These 14-3-3 $\sigma$ /K1 co-expression data were consistent with roles in epidermal differentiation, while sporadic basal-layer expression suggests 14-3-3 $\sigma$  also regulates keratinocyte commitment to differentiate.



**Fig.1 B & C: 14-3-3s, K1, p53 and p21**



**FIGURE 1** 14-3-3 $\sigma$ , p53 and p21 expression in normal and premalignant phenotypes. (A) Single-label IF/IHC analysis of normal epidermis shows supra-basal 14-3-3 $\sigma$  expression (SFN) with sporadic, expression in (narrow) basal-layer keratinocytes (arrows) committing to differentiate; while cycling basal-layer keratinocytes show low-level p53/p21. Bottom panel: normal histology. (B) IF analysis of *HK1.ras* hyperplasia displays supra-basal 14-3-3 $\sigma$  expression (SFN green), with occasional positive (yellow) differentiating basal-layer keratinocytes (arrows); Keratin K14 (red) indicates basal layer. Keratin K1 (green) and K14 (red) show similar differentiation pattern of supra-basal K1 and basal K14; with narrow, basal-layer keratinocytes committing to differentiate. IHC analysis shows sporadic, low-level p53/p21 expression. (C) *HK1.ras* papillomas show supra-basal 14-3-3 $\sigma$ , with increasing expression in basal-layer keratinocytes (orange); while supra-basal K1 indicates benign tumor. IHC analysis now shows elevated/nuclear p53 in basal layer keratinocytes. Image J quantitation of *HK1.ras* hyperplasia confirms high 14-3-3 $\sigma$ /K1 expression in supra-basal layers; with low expression in basal keratinocytes; alongside low p53/p21. *HK1.ras* papillomas show a similar supra-basal 14-3-3 $\sigma$ /K1 profile but now exhibit increased 14-3-3 $\sigma$  in basal layers paralleled by a fourfold increase in p53 and weaker twofold p21 expression. Bars (A) approximately 25–30  $\mu$ m; (B) approximately 65  $\mu$ m; (C) SFN/K14 & K1/K14 approximately 50  $\mu$ m; p53 approximately 75  $\mu$ m; p21 approximately 100  $\mu$ m.

In *HK1.ras* hyperplasia, a similar supra-basal 14-3-3 $\sigma$  expression profile was observed, alongside occasional positive basal-layer cells as keratinocytes committed to differentiate (paralleled by K1 expression; see Supporting Information: Figure S7); with sporadic p53/p21 expression in cycling cells (Figure 1B). *HK1.ras* papillomas (Figure 1C) maintained this ordered differentiation profile, with supra-basal 14-3-3 $\sigma$ /K1 expression, but now displayed a significant, almost threefold increase ( $p < 0.001$ ) in basal-layer 14-3-3 $\sigma$  expression (OD:  $61 \pm 9.4$ ), compared to hyperplasia (OD:  $29 \pm 6.4$ ); hence, an orange appearance to 14-3-3 $\sigma$ /K14 IF versus red of K1/K14 basal layers (Figure 1C). Papilloma keratinocytes now expressed uniform, nuclear p53 in both proliferative basal (OD:  $107 \pm 9.4$ ) and supra-basal keratinocytes (OD:  $105 \pm 10.4$ ) which was significantly increased (fourfold;  $p < 0.0001$ ) over earlier hyperplasia (Figure 1B) with low/cytoplasmic p53 (supra-basal OD:  $22 \pm 6.4$ ; basal OD:  $30 \pm 5.4$ ). However, there was little increase in p21 (Figure 1B: basal OD:  $17 \pm 4.4$  vs. Figure 1C: basal OD:  $29 \pm 7.4$ ). This 14-3-3 $\sigma$ -associated p53 expression increase may reflect a lack of spontaneous *HK1.ras* papilloma conversion, consistent with activated p-MDM2<sup>166</sup> being confined to supra-basal layers, alongside low p-AKT1<sup>473</sup> (below; Figures 5 and 6).

### 3.2 | *HK1.fos-Δ5PTEN*<sup>flx/flx</sup> KAs exhibit basal-layer 14-3-3 $\sigma$ associated with elevated p53/p21 and accelerated, anomalous differentiation

Previously, *HK1.fos* cooperation with inactivation of PTEN-mediated AKT regulation (*K14.creP-Δ5PTEN*<sup>flx/flx</sup>) produced keratotic hyperplasia in *HK1.fos-Δ5PTEN*<sup>flx/flx</sup> that evolved into KA (Supporting Information: Figure S2E), as compensatory p53/p21 expression halted proliferation and accelerated differentiation.<sup>28</sup> Analysis of 14-3-3 $\sigma$  in *HK1.fos-Δ5PTEN*<sup>flx/flx</sup> hyperplasia (Figure 2A,B) showed basal-layer keratinocytes exhibited strong, membranous/cytoplasmic expression, accompanied by elevated supra-basal (cytoplasmic) p53 (but not p21, which appeared later) (Figure 2A).<sup>28</sup> Image quantitation of *HK1.fos-Δ5PTEN*<sup>flx/flx</sup> hyperplasia confirmed significant increases in basal-layer 14-3-3 $\sigma$  expression (IHC:  $77 \pm 11$ ; IF:  $73 \pm 12$ ) compared to *HK1.ras* hyperplasia (Figure 1B IF:  $30 \pm 6$ ;  $p < 0.0006$ ). This was reflected by increased supra-basal p53 (Figure 2B IHC supra-basal:  $45 \pm 6.5$ ; basal:  $28 \pm 9.5$ ) versus *HK1.ras* hyperplasia (Figure 1B IHC supra-basal:  $22 \pm 5.4$ ; basal:  $30 \pm 5.4$ ) but not basal-layer p53, consistent with the previously observed requirement for a threshold level of AKT-mediated, p-GSK3 $\beta$  inactivation being necessary to trigger compensatory p53 (and p21) expression.<sup>28</sup>

This suggested basal-layer 14-3-3 $\sigma$  increases were separate to p53, possibly concerned with spatial awareness. Indeed, in the context of *HK1.fos-Δ5PTEN*<sup>flx/flx</sup> hyperplasia, such elevated basal-layer 14-3-3 $\sigma$  was not accompanied by increased keratin K1 (Figure 2B K1 basal layer:  $17 \pm 7.4$ ; see Supporting Information: Figure S7C), which remained similar to *HK1.ras* hyperplasia (Figure 1B K1 basal:  $16 \pm 4.7$ ), suggesting either roles linked to spatial awareness rather than differentiation,<sup>5</sup> or as a direct consequence of activated v-fos expression corrupting endogenous c-fos roles in differentiation.<sup>28</sup>

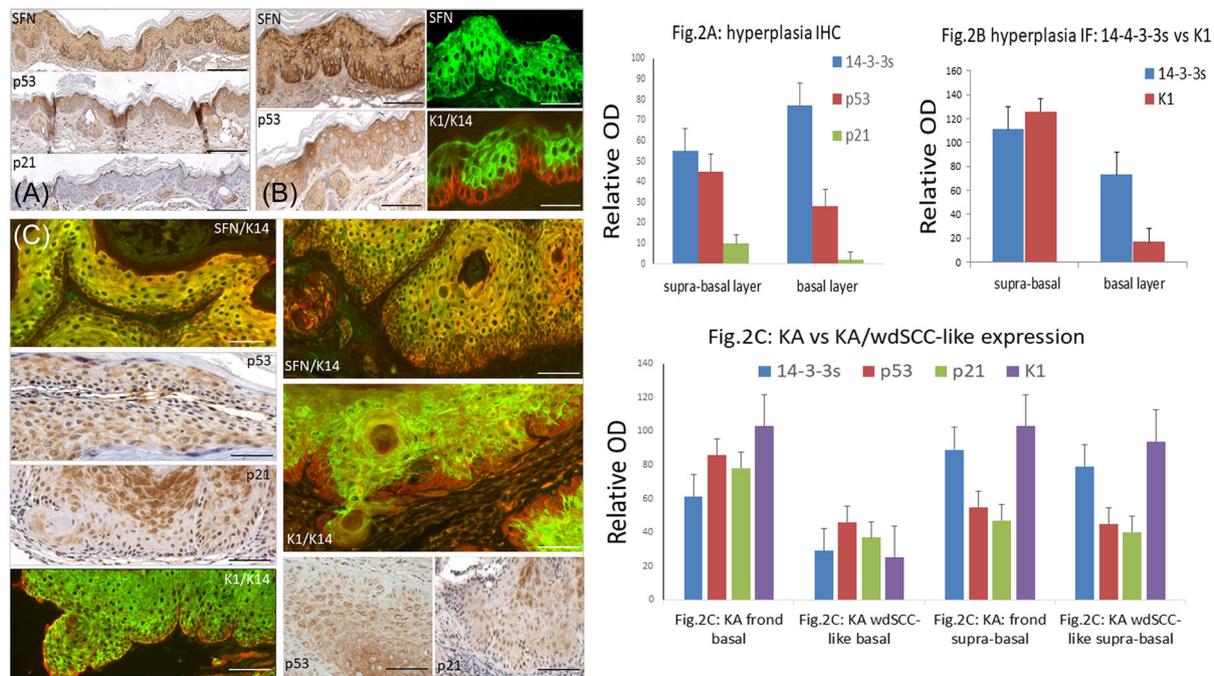
On progression to KA, two distinct *HK1.fos-Δ5PTEN*<sup>flx/flx</sup> histotypes emerged<sup>28</sup>: a massive keratosis, interspersed with fronds of keratinocytes; and an initial proliferative pathology often confused with wdSCC (Supporting Information: Figure S2E). Analysis of frond-like histotypes (Figure 2C; see Supporting Information: Figure S3), found that elevated 14-3-3 $\sigma$  in both supra-basal (OD:  $89 \pm 13.5$ ) and basal-layer keratinocytes (OD:  $61 \pm 11$ ) was paralleled by strong p53 expression in frond-like basal ( $86 \pm 11$ ) and supra-basal layers ( $55 \pm 9.4$ ). Furthermore, together with elevated p21 in basal-layer ( $78 \pm 13.5$ ) and supra-basal layers ( $47 \pm 9.5$ ), overt KA expressed anomalous keratin K1 in basal-layers (Figure 2C KA frond-like basal:  $103 \pm 19.5$ ; KA frond-like supra-basal:  $104 \pm 18.5$ ), consistent with accelerated, anomalous differentiation and the resultant keratosis observed previously.<sup>28</sup>

