Published in final edited form as:
Cell Rep. 2014 March 13; 6(5): 818–826. doi:10.1016/j.celrep.2014.01.045.

Pten loss induces autocrine FGF signaling to promote skin tumorigenesis

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SUMMARY

Inactivation of the *Pten* tumor suppressor negatively regulates the PI3K-mTOR pathway. In a model of cutaneous squamous cell carcinoma (SCC), we demonstrate that deletion of *Pten* strongly elevates Fgf10 protein levels without increasing Fgf10 transcription *in vitro* and *in vivo*. The translational activation of Fgf10 by *Pten* deletion is reversed by genetic disruption of the mTORC1 complex, which also prevents skin tumorigenesis in *Pten* mutants. We further show that ectopic expression of Fgf10 causes skin papillomas, while *Pten* deletion-induced skin tumors are inhibited by epidermal deletion of Fgfr2. Collectively, our data identify autocrine activation of FGF signaling as an essential mechanism in promoting Pten-deficient skin tumors.

INTRODUCTION

Squamous cell carcinoma (SCC) is the second most common form of skin cancer (Ridky and Khavari, 2004). Mouse genetic studies have demonstrated that skin SCC can be induced by epidermal deletion of *Pten*, a critical tumor suppresser gene implicated in multiple types of cancer (Suzuki et al., 2003). Consistent with these data, germline mutations in various types of the PTEN harmartoma tumor syndrome (PHTS) are known to predispose patients to skin tumorigenesis (Eng, 2003). In addition to gene deletion, *Pten* function can be

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manipulated in cancer cells by other mechanisms such as transcription, microRNA, translation, protein stability, promoter methylation, and by various post-translational events (Song et al., 2012). Indeed, although somatic *PTEN* mutations have not been found in human SCCs, PTEN expression is shown to be frequently down regulated by a GRHL3-mediated microRNA network (Agrawal et al., 2011; Darido et al., 2011; Stransky et al., 2011). Skin SCC observed in *Pten* deficient animals has been reproduced by transgenic overexpression of activated AKT or RHEB in the epidermis and blocked by inhibition of class Ia PI3K (Lu et al., 2010; Segrelles et al., 2007; Wang et al., 2013), demonstrating the importance of PI3K-AKT-mTOR signaling in Pten-induced tumorigenesis. Recent evidence suggests that mTOR signaling controls the translation of nearly all mRNAs to some extent, but its direct targets critical for tumorigenesis remain poorly understood (Hsieh et al., 2012; Thoreen et al., 2012).

FGF signaling is required for epidermal growth, skin barrier formation and hair cycle activation (Greco et al., 2009; Grose et al., 2007; Yang et al., 2010). Up-regulation of ligands FGF2, 7, 10 and 22, and their activation of FGFR2 signaling has been shown to initiate keratinocyte proliferation in diseased states such as acne, psoriasis and wound healing (Braun et al., 2004). On the other hand, although activating mutations in *FGFR3* have been shown to underlie benign skin tumors, including epidermal nevi or seborrhoeic keratosis, epidermal deletion of *Fgfr2* is shown to sensitize animals to chemical-induced skin papillomas and SCCs (Grose et al., 2007; Logie et al., 2005). Therefore, FGF signaling plays highly context-dependent roles in skin homeostasis and tumorigenesis (Turner and Grose, 2010). In this study, we show that Pten loss in keratinocytes activates the translation of Fgf10 in an mTOR-dependent manner. The resulting induction of FGF signaling promotes skin papilloma formation, which can be reversed by genetic ablation of *Fgfr2* in epidermis. Our results thus reveal an autocrine FGF signaling network induced by Pten-mTOR signaling essential for skin tumorigenesis.

RESULTS

A mouse model of *Pten*-regulated skin tumorigenesis

We generated an epidermal-specific ablation of *Pten* using *Le-Cre* driver, which is known to be expressed in the ocular surface but is also reportedly active in the anterior mandibular ectoderm (Ashery-Padan et al., 2000; Miller et al., 2006; Pan et al., 2008). Indeed, one month old *Le-Cre;Pten*$^{flox/flox}$ (*Pten*^{CKO}) animals exhibited skin swelling both around the eyelid and in the cheek (Fig. 1A and B, arrows). Histological sections revealed epidermal hyperplasia, hypergranulosis and hyperkeratosis, which progressed to papilloma in severely affected animals (Fig. 1C and D, arrows, and data not shown). By crossing with *R26R* Cre reporter, we showed that the Cre-positive cells stained by X-gal were restricted to the epidermis and to hair follicles, confirming the specificity of *Le-Cre* to skin (Fig. 1E and F, arrows). Consistent with these results, *Pten*^{CKO} epidermis displayed a marked reduction in Pten staining and strong increases in cell proliferation markers Ki67 and phospho-Histone H3 (Fig. 1G-J and data not shown). K14-positive keratinocytes remained within the single basal cell layer attached to the basement membrane; however, the K10-labeled suprabasal layer was greatly expanded (Fig. 1K and L, arrows). These hyperplastic proliferation

phenotypes resembled the onset of SCC previously described in a pan-keratinocyte *Pten* deletion model (Suzuki et al., 2003).

Pten deletion increased Fgf10 protein level in skin epidermis

To gain insight into the role of FGF signaling in SCC, we performed qPCR on the skin epidermal layer removed from $Pten^{CKO}$ mice but found no significant increase in the mRNA levels of *Fgfs* (*Fgf1, 2, 7,* and 10) and *Fgfrs* (*Fgfr1, 2, 3* and *4*) (Fig. 2A and S1A). Immunoblotting of the same samples confirmed depletion of Pten protein and increased phosphorylation of AKT, 4EBP1 and S6, all downstream effectors of PI3K-mTOR pathway (Fig. 2B). Interestingly, although the protein levels of Fgf1, 2 and 7 remained low, immunoblotting of $Pten^{CKO}$ epidermis revealed a significant increase in Fgf10 protein.

Fgf10 is known to be secreted by intraepithelial γδ T cells in response to epidermal wounding (Werner et al., 1994). However, crossing $Pten^{CKO}$ mouse with $TCR\delta^{-/-}$ animals which are unable to produce γδT cells not only failed to reduce Fgf10 levels but even accelerated tumorigenesis (data not shown), suggesting that activation of γδ T cells cannot account for the increase in Fgf10 protein in $Pten^{CKO}$ epidermis. We next harvested primary keratinocytes from neonatal $Pten^{flox/flox}$ pups and suppressed Pten expression using Cre-expressing adenovirus. Following viral infection, cells were either allowed to remain in a basal state by culturing in low Ca^{2+} media (basal media) or forced to differentiate into suprabasal cells by culturing in high Ca^{2+} media (suprabasal media). Similar to results observed in the *in vivo* model, qPCR of basal or suprabasal state keratinocytes showed no significant change in *Fgf10* mRNA expression upon Pten deletion but pAKT, p4EBP1, pS6 protein levels were up regulated on immunoblotting (Fig. 2C and S1B). Importantly, immunoblotting for Fgf10 showed elevated levels in Pten-depleted cells cultured in suprabasal media. Finally, immunohistochemistry of tissue sections showed intense staining of pAKT, pS6, p4EBP1 and Fgf10 in $Pten^{CKO}$ epidermis (Fig. 2D-K). Taken together, these results suggested that loss of *Pten* results in translational activation of Fgf10 in keratinocytes.

We next examined whether these pathways derived from our mouse model existed in human cells and SCCs. By siRNA mediated knockdown, we first depleted PTEN in primary human keratinocytes. The results presented in Figure 2L shows that, in both basal and suprabasal culture conditions, PTEN deficiency led to an increase in AKT and S6 phosphorylation and elevated FGF10 protein expression. We next analyzed PTEN and FGF10 expression in well-to-moderately differentiated human skin SCC that arose spontaneously (n=9) or developed after undergoing transplantation procedures (n=6) via immunohistochemistry. Normal skin tissue from patients undergoing abdominoplasty was obtained for comparison (n=6). Strikingly, 15 out of 16 of these SCC specimens showed a loss in PTEN and an increase in FGF10 in the epithelial layer when compared to normal skin tissue ($P < 0.001$) (Fig. 2M-S). This observation was further confirmed in a SCC tissue array, where 42 out of 47 samples displayed elevated levels of FGF10 (data not shown). Finally, we examined 5 poorly differentiated human SCC samples and found that they all stained weakly for PTEN but strongly for FGF10 (Fig. 2O and R). These results thereby emphasize the potential role of PI3K and FGF signaling in promoting human skin SCC.