In contrast, proliferative basal-layer keratinocytes of wdSCC-like histotypes exhibited low 14-3-3 $\sigma$  expression (Figure 2C: wdSCC-like basal OD:  $29 \pm 8$ ) compared to frond-like areas ( $p < 0.003$ ); yet with similar supra-basal expression (OD:  $79 \pm 21.9$ ). This was paralleled by lower p53 expression (wdSCC-like basal:  $46 \pm 9.9$ ; wdSCC-like supra-basal:  $45 \pm 7.9$ ) and p21 (wdSCC-like basal:  $37 \pm 21.9$ ; wdSCC-like supra-basal:  $40 \pm 9.9$ ); yet here a relatively normal K1 expression profile indicated a benign KA histotype (wdSCC-like basal layer:  $25 \pm 21.9$ ; wdSCC-like supra-basal layer:  $94 \pm 20.9$ ). Hence, increased p53/p21 now combined with 14-3-3 $\sigma$  differentiation functions and cell cycle inhibition<sup>4-6</sup> to evoke a switch from proliferation to (accelerated) differentiation,<sup>28</sup> highlighted by anomalous/premature basal layer keratin K1 expression (Figure 2C: lower end panel) and AKT inhibition (see below) thus avoiding progression to malignancy.<sup>28</sup>

### 3.3 | Early *HK1.ras/fos-Δ5PTEN*<sup>flx/flx</sup> carcinomas show persistent 14-3-3 $\sigma$ following p53 loss which may limit progression in collaboration with p21

In *HK1.ras/fos-Δ5PTEN*<sup>flx/flx</sup> mice, co-operation between *HK1.ras/fos* and loss of PTEN-mediated AKT regulation resulted in rapid papillomatogenesis, however malignant conversion required p53 loss and progression stalled at a wdSCC histotype due to persistent p21 (30; Supporting Information: Figure S2C). Analysis of 14-3-3 $\sigma$  in *HK1.ras/fos-Δ5PTEN*<sup>flx/flx</sup> papillomas (Figure 3A,B IHC; Supporting Information: Figure S7D) showed high levels in proliferative basal-layers (OD Figure 3A:  $67 \pm 9.7$ ; Figure 3B\*:  $74 \pm 9.8$ ; Figure 3B\*\*:  $57 \pm 8.8$ ); again paralleled by elevated p53 (Figure 3A:  $48 \pm 3.7$ ) and p21 (Figure 3A:  $77 \pm 6.7$ ). However, in late-stage *HK1.ras/fos-Δ5PTEN*<sup>flx/flx</sup> papillomas, p53 expression became reduced (Figure 3B\*:  $46 \pm 8.9$  [ $p < 0.0007$ ]) and expression was lost in specific areas (Figure 3B\*\*:  $25 \pm 8.7$ ); hence loss of p53 guardianship increased spontaneous mutations leading to wdSCC (Figure 3B high magnification:  $11 \pm 9$  [significance vs. Figure 3B\*:  $p < 0.0003$ ; vs. Figure 3B\*\*:  $p < 0.001$ ], see Supporting Information: Figure S4A).

In contrast, in both late-stage papilloma and wdSCC histotypes, 14-3-3 $\sigma$  and p21 remained elevated (Figure 3B\* 14-3-3 $\sigma$ :  $74 \pm 9.4$ ; Figure 3B\* p21:  $89 \pm 8.1$ ); and moreover, 14-3-3 $\sigma$  expression remained in areas of reduced p53 (Figure 3B\*\*:  $55 \pm 6.7$



**FIGURE 2** 14-3-3 $\sigma$ , p53 and p21 expression in *HK1.fos- $\Delta$ 5PTEN<sup>flx/flx</sup>* keratoacanthoma etiology. (A) *HK1.fos/ $\Delta$ 5PTEN<sup>flx/flx</sup>* hyperplasia shows elevated basal layer 14-3-3 $\sigma$  expression with mainly cytoplasmic p53 expression in supra-basal keratinocytes, whereas p21 is expressed at low levels. (B) At higher magnification, both IHC and IF analysis show elevated basal-layer 14-3-3 $\sigma$  expression is cytoplasmic and membranous; similarly elevated p53 appears mainly cytoplasmic with sporadic nuclear expression and K1 expression remains normal. (C) Left panels: *HK1.fos/ $\Delta$ 5PTEN<sup>flx/flx</sup>* keratoacanthoma shows the highly differentiated, frond-like histopathology (see Supporting Information: Figure S2E) exhibits strong basal- and supra-basal 14-3-3 $\sigma$  expression, paralleled by elevated, nuclear p53 and p21; where anomalous basal-layer K1 expression indicates premature differentiation.<sup>28</sup> In contrast, less-differentiated, wdSCC-like keratoacanthoma histopathology (right panels) exhibits weaker and mainly supra-basal 14-3-3 $\sigma$ /K1 expression, paralleled by lower p53/p21. Right: Image J quantitation of hyperplasia confirms elevated basal- and supra-basal 14-3-3 $\sigma$  expression, with elevated p53 but little p21. Analysis of KA histotypes shows high expression of each protein in areas of highly differentiated histotypes – including anomalous K1 expression in basal layers; whereas wdSCC-like histotypes exhibit lower basal-layer expression, yet 14-3-3 $\sigma$  and K1 expression persists in the supra-basal layers as observed in wdSCC. Bars: (A) approximately 120–150  $\mu$ m; (B) IHC: 14-3-3 $\sigma$ /p53 approximately 75  $\mu$ m; IF: 14-3-3 $\sigma$  approximately 50  $\mu$ m; K1/K14 approximately 30  $\mu$ m; (C) Left column: 14-3-3 $\sigma$ /p53/p21 approximately 60–75  $\mu$ m; K1/K14 approximately 100  $\mu$ m. Right column: 14-3-3 $\sigma$ /p53 approximately 60–75  $\mu$ m; K1/K14 approximately 50  $\mu$ m; p21 approximately 100  $\mu$ m. wdSCC, well-differentiated squamous cell carcinoma.

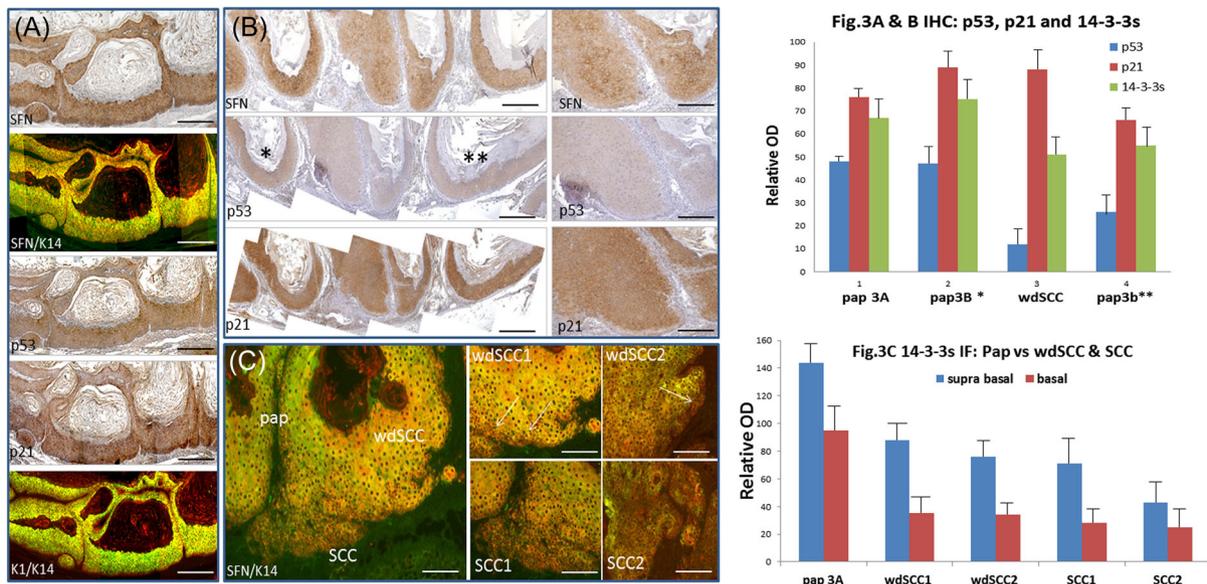
[ $p < 0.0005$ ]) and persisted in p53-negative wdSCC (Figure 3B:  $52 \pm 5.7$  [ $p < 0.0001$ ]); albeit at reduced levels, possibly reflecting 14-3-3 $\sigma$  expression regulation by p53.<sup>4</sup> Similarly, p21 persisted (Figure 3B<sup>\*\*</sup>:  $67 \pm 6.7$  [vs. p53:  $p < 0.0005$ ]) and expression actually increased following conversion to p53-negative wdSCC (Figure 3B:  $87 \pm 8.7$  [vs. p53 wdSCC:  $p < 0.0001$ ]; see Supporting Information: Figure S4A); again suggesting a feedback to p53 loss that, alongside persistent 14-3-3 $\sigma$ , helped inhibit progression<sup>29</sup> until lost during development of aggressive SCC (see Figure 6 and Supporting Information: Figure S4A, lane 14).

Immunofluorescence analysis of *HK1.ras/fos- $\Delta$ 5PTEN<sup>flx/flx</sup>* tumors possessing papilloma, wdSCC and SCC histotypes (Figure 3C, see Supporting Information: Figure S7E) also showed that strong 14-3-3 $\sigma$  expression in papillomas (Figure 3A OD:  $125 \pm 15.1$ ) became reduced in late-stage papilloma (Figure 3C [low mag. green] OD:  $107 \pm 13.3$  [vs. 3A:  $p < 0.02$ ]), yet expression persisted following conversion to (p53-negative) wdSCC (Figure 3C wdSCC [low mag. yellow area] OD:  $64 \pm 10.1$  [ $p < 0.005$ ]); until lost on progression to SCC (Figure 3C [low mag.] OD:  $34 \pm 7.6$  [ $p < 0.0005$ ]). At higher magnification,

compared to papilloma basal-layer 14-3-3 $\sigma$  expression (Figure 3A IF:  $91 \pm 20.5$ ), wdSCCs showed invasive basal-layer keratinocytes exhibited significantly reduced 14-3-3 $\sigma$  levels (Figure 3C [arrows] wdSCC1:  $37 \pm 8.6$ ; wdSCC2:  $35.5 \pm 6.6$  [ $p < 0.0001$ ]) consistent with loss of a TSG role during invasion and progression (see K1 analysis for histotype conformation; Supporting Information: Figure S5). Hence, larger areas of reduced 14-3-3 $\sigma$  expression and eventual loss (Figure 3C SCC1:  $29 \pm 12.2$ ; SCC2:  $26 \pm 11.9$ ) drove SCC progression. Of note, 14-3-3 $\sigma$  loss was paralleled by p21 loss and increased p-AKT1<sup>473</sup>; suggesting this was a key interaction during malignant progression – a result also repeated in TPA promotion experiments.