mTOR signaling in the epidermis regulates Fgf10 expression and tumorigenesis

Pten-PI3K signaling is known to promote protein translation via mTOR-mediated phosphorylation of translational suppressor 4EBP1. 4EBP1 phosphorylation activates cap-dependent translation of mRNA sequences containing a TOP-like motif, which is defined as containing *at least* five pyrimidines near the transcriptional start site (Hsieh et al., 2012; Thoreen et al., 2012). Interestingly, among *Fgf* mRNAs we detected in keratinocytes, only *Fgf10* contains a TOP-like sequence immediately following its transcriptional start site (Fig. 3A), suggesting that translation of *Fgf10* may be uniquely regulated by mTOR signaling. It was recently demonstrated that the allosteric inhibitor of mTOR, rapamycin, suppresses phosphorylation of only a subset of mTOR downstream targets, most notably S6K1 and its substrate S6 (Kang et al., 2013). In contrast, the ATP-competitive mTOR inhibitor, Torin, further blocks phosphorylation of T37 and T46 residues of 4E-BP1, sites that are rapamycin-resistant. Since TOP-like mRNA translation is mainly regulated through 4EBPs, but not S6Ks (Kang et al., 2013; Pende et al., 2004), we hypothesized that the *Fgf10* TOP-like sequence may be sensitive to Torin but not to rapamycin. To test this idea, we cloned *Fgf10* 5'UTR into a GFP reporter, carrying either wild type or 6 bp transversions within the TOP-like sequence (Fig. 3A). As expected, Torin treatment of NIH-3T3 cells abolished both S6 and 4EBP1 phosphorylation and prevented expression of the wild type *Fgf10* 5'UTR-GFP reporter (wtTOP) (Fig. 3B). However, both the vector control and the mutated *Fgf10* 5'UTR-GFP reporter (mutTOP) were unaffected, demonstrating the critical role of *Fgf10* TOP-like sequence in mTOR-induced translation. Although rapamycin treatment eliminated S6 phosphorylation, there was still significant 4EBP1 phosphorylation in transfected NIH-3T3 cells. As a result, neither the wild type nor the mutant GFP reporters exhibit reduced expression. These results support that 4EBPsmediated mTOR signaling controls the translational activity of *Fgf10* TOP-like sequence.

Torin inhibits activities of both mTORC1 and mTORC2, two distinct complexes of mTOR kinase. To identify which mTOR complexes regulates Fgf10 expression in vivo, we crossed $Pten^{CKO}$ mice with epidermal-specific knockouts of *Raptor* and *Rictor*, which are obligatory components of mTORC1 and mTORC2 respectively (Zoncu et al., 2011). Consistent with the known function of mTORC2 as an AKT S473 kinase, *Le-Cre;*$Pten^{flox/flox}$*;*$Rictor^{flox/flox}$ ($Pten^{CKO}$*;*$Rictor^{CKO}$) epidermis completely lost AKT^{S473} phosphorylation (Fig. 3C). The levels of p4EBP1 and pS6, however, were unaffected by *Rictor* deletion, while Fgf10 expression was moderately down regulated. In contrast, *Le-Cre;*$Pten^{flox/flox}$*;*$Raptor^{flox/flox}$ ($Pten^{CKO}$*;*$Raptor^{CKO}$) epidermis exhibited not only attenuated AKT^{S473} phosphorylation but also significant reduction in p4EBP1, pS6 and Fgf10 levels (Fig. 3C). As a result, epidermal hyperplasia observed in one month old $Pten^{CKO}$ mice was reduced in $Pten^{CKO}$*;*$Rictor^{CKO}$ littermates and completely prevented in $Pten^{CKO}$*;*$Raptor^{CKO}$ animals (Fig. 3D-P). Thus, mTORC1 signaling is critically required for Fgf10 expression and tumorigenesis in *Pten* mutants.

Autocrine Fgf10-Fgfr2 signaling mediates *Pten* deletion-induced tumorigenesis

We next explored the functional significance of increased Fgf10 expression in *Pten* deficient epidermis. We utilized an inducible *Fgf10* transgene (*pMes-Fgf10*) that places a *LoxP*-flanked transcription STOP cassette between a constitutively active chick β-actin promoter

and an Fgf10 cDNA (Fig. 4A) (Song et al., 2013). Preliminary characterization of this transgene indicated that, due to positional variegation, Cre-mediated excision of STOP cassette typically results in a mosaic expression of Fgf10 (data not shown). Nevertheless, all *Le-Cre*; *pMes-Fgf10* mice at 3 weeks of age developed spontaneous papilloma, which appeared as finger-like protrusions arising from epidermis (Fig.4C-F, arrows). Importantly, immunoblotting of tissues lysates indicated that these nodules expressed higher levels of Fgf10 than their adjacent skin control (Fig. 4B). These results demonstrate that overexpression of Fgf10 is sufficient to induce epidermal hyperplasia and papilloma formation in a dose-dependent manner.