### 3.4 | Rapid tumor progression in TPA-treated *HK1.ras- $\Delta$ 5PTEN<sup>flx/flx</sup>* carcinogenesis associates with early loss of 14-3-3 $\sigma$ , p53 and p21

Previously, a lack of tumor progression in *HK1.ras- $\Delta$ 5PTEN<sup>flx/flx</sup>* studies<sup>27</sup> had prompted TPA promotion experiments, which resulted



**FIGURE 3** 14-3-3 $\sigma$ , p53 and p21 expression in *HK1.ras/fos-Δ5PTEN<sup>flx/flx</sup>* SCC etiology. *Left panel:* (A) Early *HK1.ras/fos-Δ5PTEN<sup>flx/flx</sup>* papillomas exhibit strong basal and granular layer 14-3-3 $\sigma$  expression; with a hiatus in the intermediate, acanthotic layers. Strong p53 is observed in all layers; while basal p21 expression parallels 14-3-3 $\sigma$ , with lower levels in acanthotic layers. Supra-basal K1 expression indicates benign tumor. (B) IHC analysis of typical mixed histotype *HK1.ras/fos-Δ5PTEN<sup>flx/flx</sup>* tumor shows elevated 14-3-3 $\sigma$ /p21 co-expression; whereas p53 is reduced in papilloma histotypes (\*) and absent in areas of converting papilloma (\*\*). Higher magnification (used for quantitation) of wdSCC histotype shows persistent (if lower) 14-3-3 $\sigma$  expression alongside maintained p21 expression and p53 loss. (C) IF analysis of mixed *HK1.ras/fos-Δ5PTEN<sup>flx/flx</sup>* tumor shows elevated 14-3-3 $\sigma$  expression in papilloma histotypes (green) persists in wdSCC histotypes (yellow) but weakens in invasive SCC areas (red due to K14 counterstain). At higher magnification wdSCC histotypes exhibit reduced 14-3-3 $\sigma$  in the invasive basal layers (arrows wdSCC 1 and 2) with further, more uniform regression in SCC1 which is finally lost on progression to more aggressive SCC (SCC2) (Quantitation shown in Figure 4 below). *Right panel:* Image J quantitation of IHC analysis shown in (B), confirms persistence of 14-3-3 $\sigma$  and p21 following conversion to wdSCC whereas p53 expression diminished in papillomas (\*\*) and was lost in wdSCC. Lower: Image J quantitation of IF analysis shown in (C) demonstrates reduction and loss of 14-3-3 $\sigma$  expression in basal versus suprabasal keratinocytes of papillomas, wdSCC and SCC. Bars (A) approximately 120–150  $\mu$ m; (B) Left: approximately 120–150  $\mu$ m. Right: approximately 100  $\mu$ m; (C) approximately 100 and 75  $\mu$ m. SCC, squamous cell carcinoma; wdSCC, well-differentiated SCC.

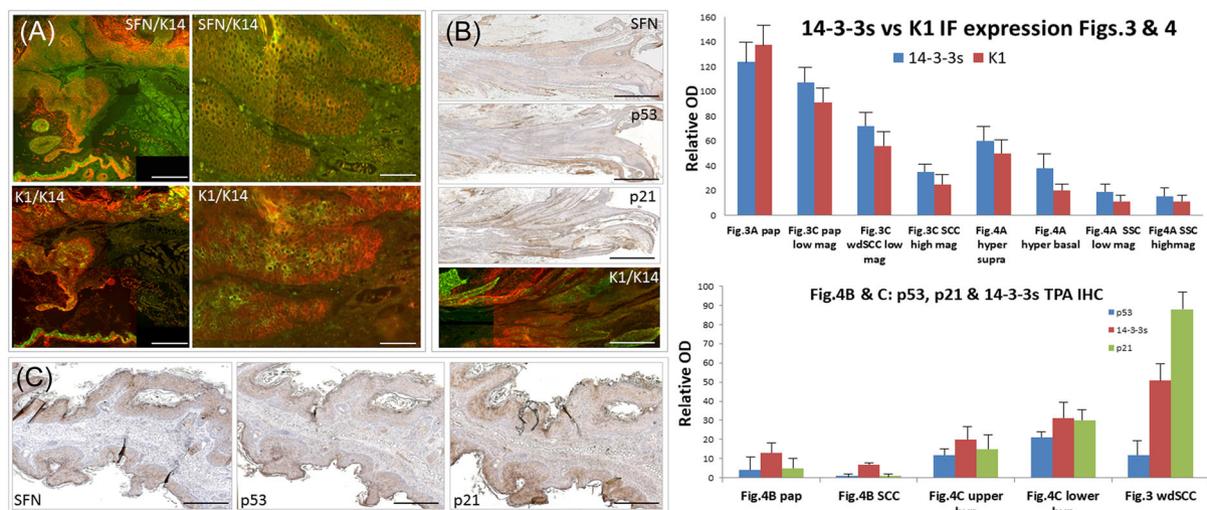
in rapid progression to aggressive SCC/pdSCC<sup>27</sup> (see Supporting Information: Figure S2D). Immunofluorescence analysis (Figure 4), demonstrated that SCC/pdSCC tumors lacked detectable 14-3-3 $\sigma$  expression (Figure 4A:  $17 \pm 8.5$ ), while the areas of TPA-promoted *HK1.ras-Δ5PTEN<sup>flx/flx</sup>* hyperplasia already exhibited reduced expression (Figure 4A supra-basal:  $63 \pm 10.5$ ; basal:  $38 \pm 11.6$ ; see Supporting Information: Figure S5), compared to typical papillomas (Figure 3A IF supra-basal:  $142 \pm 23.5$  [ $p < 0.001$ ]; basal:  $91 \pm 20.5$  [ $p < 0.0007$ ]). Indeed, hyperplastic basal-layer 14-3-3 $\sigma$  expression was already reduced to levels observed in wdSCCs (e.g., wdSCC1:  $37 \pm 8.6$ ).

Thus, any initial 14-3-3 $\sigma$ -mediated suppressive responses to inhibit papillomatogenesis (alongside p53 and p21) were short lived, as more sensitive IHC analysis of TPA-promoted *HK1.ras-Δ5PTEN<sup>flx/flx</sup>* tumors (Figure 4B) showed 14-3-3 $\sigma$  loss in K1-positive papilloma ( $17 \pm 2.6$ ), resulting in 14-3-3 $\sigma$ -negative pdSCC ( $11 \pm 3.6$ ), was paralleled by complete p53 ( $4.3 \pm 6.6$ ) and p21 ( $7 \pm 3.6$ ) loss (Figure 4B). Similarly, IHC analysis of TPA-promoted *HK1.ras-Δ5PTEN<sup>flx/flx</sup>* hyperplasia showed early loss of TSG expression (Figure 4C: 14-3-3 $\sigma$  upper:  $19 \pm 6.6$ ; lower:  $31 \pm 6.4$ ; p53 upper:  $14 \pm 4.6$ ; lower:  $21 \pm 3.4$ ; p21 upper:  $16 \pm 6.6$ ; lower:  $28 \pm 6.4$ ),

consistent with rapid progression to pdSCC (see Supporting Information: Figure S5 for quantitation and K1 expression analysis).

### 3.5 | *HK1.ras/fos-Δ5PTEN<sup>flx/flx</sup>* tumor progression highlights 14-3-3 $\sigma$ /p-MDM2<sup>166</sup> antagonism in p53-associated malignant conversion

Given the antagonistic 14-3-3 $\sigma$  and MDM2 interactions involved in p53 regulation,<sup>3,4,22</sup> activated p-MDM2<sup>166</sup> status was investigated in stage-specific tumor etiology (Figure 5). In normal epidermis, activated p-MDM2<sup>166</sup> expression paralleled p53 and 14-3-3 $\sigma$  in being supra-basal, with occasional positive basal expression in cycling keratinocytes alongside sporadic p53/14-3-3 $\sigma$  (Figure 5A: p-MDM2<sup>166</sup> supra-basal:  $48 \pm 3.5$ ; basal:  $26 \pm 3.5$ ; 14-3-3 $\sigma$  [IF] supra-basal:  $42 \pm 6.5$ ; basal:  $24 \pm 3.5$ ; p53 supra-basal:  $28 \pm 6.5$ ; basal:  $28 \pm 8.5$ ). Similarly, *HK1.ras* papillomas displayed elevated, supra-basal p-MDM2<sup>166</sup> (Figure 5B: supra-basal:  $107 \pm 29.5$ ), alongside elevated 14-3-3 $\sigma$  expression ( $108 \pm 16.5$ ); possibly geared to maintain lower p53 levels ( $30 \pm 7.5$ ) and avoid anomalous p53-mediated apoptosis that might compromise barrier functions.<sup>27,28</sup>



**FIGURE 4** 14-3-3 $\sigma$ , p53 and p21 expression in TPA-promoted, poorly differentiated SCC. (A) Low and high magnification immunofluorescence analysis of a typical TPA-promoted *HK1.ras-Δ5PTEN<sup>flx/flx</sup>* pdSCC shows loss of 14-3-3 $\sigma$  expression, alongside K1 loss, compared to adjacent epidermal hyperplasia (bottom left). (B) IHC analysis of earlier TPA-promoted *HK1.ras-Δ5PTEN<sup>flx/flx</sup>* papilloma/wdSCC/SCC also shows 14-3-3 $\sigma$  loss parallels loss of p53 and p21; even in areas of K1-positive papilloma. (C) IHC analysis of TPA-promoted *HK1.ras-Δ5PTEN<sup>flx/flx</sup>* hyperplasia already shows loss of 14-3-3 $\sigma$  parallels p53 loss and reduced p21 (see Supporting Information: Figure S2D). Right panels: Upper: Image J quantitation of IF analysis tumor histotypes in Figures 3 and 4A confirms reduced expression of 14-3-3 $\sigma$  and keratin K1 as papillomas (Figure 3A) convert to wdSCC (Figure 3C) and such reduced levels were already demonstrated in TPA-promoted hyperplasia (Figure 4A; Supporting Information: Figure S5). Lower panel: Quantitation of Figure 4B,C IHC analysis shows TPA treated papillomas and SCCs lacked 14-3-3 $\sigma$ , p53 and p21; while TPA-promoted *HK1.ras-Δ5PTEN<sup>flx/flx</sup>* hyperplasia again expressed loss compared to persistent 14-3-3 $\sigma$  and p21 observed in wdSCC (Figure 3B; see also Supporting Information: Figure S5). Bars (A) approximately 120–150 and 50  $\mu$ m; (B) approximately 150  $\mu$ m; (C) approximately 100  $\mu$ m. pdSCC, poorly differentiated SCC; SCC, squamous cell carcinoma; wdSCC, well-differentiated SCC.

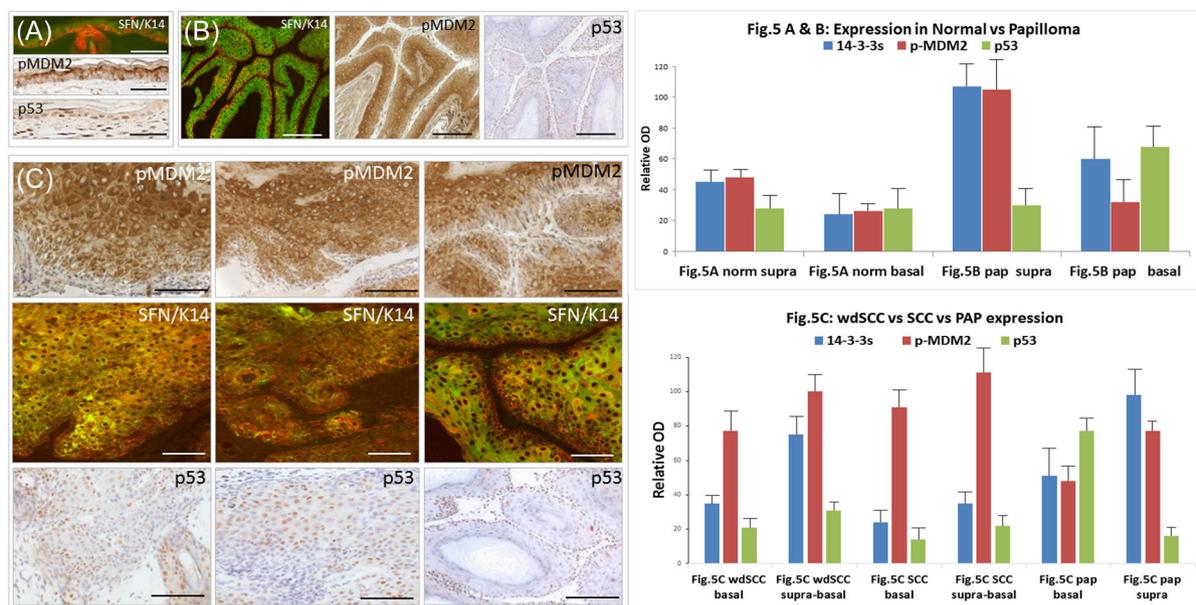
In larger *HK1.ras* papillomas where p-MDM2<sup>166</sup> was detectable in strands of basal-layer keratinocytes, expression was mainly cytoplasmic not nuclear (Figure 5B: 42 ± 13.5), reflecting increased 14-3-3 $\sigma$  (Figure 5B: 60 ± 3.5), which acted to chaperone MDM2 into the cytoplasm for ubiquitination.<sup>3,4,22</sup> Hence, *HK1.ras* papillomas lacked activated, nuclear p-MDM2<sup>166</sup> which resulted in elevated nuclear p53 (Figure 5B: 68 ± 10.5) and this significant increase over normal ( $p < 0.0001$ ) helped prevent malignant conversion.<sup>27,33</sup> In triple *HK1.ras/fos-Δ5PTEN<sup>flx/flx</sup>* papillomas (Figure 5C), elevated basal-layer 14-3-3 $\sigma$  (51 ± 20.4) again appears geared to sequester activated p-MDM2<sup>166</sup> to supra-basal keratinocytes (77 ± 7.4); and any potential increase in basal-layer p-MDM2<sup>166</sup> expression (48 ± 8.4) remained destined for cytoplasmic ubiquitination. Thus, basal-layer keratinocytes were mainly negative for nuclear p-MDM2<sup>166</sup> (Figure 5C; papilloma panels) and consequently at this stage, nuclear p53 levels remained high (77 ± 5.4).

However, with time, in *HK1.ras/fos-Δ5PTEN<sup>flx/flx</sup>* tumors comprised of papilloma, wdSCC and SCC histotypes (above, Figure 3C), strong nuclear p-MDM2<sup>166</sup> expression appeared in all layers of wdSCC and SCC histotypes (Figure 5C: left and middle panels: wdSCC: 77 ± 11.4; SCC: 91 ± 9.4 [vs. papilloma  $p < 0.0002$ ]). This supra-basal-to-basal expression of activated p-MDM2<sup>166</sup> in wdSCC was paralleled by a reverse, basal-to-supra-basal reduction in 14-3-3 $\sigma$  (Figure 5C wdSCC basal: 35 ± 5.4 [vs. papilloma  $p < 0.0005$ ]) and loss of p53 (21 ± 4.4

[ $p < 0.0003$ ]); while continued expression of p-MDM2<sup>166</sup> in SCCs corresponded loss of 14-3-3 $\sigma$  expression (basal: 24 ± 6.9 [ $p < 0.0001$ ]) and virtually undetectable p53 (basal: 14 ± 4.1 [ $p < 0.0001$ ]). Similar 14-3-3 $\sigma$  loss was observed in vitro, where 3D raft cultures of invasive SCC and melanoma cells were negative for 14-3-3 $\sigma$  expression, unlike their cultured 2D counterparts (see Figure 7). Thus, in *HK1.ras/fos-Δ5PTEN<sup>flx/flx</sup>* carcinogenesis, reduced basal-layer 14-3-3 $\sigma$  and subsequent increased p-MDM2<sup>166</sup> activity, lowered p53 levels giving a susceptibility to malignant conversion/progression.

### 3.6 | 14-3-3 $\sigma$ /p21 co-operation inhibits AKT to limit malignant progression

Analysis of *HK1.ras/fos-Δ5PTEN<sup>flx/flx</sup>* SCC and TPA-promoted *HK1.ras-Δ5PTEN<sup>flx</sup>* pdSCCs etiology showed a distinct 14-3-3 $\sigma$ /p21 co-expression profile that appeared to antagonize p-AKT1<sup>473</sup> and limit malignant progression (Figure 6). Initially, in *HK1.ras/fos-Δ5PTEN<sup>flx/flx</sup>* papillomas (Figure 6A), elevated 14-3-3 $\sigma$ /p21 co-expression in basal and supra basal layers (14-3-3 $\sigma$  basal: 88 ± 4.4; 14-3-3 $\sigma$  supra-basal: 75 ± 3.6; p21 basal: 97 ± 7.4; p21 supra-basal: 72 ± 9.4) was associated with low p-AKT1<sup>473</sup> expression (basal: 22 ± 12.4; supra-basal: 18 ± 5.4). This suggested initial responses to loss of PTEN-mediated AKT regulation, including



**FIGURE 5** p-MDM2<sup>166</sup> and 14-3-3σ/p53 expression in *HK1.ras* papilloma and *HK1.ras/fos-Δ5PTEN<sup>flx/flx</sup>* SCC etiology. (A) Normal epidermis shows supra-basal 14-3-3σ parallels activated p-MDM2<sup>166</sup> expression, with sporadic positive basal-layer keratinocytes also observed for p53. (B) *HK1.ras* papilloma sections show supra-basal 14-3-3σ parallels supra-basal p-MDM2<sup>166</sup> expression, but with increasing nuclear p53 appearing in basal layer keratinocytes. (C) Left column: *HK1.ras/fos-Δ5PTEN<sup>flx/flx</sup>* wdSCCs show elevated basal layer p-MDM2<sup>166</sup> expression, while 14-3-3σ remains essentially supra-basal and p53 becomes sporadic. Middle column: Aggressive *HK1.ras/fos-Δ5PTEN<sup>flx/flx</sup>* SCC/pdSCC shows strong p-MDM2<sup>166</sup> expression in invasive SCC keratinocytes; while 14-3-3σ becomes sporadic alongside occasional p53-positive cells. Right column: For comparison *HK1.ras* papilloma exhibits supra-basal 14-3-3σ/p-MDM2<sup>166</sup> and nuclear p53-positive basal-layer keratinocytes. Bars (A) approximately 25–30 μm; (B) approximately 85–100 μm; (C) wdSCC and pdSCC approximately 50–75 μm. pdSCC, poorly differentiated SCC; SCC, squamous cell carcinoma; wdSCC, well-differentiated SCC.

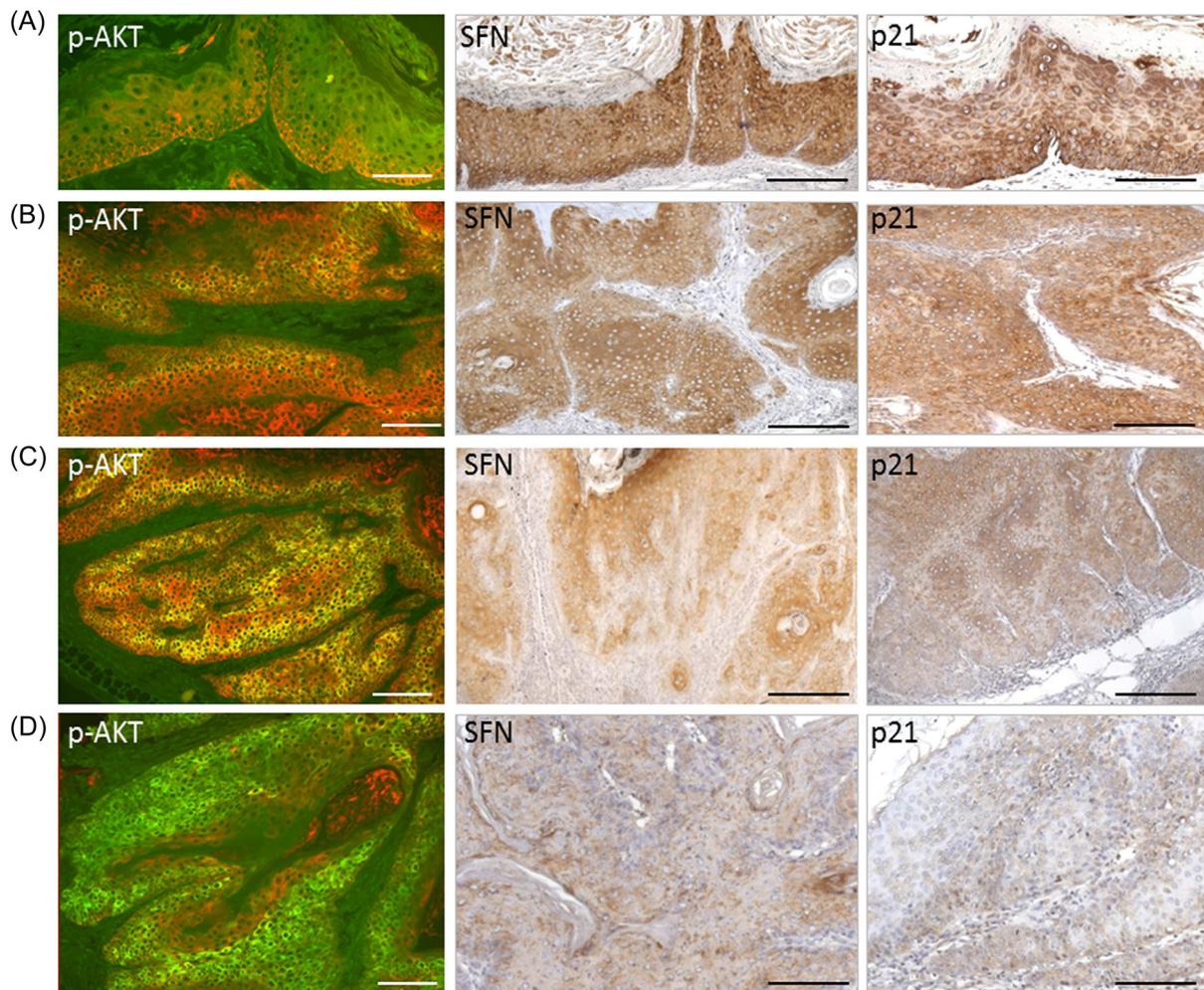
high p53 levels (above; Figure 3A), inhibited p-AKT1<sup>473</sup> expression. Further, despite p53 loss in *HK1.ras/fos-Δ5PTEN<sup>flx/flx</sup>* wdSCCs (Figure 6B), persistent basal/suprabasal-layer 14-3-3σ/p21 co-expression (14-3-3σ basal: 51 ± 2.4; supra-basal: 86 ± 3.2; p21 basal: 70 ± 13.9; supra-basal: 64 ± 5.5) appeared to confine any increase in p-AKT1<sup>473</sup> expression to supra-basal wdSCC layers (basal: 17 ± 4.3; supra-basal: 53 ± 5.4).