Fgf10 is known to signal through primarily Fgfr2 and Fgfr1 to a lesser extent (Zhang et al., 2006). We thus reasoned that if Fgf10 was a key downstream target of Pten-PI3K signaling, the tumorigenic phenotype in $Pten^{CKO}$ mice may be rescued by genetic ablation of *Fgfr2*. Indeed, epidermal hyperplasia was only slightly reduced in $Le\text{-}Cre;Pten^{flox/flox};Fgfr1^{flox/flox}$ ($Pten^{CKO};Fgfr1^{CKO}$) mice, which still maintained high levels of AKT phosphorylation and Fgf10 expression (Fig. 4G, H, J, K and M, arrows). In contrast, pAKT and Fgf10 in $Le\text{-}Cre;Pten^{flox/flox};Fgfr2^{flox/flox}$ ($Pten^{CKO};Fgfr2^{CKO}$) animals were significantly reduced as compared to $Pten^{CKO}$ mice, suggesting a Fgf10-Fgfr2 autocrine feedback loop (Fig. 4M). Most importantly, $Pten^{CKO};Fgfr2^{CKO}$ animals displayed a thin layer of epidermis similar to that of wild type mice, and no tumor was ever observed during their lifespan for up to two years (Fig. 4I-N, arrows). Therefore, *Pten* deficient epidermis tumorigenesis requires Fgf10-Fgfr2 signaling.

DISCUSSION

In this study, we showed that hyperactivation of mTOR in *Pten*-deficient epidermis translationally stimulated expression of Fgf10 protein. The resulting activation of Fgfr2 in keratinocytes forms a positive autocrine feedback loop that is both necessary and sufficient for development of skin SCC. FGF ligands are generally thought to be regulated transcriptionally to achieve tissue specific expression patterns. In particular, it has been well established that stromal induction of Fgf10 and its binding to Fgfr2 in the epithelia are critical for limb induction, branching morphogenesis and skin development (Beenken and Mohammadi, 2009; Turner and Grose, 2010). However, in contrast to the paracrine function of Fgf10 common in embryonic development, we showed that Pten deletion led to an autocrine Fgf10 signaling in skin SCC. The presence of an mTORC1 regulated TOP-like sequence in *Fgf10* mRNA suggests that ectopic Fgf10 expression in skin keratinocytes is induced by constitutive mTOR signaling. Indeed, by exploring the different target specificities of mTORC1 inhibitors Torin and rapamycin, we showed that phosphorylation of 4EBPs but not S6Ks by mTORC1 is critical for translational activation of the Fgf10 TOP-like sequence. Furthermore, genetic disruption of mTORC1 complex not only prevented overexpression of Fgf10 protein but also suppressed tumorigenesis in Pten deficient epidermis. Our results thus revealed a novel translational mechanism of FGF signaling activation by Pten-PI3K-mTOR signaling in skin SCC. To date, FGF signaling deregulation in cancer has been primarily scrutinized at genomic level for genetic mutations or at transcriptomic level for mRNA misexpression (Turner and Grose, 2010). Our findings

suggest that proteomic analysis of FGF signaling is a promising venue to identify aberrant FGF signaling in carcinogenesis.

To ensure cutaneous tissue homeostasis, a fine balance of FGF signaling and its consequent mitogenic effects must be maintained. There is compelling evidence that Fgfr2 has tumor suppressive function in normal keratinocytes, as *Fgfr2*-deficient animals are more prone to develop skin tumors in response to chemical carcinogens (Grose et al., 2007). On the other hand, previous studies have showed that excessive expression of Fgfr2 ligand, Fgf7, could lead to epidermal hyperplasia and gross transformation, and p63-induced *Fgfr2* overexpression was essential for survival of autochthonous SCCs (Chikama et al., 2008; Guo et al., 1993; Ramsey et al., 2013). We now present genetic evidence that loss of Fgfr2 signaling completely blocked tumorigenesis in *Pten*-deficient epidermis, demonstrating that Fgfr2 is important for pathogenesis of skin SCC. These results thus raise the conundrum of how FGF signaling can be both tumor-suppressive and oncogenic in the epidermis. We would like to suggest that these two opposing functions of FGF signaling may be explained by different magnitudes of signaling activity. In this view, FGF signaling at the physiological level is required for proper skin development and homeostasis, lack of which results in abnormal skin barrier, pathological inflammation and deficient tumor surveillance. The *Fgfr2* deficiency could be exacerbated by the normal function of Pten, which is expected to further dampen overall mitogenic signaling. This explains the apparent paradox that *Fgfr2* deletion induces epidermal hyperplasia only in the presence of wild type *Pten*, an archetypical tumor suppressor gene. Excessive FGF signaling, however, promotes cell proliferation and survival, potentiates cell migration and invasion, and attracts angiogenesis. These oncogenic effects may be especially potent in *Pten*-deficient epidermis, where an Fgf10-Fgfr2 autocrine feedback loop greatly amplifies FGF signaling. Therefore, FGF signaling can act as an important potentiating factor in skin SCC.