As wdSCC *HK1.ras/fos-Δ5PTEN<sup>flx/flx</sup>* tumors progressed to aggressive SCC (Figure 6C), reduced 14-3-3σ was paralleled by reduced p21 expression, particularly in the invasive basal layers (14-3-3σ basal: 13 ± 12.4; supra-basal: 38 ± 8.2; p21 basal: 17 ± 5.3; supra-basal: 48 ± 2.5), which corresponded to increased, activated p-AKT1<sup>473</sup> becoming widely expressed in all layers (basal: 48 ± 12.5; supra-basal: 70 ± 11.9). This antagonism was also demonstrated in TPA-promoted, *HK1.ras-Δ5PTEN<sup>flx/flx</sup>* pdSCC etiology (Figure 6D). Here, early loss of 14-3-3σ/p21 expression (14-3-3σ basal: 10 ± 2.4; supra-basal: 38 ± 5.4; p21 basal: 20 ± 4.3; supra-basal: 7 ± 4.1) coupled to p53 loss (Figure 4C p53: basal 14 ± 4.6), gave high levels of p-AKT1<sup>473</sup> expression (basal: 71 ± 5.5; supra-basal: 89 ± 12.9) consistent with rapid papillomatogenesis, conversion and progression to pdSCC. Taken collectively, these data indicate a strong synergism exists between 14-3-3σ and p21, possibly geared to inhibit AKT-mediated progression following p53 loss; and this aspect is under further investigation in p21 knockout and inducible 14-3-3σ knockout models.

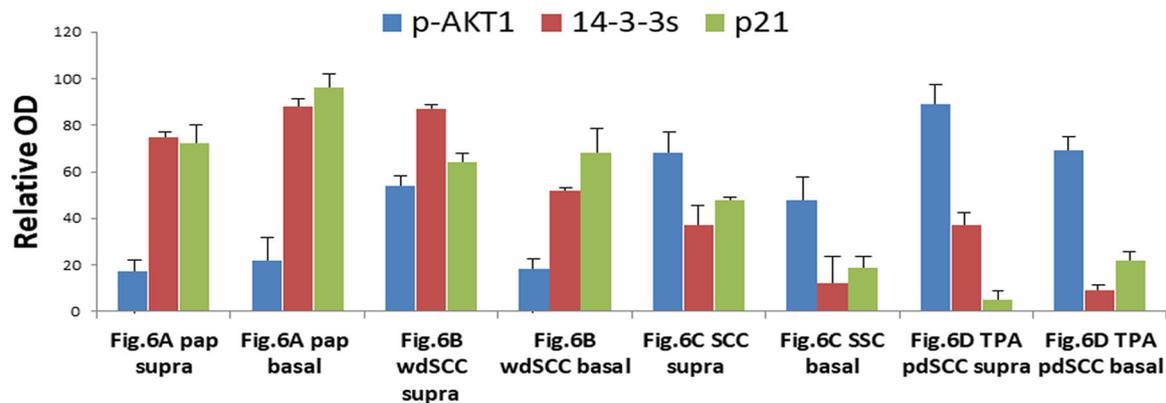
### 3.7 | 14-3-3σ persists in malignant SCC cells grown in 2D but not invasive cells of 3D-cultures

To compare in vivo findings to cultured cells (Figure 7), 14-3-3σ expression was assessed in immortalized HaCaT keratinocytes<sup>34</sup>; ras<sup>Ha</sup>-transformed (DMBA/TPA) SP1 papilloma cells (SP1<sup>ras61</sup>); and T52 SCC cells, derived from SP1 keratinocytes transformed by v-fos (T52<sup>ras61/HVVfos</sup>).<sup>37</sup> Initially, 14-3-3σ was assessed in transformation assays via resistance to calcium induced differentiation. Here, SP1<sup>ras61</sup> cells adopted a flattened morphology consistent with attempts to differentiate; while T52<sup>ras61/HVVfos</sup> cells were indifferent to raised calcium levels, consistent with their SCC phenotype (Supporting Information: Figure S6).

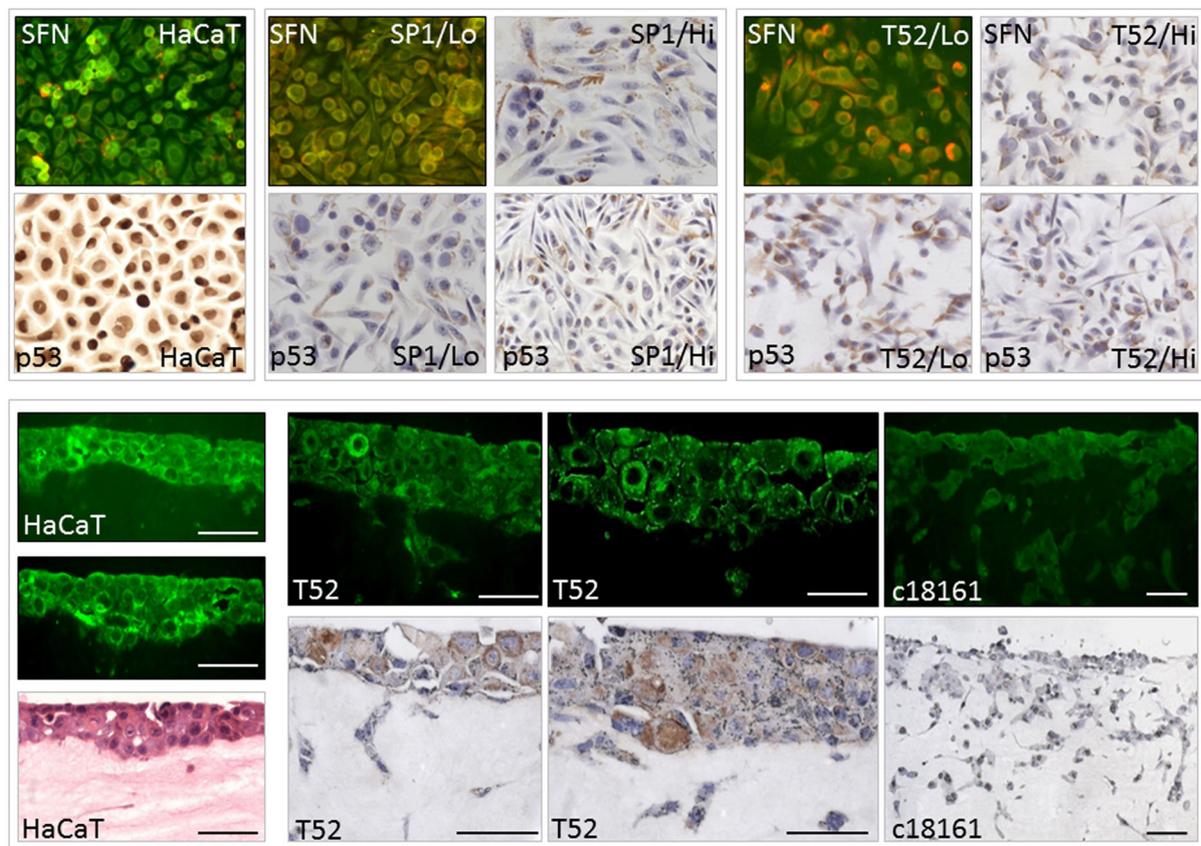
In 2D cultures, membranous/cytoplasmic 14-3-3σ expression was detected in immortalized HaCaT and SP1<sup>ras61</sup> papilloma keratinocytes cultured in proliferative Low Calcium (0.05 mM Ca<sup>2+</sup>) media, giving a halo appearance (upper panel; Figure 7), consistent with roles in spatial awareness/contact inhibition<sup>19,20,22</sup> and immortalized/benign tumor phenotypes observed in vivo. However, 14-3-3σ expression was also observed in malignant T52<sup>ras61/v-fos</sup> SCC keratinocytes, with a similar cytoplasmic halo appearance, which may reflect expression observed in malignant *HK1.ras/fos-Δ5PTEN<sup>flx/flx</sup>* wdSCCs (above). When challenged to differentiate in high calcium media (>0.12 mM Ca<sup>2+</sup>), increased numbers of SP1<sup>ras61</sup> keratinocytes exhibited 14-3-3σ with expression in distinct cytoplasmic areas; consistent with roles in spatial awareness. T52<sup>ras61/v-fos</sup> also



**Fig.6: 14-3-3s, p-AKT1 and p21 in progression**



**FIGURE 6** p-AKT1<sup>473</sup> and 14-3-3σ/p21 expression in *HK1.ras/fos-Δ5PTEN<sup>flx/flx</sup>* wdSCC/SCC and TPA-promoted pdSCC etiology. (A) *HK1.ras/fos-Δ5PTEN<sup>flx/flx</sup>* hyperplasia lacks p-AKT1<sup>473</sup> expression alongside basal layer 14-3-3σ and p21 co-expression which become reduced in the upper acanthotic layers. (B) Early, well-differentiated *HK1.ras/fos-Δ5PTEN<sup>flx/flx</sup>* SCCs exhibit increasing p-AKT1<sup>473</sup> but expression is confined to supra-basal layers given continued basal-layer 14-3-3σ/p21 co-expression. (C) Aggressive *HK1.ras/fos-Δ5PTEN<sup>flx/flx</sup>* SCCs exhibit uniform p-AKT1<sup>473</sup> expression in basal layers, paralleled by reduction/loss of 14-3-3σ/p21 expression in invasive keratinocytes. (D) TPA-promoted *HK1.ras-Δ5PTEN<sup>flx/flx</sup>* pdSCC etiology exhibits high uniform p-AKT1<sup>473</sup> expression (i.e., levels mask K14 counterstain); paralleled by loss of 14-3-3σ and p21 expression. Bars approximately 85–100 μm. pdSCC, poorly differentiated SCC; SCC, squamous cell carcinoma; wdSCC, well-differentiated SCC.



**FIGURE 7** In vitro analysis of 14-3-3 $\sigma$  expression in normal, papilloma, and malignant cells. Upper panel: 2D culture shows immortalized HaCaT keratinocytes express membranous 14-3-3 $\sigma$  and high levels of nuclear p53. Benign SP1<sup>ras61</sup> papilloma cells express membranous 14-3-3 $\sigma$  in proliferative, low Ca<sup>2+</sup> media with spatially localized cytoplasmic expression when differentiating in high Ca<sup>2+</sup> media; accompanied by sporadic, mainly cytoplasmic p53. Malignant T52<sup>ras61/HV<sup>v</sup>fos</sup> cells express less membranous 14-3-3 $\sigma$ /SFN in proliferative low Ca<sup>2+</sup> media; but retained this distinct cytoplasmic localization in high Ca<sup>2+</sup> media, despite being resistant to Ca<sup>2+</sup>-induced differentiation. In both proliferative and differentiating media, T52<sup>ras61/HV<sup>v</sup>fos</sup> cells exhibit cytoplasmic/nuclear levels of p53 (Supporting Information: Figure S3 gives cell morphology). Lower panel: 14-3-3 $\sigma$  expression in 3D tumor invasion assays. HaCaT cells produce a relatively normal skin on organotypic rafts, with membranous 14-3-3 $\sigma$  expression in basal and supra-basal keratinocytes. T52<sup>ras61/v-<sup>fos</sup></sup> SCC cells exhibit weaker expression in sporadic basal layer cells; however, both IF and IHC analysis show invasive T52<sup>ras61/v-<sup>fos</sup></sup> cells lack 14-3-3 $\sigma$  expression. Similarly, the positive invasive control, c18161 melanoma cells are negative for 14-3-3 $\sigma$  expression. Bars: approximately 30–40  $\mu$ m. SCC, squamous cell carcinoma.

exhibited this profile, thus maintaining a spatial awareness, yet suggesting 14-3-3 $\sigma$  had become uncoupled from suppressive functions or had adopted an oncogenic role.

For comparison, p53 expression was strong and nuclear in HaCaT cells,<sup>33</sup> whereas in proliferative Low Cal conditions, sporadic SP1<sup>ras61</sup> cells expressed mainly cytoplasmic p53 which increased in differentiating Hi Cal media. Oddly, malignant T52<sup>ras61/v-<sup>fos</sup></sup> cells expressed p53, but expression was assumed to be a mutant version, based on initiation with DMBA<sup>33</sup> such as the p53<sup>175</sup> GOF mutant.<sup>21</sup> Given that p53 directly induces 14-3-3 $\sigma$  expression,<sup>3,4,22</sup> this observation may also account for 14-3-3 $\sigma$  detection in SCC cells.

In organotypic 3D rafts, the normal skin produced by HaCaT cells displayed 14-3-3 $\sigma$  in basal and supra-basal keratinocytes; with a distinct membranous location indicative of spatial awareness (Figure 7 left panel: single IF staining). Unfortunately, SP1<sup>ras61</sup> cells attempted to create (fragile) papilloma at the air/liquid interface, which were lost in sample

processing. Indeed, collagen-based rafting protocols<sup>36</sup> appeared less optimum for murine SP1/T52 cells compared to human HaCaT or c18161 melanoma cells. However, invasive T52<sup>ras61/v-<sup>fos</sup></sup> SCC cells essentially nailed this model epidermis to the collagen raft (Figure 7: lower panel) where T52 cells exhibited 14-3-3 $\sigma$  expression but this was lost in the invasive cells; as observed for invasive c18161 melanoma cells (Figure 7: lower panel; right). Thus, in vitro data suggest that while TSG functions may be lost during progression/invasion; in certain malignant contexts, persistent 14-3-3 $\sigma$  expression may act to influence tumor outcome.

## 4 | DISCUSSION

In *HK1.ras/fos-Δ5PTEN<sup>flx/flx</sup>* mice overall the in vivo observations were consistent with tumor suppressive roles for 14-3-3 $\sigma$ , where elevated expression interacted with p53/MDM2 during papillomatogenesis to

inhibit malignant conversion; while lower, persistent expression cooperated with p21 to initially inhibit AKT-associated malignant progression. Additionally, as 14-3-3 $\sigma$  acts with CDK-inhibitors p21, p15 and p27 to limit G1/S progression,<sup>38</sup> normal epidermis displayed sporadic 14-3-3 $\sigma$  in dividing (p53/p21 positive) basal-layer keratinocytes; hence, development of epidermal hypoplasia when overexpressed in transgenic mice.<sup>39</sup> Consistent with roles in differentiation, normal epidermis and *HK1.ras* hyperplasia/papillomas expressed supra-basal 14-3-3 $\sigma$ ; further, expression appeared in sporadic populations of narrow, basal-layer keratinocytes that co-expressed keratin K1 (Figure 1A,B); an early marker of epidermal differentiation lost during malignant conversion.<sup>27-29</sup> This suggests roles in keratinocyte commitment to differentiate,<sup>17-19</sup> which require abilities to alter cell shape and migrate,<sup>5</sup> consistent with findings that 14-3-3 $\sigma$  stabilized complexes of keratin intermediate filaments with the actomyosin cytoskeleton<sup>36</sup> to alter cell shape, rigidity and motility; a role potentially subverted in localized tumor invasion.<sup>40</sup> In *HK1.fos- $\Delta$ 5PTEN<sup>flx/flx</sup>* KA etiology, 14-3-3 $\sigma$  expression reinforced roles in differentiation with elevated, membranous expression in hyperplastic *HK1.fos- $\Delta$ 5PTEN<sup>flx/flx</sup>* basal-layer keratinocytes (Figure 2A,B), which resulted in accelerated/premature keratinocyte differentiation,<sup>28</sup> culminating in classic KA keratosis (Supporting Information: Figure S2E). This suggests 14-3-3 $\sigma$  co-operation with *fos* and *PTEN* in regulation of the proliferation/differentiation balance. Indeed given the well-characterized interactions between 14-3-3 $\sigma$ /p53/p21,<sup>3,4,22</sup> as elevated basal-layer 14-3-3 $\sigma$  expression preceded that of p53/p21 (Figure 2A,B), it may help trigger compensatory p53/p21 responses that switched hyperproliferation into the differentiation that dominates KA etiology.<sup>28</sup> Hence, the initial proliferative KA bulb (Supporting Information: Figure S2E) exhibited supra-basal 14-3-3 $\sigma$ /K1 expression associated with weak p53/p21 (Figure 2C<sup>28</sup>); whereas overt KAs exhibited uniform 14-3-3 $\sigma$ /p53/p21, accompanied by premature basal-layer K1 expression in keratotic frond histotypes (Figure 2C).<sup>28</sup> These links between 14-3-3 $\sigma$  and *fos*/*PTEN* which triggered p53/p21, may also account for basal-to-supra-basal changes in p-AKT<sup>ser473</sup> expression,<sup>28</sup> where differentiation to inhibit AKT and/or restrict expression to supra-basal layers (Figure 6)<sup>30-32,41</sup> contributed to a KA outcome rather than malignant progression.

Additional *ras<sup>Ha</sup>* activation in *HK1.ras/fos- $\Delta$ 5PTEN<sup>flx/flx</sup>* mice altered papillomatogenesis, giving rise to tumors that converted to malignancy, which centered on 14-3-3 $\sigma$  interactions with MDM2 leading to p53 loss.<sup>3,4,22</sup> Here, tri-genic *HK1.ras/fos- $\Delta$ 5PTEN<sup>flx/flx</sup>* mice also identified 14-3-3 $\sigma$  interactions with p21, that appeared to limit early-stage malignant progression via AKT inhibition. Initially, in normal epidermis and preneoplastic *HK1.ras* or *HK1.ras/fos* hyperplasia, activated p-MDM2<sup>166</sup> expression was supra-basal alongside 14-3-3 $\sigma$ , and in basal-layers, sporadic p-MDM2<sup>166</sup> was also associated with p53/p21 co-expression in keratinocytes completing the cell cycle (Figure 1A,B).<sup>3,4,22,38</sup> Such supra-basal 14-3-3 $\sigma$ /p-MDM2<sup>166</sup> may be a feature of epidermal homeostasis where p-MDM2<sup>166</sup>-mediated removal of p53, alongside supra-basal AKT activation, facilitates terminal differentiation to prevent deregulated apoptosis and maintain barrier functions.<sup>28,41</sup> Indeed, this aspect of barrier homeostasis<sup>17-19,42</sup> may underlie the minimal p53/p21 responses

observed in *HK1.ras/fos* hyperplasia; possibly reflecting an epidermal tolerance to such *ras/fos*-mediated proliferation (Figure 1A,B).<sup>27-29</sup>

In overt *HK1.ras*, *HK1.ras/fos* and *HK1.ras- $\Delta$ 5PTEN<sup>flx/flx</sup>* papillomas, this supra-basal 14-3-3 $\sigma$ /p-MDM2<sup>166</sup> profile continued yet increasing 14-3-3 $\sigma$  appeared in strands of basal papilloma keratinocytes and was accompanied by strong, uniform nuclear p53 expression. As 14-3-3 $\sigma$  is a chaperone that removes MDM2 from the nucleus for ubiquitination, increased 14-3-3 $\sigma$  expression led to elevated, nuclear p53<sup>3,4,22</sup> that subsequently inhibited malignant conversion; a feature of these bi-genic mice.<sup>26-29</sup> This was consistent with rapid papillomatogenesis observed in 14-3-3 $\sigma$  knockout mice following two-stage chemical carcinogenesis<sup>26</sup> and susceptibility to skin carcinogenesis in repeated epilation mice (*Er/Er*) that express a truncated 14-3-3 $\sigma$  protein.<sup>23,24</sup>

In tri-genic *HK1.ras/fos- $\Delta$ 5PTEN<sup>flx/flx</sup>* hyperplasia and papillomas, elevated 14-3-3 $\sigma$ , p53 and p21 expression responses were immediate, appearing in all layers; and with a noted diminished 14-3-3 $\sigma$ /p21 co-expression in the acanthotic regions (Figure 3A,6A) suggesting direct 14-3-3 $\sigma$ /p21 interactions (see below). Such 14-3-3 $\sigma$ /p53/p21 responses helped maintained supra-basal p-MDM2<sup>166</sup> expression (Figure 5B,C) together with AKT inhibition (Figure 6A); thus, despite significant increased hyperplasia and rapid papilloma formation, malignant conversion was delayed, for example, in contrast to TPA promotion (below). However, with time basal-layer 14-3-3 $\sigma$  expression decreased (Figure 3C: wdSCC1 and 2), and this was paralleled by supra-basal-to-basal increases in activated p-MDM2<sup>166</sup> (Figure 5C), resulting in p53 down regulation and increased spontaneous mutations in proliferative basal cells resulting in conversion to wdSCCs.<sup>29</sup>

Of note, this wdSCC histotype was maintained over the medium term and thus mimics persistent p53-independent, 14-3-3 $\sigma$  expression observed in many human tumors.<sup>11-16</sup> Indeed, this remained the case until 14-3-3 $\sigma$  expression was lost during progression to more aggressive SCC (Figures 5C and 6C). Here, despite reduced 14-3-3 $\sigma$  in wdSCC basal layer cells, supra-basal expression continued, probably trans-activated by p63 (primarily TAp63 $\gamma$ ) in the absence of p53.<sup>43</sup> This supports the idea that 14-3-3 $\sigma$  roles in differentiation (above<sup>18,19</sup>; also contribute to maintenance of wdSCC histotypes, such as maintaining persistent (albeit reduced) keratin K1 expression; as keratin K1 (or K10) expression inhibits transformation<sup>44,45</sup> and were rapidly lost in TPA promotion experiments (Figure 4). In addition, 14-3-3 $\sigma$ /p21 interactions<sup>32,46</sup> may also inhibit AKT activity to limit early-stage malignant progression (below<sup>30,31,47</sup>). Nonetheless, persistent 14-3-3 $\sigma$  in p53-negative wdSCCs cannot exclude oncogenic roles, as suggested in vitro by malignant T52 carcinoma cells (below); that is, in cells lacking p53, once protection failed, spontaneous mutations increased with potential to cooperate with persistent, deregulated 14-3-3 $\sigma$  expression and dictate the outcome as observed in human tumor progression and patient prognosis.<sup>8,15,16</sup>

In terms of *HK1.ras/fos- $\Delta$ 5PTEN<sup>flx/flx</sup>* progression, previous studies showed that p53-independent p21 expression limited malignant progression,<sup>29,46</sup> and as 14-3-3 $\sigma$ /p21 co-expression in hyperplasia/papilloma varied with the degree of acanthosis

(Figures 3A and 6A) this strengthens their direct interactions.<sup>32,38</sup> Moreover, 14-3-3 $\sigma$  and p21 are known inhibitors of AKT,<sup>31–33,46,47</sup> which maybe a key activity that limited early-stage malignant progression following p53 loss. Previously, western blot analysis of TPA-promoted *HK1.ras- $\Delta$ PTEN<sup>flx/flx</sup>* mice showed immediate increases in p-AKT expression in hyperplasia and papillomas, culminating in strong expression in aggressive SCC.<sup>27</sup> Subsequently, p-AKT inhibition appeared to be a direct target of compensatory p53/p21 expression in *HK1.fos- $\Delta$ PTEN<sup>flx/flx</sup>* mice<sup>28</sup> and, as shown in *HK1.ras/fos- $\Delta$ PTEN<sup>flx/flx</sup>* mice following p53 loss,<sup>29</sup> persistent p21 was again associated with low-level p-AKT1<sup>ser473</sup>; now appearing alongside 14-3-3 $\sigma$  expression (Figure 6).

Thus, given the levels of 14-3-3 $\sigma$ /p21 observed during *HK1.ras/fos- $\Delta$ PTEN<sup>flx/flx</sup>* papillomatogenesis (Figure 6A), their combined co-expression (alongside that of p53; Figures 1–3), may help account for the distinct lack of p-AKT1<sup>ser473</sup> despite PTEN ablation. This lack had been an intriguing result, given loss of PTEN-mediated AKT signaling regulation had been expected to result in elevated p-AKT1<sup>473</sup> expression.<sup>27–29</sup> Further, it may be that such persistent basal-layer 14-3-3 $\sigma$ /p21 still restricted the increasing levels of p-AKT1<sup>473</sup> expression to supra-basal layers of early *HK1.ras/fos- $\Delta$ PTEN<sup>flx/flx</sup>* wdSCCs (Figure 6B). However, with time, beginning with 14-3-3 $\sigma$ , both 14-3-3 $\sigma$  and p21 expression faded and increased, uniform basal-layer p-AKT<sup>ser473</sup> expression was associated with progression to aggressive SCC, presumably combined with increasing spontaneous mutations given the lack of p53 surveillance.

Furthermore, the protective nature of this 14-3-3 $\sigma$ /p21/p53 TSG triad was clearly demonstrated by rapid progression to pdSCCs following TPA promotion of *HK1.ras- $\Delta$ PTEN<sup>flx/flx</sup>* mice.<sup>27</sup> Here, *HK1.ras- $\Delta$ PTEN<sup>flx/flx</sup>* pdSCCs were devoid of 14-3-3 $\sigma$  and K1 (Figure 4A,B; Supporting Information: Figure S5); and deployment of protective 14-3-3 $\sigma$ /p53/p21 expression was short lived, as analysis of TPA-promoted *HK1.ras- $\Delta$ PTEN<sup>flx/flx</sup>* papillomas and the earliest hyperplasia, were already negative for 14-3-3 $\sigma$ /p53/p21 expression (Figure 4). Thus, TPA-promoted *HK1.ras- $\Delta$ PTEN<sup>flx/flx</sup>* papillomatogenesis was rapid, as also observed in two-stage chemical carcinogenesis employing 14-3-3 $\sigma$  knockouts<sup>26</sup>; with uniform p-AKT1<sup>ser473</sup> helping drive early papillomatogenesis, consistent with previous western blot analysis<sup>27</sup> with rapid malignant conversion and progression to pdSCC (Figure 6). These data suggest important roles for AKT activation in malignant progression and, together with that of p-mTOR, are under further investigation in 14-3-3 $\sigma$  and p21 knockout models to confirm the significance and causality of stage-specific 14-3-3 $\sigma$  and/or p21 loss and subsequent AKT/p-mTOR overexpression.

In vitro, membranous 14-3-3 $\sigma$  expression profiles observed in proliferative HaCaT and benign SP1 papilloma cells were consistent with suppressive roles<sup>5,17–19,42</sup>; while the distinct cytoplasmic polarity of 14-3-3 $\sigma$  expression in SP1<sup>ras61</sup> papilloma keratinocytes again suggested a degree of spatial awareness<sup>5,19,42</sup>; as seen in *HK1.fos- $\Delta$ PTEN<sup>flx/flx</sup>* hyperplasia (Figure 2B). Unexpectedly in 2D cultures, malignant T52<sup>ras61/HVVfos</sup> SCC cells retained membranous 14-3-3 $\sigma$  and cytoplasmic polarity, despite their resistance to terminal

differentiation.<sup>33</sup> This raised oncogenic implications for persistent (or secreted) 14-3-3 $\sigma$  expression<sup>11–14</sup> that may contribute to cellular migration during early invasion, as observed in mammary carcinogenesis<sup>40</sup>; however, in 3D cultures, 14-3-3 $\sigma$  expression was sporadic and was lost in individual invading T52<sup>ras61/HVVfos</sup> SCC cells (Figure 7).

This alternate view of potential oncogenic roles regarding persistent, p53-independent 14-3-3 $\sigma$  expression likely reflect the multitude of interactions assigned to 14-3-3 $\sigma$  activities and are clearly context dependent.<sup>1,2,7</sup> For instance, while elevated 14-3-3 $\sigma$  counters ras<sup>Ha</sup><sup>26</sup> and fos activation,<sup>33</sup> and alongside p21, responds to loss of PTEN-mediated AKT regulation,<sup>26–32,46,47</sup> the roles for 14-3-3 $\sigma$  in cell rigidity and migration observed in mammary carcinogenesis<sup>40</sup> may interact with the loss of PTEN functions in cell spreading and migration.<sup>48,49</sup> Further, during wound healing, 14-3-3 $\sigma$  is known to stimulate the PI3K/mTOR pathway increasing cell growth and migration.<sup>36,50</sup> Here, alternate keratins are expressed such as K17,<sup>51</sup> that aid keratinocyte migration via direct cytoplasmic interactions with 14-3-3 $\sigma$  to stimulate mTOR activities.<sup>50,51</sup> This fits in well with largely subcellular cytoplasmic location of 14-3-3 $\sigma$  in the tumor cell lines and potentially invasion roles in graft assays<sup>35,40</sup> together with the membranous expression and changes in cell shape as keratinocytes committed to differentiate.

Finally, in p53-positive tumors,<sup>9–16</sup> the oncogenic contribution of persistent 14-3-3 $\sigma$  expression maybe indirect, for example, reduced MDM2 leading to elevated expression of (UVB-mediated) gain-of-function p53 mutants.<sup>21</sup>

Taken collectively, in vivo data highlight 14-3-3 $\sigma$  suppressive roles geared to limit ras<sup>Ha</sup>-activated papillomatogenesis via increased p53<sup>26,27</sup> or exploit differentiation roles as in KA etiology that link 14-3-3 $\sigma$  to fos and PTEN. On tumor progression, decreased 14-3-3 $\sigma$  and increased p-MDM2<sup>166</sup> activity reduced p53 leading to malignant conversion; yet 14-3-3 $\sigma$ /p21 co-expression continued to limit early-stage malignant progression via p-AKT<sup>473</sup> inhibition; until circumvented as observed in TPA promotion experiments. Given the diverse, context-dependent nature of 14-3-3 $\sigma$  activities in human tumors, multistage transgenic models are likely to help validate the tumor suppressive or potentially oncogenic roles for 14-3-3 $\sigma$  deregulation.

## AUTHOR CONTRIBUTIONS

*The conception and design of the study:* David A. Greenhalgh. *Wrote the manuscript:* David A. Greenhalgh. *Critiqued the output for important intellectual content:* David A. Greenhalgh. *The organization of the conduct of the study:* David A. Greenhalgh, Carol M. McMenemy, and Jean A.Quinn. *Carrying out the study:* David A. Greenhalgh, Carol M. McMenemy, Dajiang Guo, and Jean A.Quinn. *The analysis and interpretation of study data:* David A. Greenhalgh, Carol M. McMenemy, Dajiang Guo, and Jean A.Quinn.

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### CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request and are also deposited in Enlighten Database Glasgow University. <https://www.gla.ac.uk/myglasgow/openaccess/Greenhalgh> For the purpose of open access, the author(s) has applied a Creative Commons Attribution (CC BY) licence to any Author Accepted Manuscript version arising from this submission.

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### REFERENCES

- Pennington KL, Chan TY, Torres MP, Andersen JL. The dynamic and stress-adaptive signaling hub of 14-3-3: emerging mechanisms of regulation and context-dependent protein-protein interactions. *Oncogene*. 2018;37:5587-5604.
- Aghazadeh Y, Papadopoulos V. The role of the 14-3-3 protein family in health, disease, and drug development. *Drug Discov Today*. 2016;21:278-287.
- Hermeking H, Benzinger A. 14-3-3 proteins in cell cycle regulation. *Sem Cancer Biol*. 2006;16:183-192.
- Hermeking H, Lengauer C, Polyak K, et al. Is a p53-regulated inhibitor of G2/M progression. *Mol Cell*. 1997;1:3-11.
- Ling C, Zuo D, Xue B, Muthuswamy S, Muller WJ. A novel role for 14-3-3 $\sigma$  in regulating epithelial cell polarity. *Genes Dev*. 2010;24:947-956.
- Lodygin D, Hermeking H. The role of epigenetic inactivation of 14-3-3 $\sigma$  in human cancer. *Cell Res*. 2005;15:237-246.
- Phan L, Chou PC, Velazquez-Torres G, et al. The cell cycle regulator 14-3-3 $\sigma$  opposes and reverses cancer metabolic reprogramming. *Nat Commun*. 2015;6:7530.
- Ko S, Kim JY, Jeong J, Lee JE, Yang WI, Jung WH. The role and regulatory mechanism of 14-3-3 sigma in human breast cancer. *J Breast Cancer*. 2014;17:207-218.
- Iwata N, Yamamoto H, Sasaki S, et al. Frequent hypermethylation of CpG islands and loss of expression of the 14-3-3 $\sigma$  gene in human hepatocellular carcinoma. *Oncogene*. 2000;19:5298-5302.
- Yatabe Y, Osada H, Tatematsu Y, Mitsudomi T, Takahashi T. Decreased expression of 14-3-3 $\sigma$  in neuroendocrine tumors is independent of origin and malignant potential. *Oncogene*. 2002;21:8310-8319.
- Shiba-Ishii A, Noguchi M. Aberrant stratifin overexpression is regulated by tumor-associated CpG demethylation in lung adenocarcinoma. *Am J Pathol*. 2012;180:1653-1662.
- Radhakrishnan VM, Jensen TJ, Cui H, Futscher BW, Martinez JD. Hypomethylation of the 14-3-3 $\sigma$  promoter leads to increased expression in non-small cell lung cancer. *Genes Chromosomes Cancer*. 2011;50:830-836.
- Perathoner A, Pirkebner D, Brandacher G, et al. 14-3-3 $\sigma$  expression is an independent prognostic parameter for poor survival in colorectal carcinoma patients. *Clin Cancer Res*. 2005;11:3274-3279.
- Liu CC, Chang TC, Lin YT, et al. Paracrine regulation of matrix metalloproteinases contributes to cancer cell invasion by hepatocellular carcinoma-secreted 14-3-3 $\sigma$ . *Oncotarget*. 2016;7:36988-36999.
- Tanaka K, Hatada T, Kobayashi M, et al. The clinical implication of 14-3-3 sigma expression in primary gastrointestinal malignancy. *Int J Oncol*. 2004;25:1591-1597.
- Winter M, Rokavec M, Hermeking H. 14-3-3 $\sigma$  Functions as an intestinal tumor suppressor. *Cancer Res*. 2021;81:3621-3634.
- Hammond NL, Headon DJ, Dixon MJ. The cell cycle regulator protein 14-3-3 $\sigma$  is essential for hair follicle integrity and epidermal homeostasis. *J Invest Dermatol*. 2012;132:1543-1553.
- Sun BK, Boxer LD, Ransohoff JD, et al. CALML5 is a ZNF750- and TINC2-induced protein that binds stratifin to regulate epidermal differentiation. *Genes Dev*. 2015;29:2225-2230.
- Sambandam SAT, Kasetti RB, Xue L, Dean DC, Lu Q, Li Q. 14-3-3 $\sigma$  regulates keratinocyte proliferation and differentiation by modulating Yap1 cellular localization. *J Invest Dermatol*. 2015;135:1621-1628.
- Lodygin D, Yazdi AS, Sander CA, Herzinger T, Hermeking H. Analysis of 14-3-3 $\sigma$  expression in hyperproliferative skin diseases reveals selective loss associated with CpG-methylation in basal cell carcinoma. *Oncogene*. 2003;22:5519-5524.
- Caulin C, Nguyen T, Lang GA, et al. An inducible mouse model for skin cancer reveals distinct roles for gain- and loss-of-function p53 mutations. *J Clin Invest*. 2007;117:1893-1901.
- Lee MH, Lozano G. Regulation of the p53-MDM2 pathway by 14-3-3 $\sigma$  and other proteins. *Sem Cancer Biol*. 2006;16:225-234.
- Herron BJ, Liddell RA, Parker A, et al. A mutation in stratifin is responsible for the repeated epilation (Er) phenotype in mice. *Nat Genet*. 2005;37:1210-1212.
- Lutzner MA, Guenet JL, Breitburd F. Multiple cutaneous papillomas and carcinomas that develop spontaneously in a mouse mutant, the repeated epilation heterozygote Er/+. *J Natl Cancer Inst*. 1985;75:161-166.
- Ling C, Su VMT, Zuo D, Muller WJ. Loss of the 14-3-3 $\sigma$  tumor suppressor is a critical event in ErbB2-mediated tumor progression. *Cancer Discov*. 2012;2:68-81.
- Winter M, Lodygin D, Verdoodt B, Hermeking H. Deletion of 14-3-3 $\sigma$  sensitizes mice to DMBA/TPA-induced papillomatosis. *Oncotarget*. 2016;7:46862-46870.
- Yao D, Alexander CL, Quinn JA, Porter MJ, Wu H, Greenhalgh DA. PTEN loss promotes rasHa-mediated papillomatogenesis via dual up-regulation of AKT activity and cell cycle deregulation but malignant conversion proceeds via PTEN-associated pathways. *Cancer Res*. 2006;66:1302-1312.
- Yao D, Alexander CL, Quinn JA, Chan WC, Wu H, Greenhalgh DA. Fos cooperation with PTEN loss elicits keratoacanthoma not carcinoma, owing to p53/p21WAF-induced differentiation triggered by GSK3 $\beta$  inactivation and reduced AKT activity. *J Cell Sci*. 2008;121:1758-1769.
- Macdonald FH, Yao D, Quinn JA, Greenhalgh DA. PTEN ablation in Ras(Ha)/Fos skin carcinogenesis invokes p53-dependent p21 to delay conversion while p53-independent p21 limits progression via cyclin D1/E2 inhibition. *Oncogene*. 2014;33:4132-4143.
- Yang H, Wen Y-Y, Zhao R, et al. DNA damage-induced protein 14-3-3 $\sigma$  inhibits protein kinase B/Akt activation and suppresses Akt-activated cancer. *Cancer Res*. 2006;66:3096-3105.
- Shao Z, Cai Y, Xu L, et al. Loss of the 14-3-3 $\sigma$  is essential for LASP1-mediated colorectal cancer progression via activating PI3K/AKT signaling pathway. *Sci Rep*. 2016;6:25631.

32. Hemmati PG, Normand G, Gillissen B, Wendt J, Dörken B, Daniel PT. Cooperative effect of p21Cip1/WAF-1 and 14-3-3 $\sigma$  on cell cycle arrest and apoptosis induction by p14ARF. *Oncogene*. 2008;27:6707-6719.
33. Greenhalgh DA, Quintanilla MI, Orengo CC, et al. Cooperation between v-fos and v-ras<sup>H</sup>a induces autonomous papillomas in transgenic epidermis but not malignant conversion. *Cancer Res*. 1993;53:5071-5075.
34. Berton TR, Wang XJ, Zhou Z, et al. Characterization of an inducible, epidermal-specific knockout system: differential expression of lacZ in different Cre reporter mouse strains. *Genesis*. 2000;26:160-161.
35. Greenhalgh DA, Yuspa SH. Malignant conversion of murine squamous papilloma cell lines by transfection with the fos oncogene. *Mol Carcinog*. 1988;1:134-143.
36. Edward M, Quinn JA, Sands W. Keratinocytes stimulate fibroblast hyaluronan synthesis through the release of stratifin: a possible role in the suppression of scar tissue formation. *Wound Repair Regen*. 2011;19:379-386.
37. Henseleit U, Zhang J, Wanner R, Haase I, Kolde G, Rosenbach T. Role of p53 in UVB-induced apoptosis in human HaCaT keratinocytes. *J Invest Dermatol*. 1997;109:722-727.
38. Laronga C, Yang HY, Neal C, Lee MH. Association of the cyclin-dependent kinases and 14-3-3 sigma negatively regulates cell cycle progression. *J Biol Chem*. 2000;275:23106-23112.
39. Cianfarani F, Bernardini S, De Luca N, et al. Impaired keratinocyte proliferative and clonogenic potential in transgenic mice overexpressing 14-3-3 $\sigma$  in the epidermis. *J Invest Dermatol*. 2011;131:1821-1829.
40. Boudreau A, Tanner K, Wang D, Geyer FC, Reis-Filho JS, Bissell MJ. 14-3-3 $\sigma$  stabilizes a complex of soluble actin and intermediate filament to enable breast tumor invasion. *Proc Natl Acad Sci*. 2013;110:E3937-E3944.
41. Calautti E, Li J, Saoncella S, Brissette JL, Goetinck PF. Phosphoinositide 3-kinase signaling to Akt promotes keratinocyte differentiation versus death. *J Biol Chem*. 2005;280:32856-32865.
42. Dellambra E, Golisano O, Bondanza S, et al. Downregulation of 14-3-3 $\sigma$  prevents clonal evolution and leads to immortalization of primary human keratinocytes. *J Cell Biol*. 2000;149:1117-1130.
43. Trink B, Osada M, Ratovitski EA, Sidransky D. p63 transcriptional regulation of epithelial integrity and cancer. *Cell Cycle*. 2007;6:240-245.
44. Kartasova T, Roop DR, Yuspa SH. Relationship between the expression of differentiation-specific keratins 1 and 10 and cell proliferation in epidermal tumors. *Mol Carcinog*. 1992;6:18-25.
45. Santos M, Paramio JM, Bravo A, Ramirez A, Jorcano JL. The expression of keratin K10 in the basal layer of the epidermis inhibits cell proliferation and prevents skin tumorigenesis. *J Biol Chem*. 2002;277:19122-19130.
46. Topley GI, Okuyama R, Gonzales JG, Conti C, Dotto GP. p21(WAF1/Cip1) functions as a suppressor of malignant skin tumor formation and a determinant of keratinocyte stem-cell potential. *Proc Natl Acad Sci*. 1999;96:9089-9094.
47. Zhou BP, Liao Y, Xia W, Spohn B, Lee MH, Hung MC. Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nat Cell Biol*. 2001;3:245-252.
48. Kotelevets L, Hengel J, Bruyneel E, Mareel M, Roy F, Chastre E. Implication of the MAGI-1b/PTEN signalosome in stabilization of adherens junctions and suppression of invasiveness. *FASEB J*. 2005;19:115-117.
49. Subauste MC, Nalbant P, Adamson ED, Hahn KM. Vinculin controls PTEN protein level by maintaining the interaction of the adherens junction protein  $\beta$ -catenin with the scaffolding protein MAGI-2. *J Biol Chem*. 2005;280:5676-5681.
50. Kim S, Wong P, Coulombe PA. A keratin cytoskeletal protein regulates protein synthesis and epithelial cell growth. *Nature*. 2006;441:362-365.
51. Mikami T, Maruyama S, Abé T, et al. Keratin 17 is co-expressed with 14-3-3 sigma in oral carcinoma in situ and squamous cell carcinoma and modulates cell proliferation and size but not cell migration. *Virchows Arch*. 2015;466:559-569.

#### SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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