EXPERIMENTAL PROCEDURES

Mice

Pten^{flox}, *Fgfr1*^{flox}, *TCR*$\delta^{-/-}$ and *R26R* mice were obtained from Jackson Laboratory (Bar Harbor, ME) (Hoch and Soriano, 2006; Itohara et al., 1993; Lesche et al., 2002; Soriano, 1999). *Rictor*^{flox} and *Raptor*^{flox} mice were kindly provided by Drs. Markus A. Rüegg and Michael N. Hall (Biozentrum, University of Basel, Basel, Switzerland) and Estela Jacinto (UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ) (Bentzinger et al., 2008). *Le-Cre* and *Fgfr2*^{flox} mice were kindly provided by Dr. Ruth Ashery-Padan (Tel Aviv University, Tel Aviv, Israel) and Dr. David Ornitz (Washington University Medical School, St Louis, MO), respectively (Ashery-Padan et al., 2000; Yu et al., 2003). *Fgf10* conditional transgenic mouse (*pMes-Fgf10*) was previously described (Song et al., 2013). All animal experiments were performed according to IACUC regulations.

Histology and immunohistochemistry

Histology and immunohistochemistry were performed as previously described (Pan et al., 2010). Human specimens of normal skin, SCC, and SCC occurring after solid organ transplantation (SOT) were obtained after approval from IRB at Indiana University School

of Medicine. For histopathological analysis, 5 μm sections obtained from formalin fixed paraffin embedded skin tissue were used. Image analysis of histology data was performed using ImageJ. The relative pixel intensity in gray scale ranging from 0–255 was obtained by measuring a specified area at three different locations on the same section. Each data point was calculated by taking the average of pixel intensity for three separate images of the same section, and analyzed by one-way ANOVA analysis.

Western Blot

Facial hair was removed using Nair® hair removal cream and skin around the cheek and eyelid area was dissected out in one piece, snap frozen and stored at −80°C until usage. The dissected skin samples were then carefully scraped with a curette to selectively remove only the epidermal layer, which was lysed in RIPA buffer containing protease inhibitors. Equal amounts of the lysates were separated on SDS-PAGE and the blots stained with primary antibodies were visualized using IRDye linked secondary antibody in Odyssey SA scanner (LICOR Biosciences, Lincoln, NE) as previously described (Qu et al., 2011). Antibodies used are specific to Actin (Abcam, Cambridge, MA), GFP (a gift from Dr. Pamela Silver, Harvard Medical School, Boston, MA), Ki67 (BD Pharmingen San Diego, CA), Keratin 10, Keratin 14, phospho-4EBP1 $^{T37/46}$, phospho-S6$^{S235/236}$, phospho-Akt^{S473}, Pten (all from Cell Signaling Technology, Beverly, MA), phospho-Histone H3 (Upstate, Temecula, CA), Fgf1, Fgf2, Fgf7, and Fgf10 (all from Santa Cruz Biotechnology, Santa Cruz, CA). At least three mice of each genotype were analyzed.

Keratinocyte Culture

Skins removed from neonatal pups were washed in DPBS plus 2% antibiotics, stretched out (dermis side down) on autoclaved filter paper in a 100 mm culture plate and digested overnight at 4°C in 0.25% trypsin. The epidermis was next gently separated from the dermis, finely minced, washed once and filtered through a 70μm cell strainer. Isolated keratinocytes were plated in KSFM media supplemented with growth factors and 0.2 mM $CaCl^2$ (Invitrogen, Carlsbad, CA) and maintained at 37^0C/5% CO_2 with media changed every 2 days. Keratinocyte differentiation from basal to suprabasal stage was induced by increasing the Ca^{2+} concentration to 1.2 mM in the culture media. At 70% confluency, keratinocytes were infected overnight with Adeno-Cre GFP virus (Gene Transfer Vector Core, University of Iowa, IA) at 100 MOI virus parts per cell. At about 90–100% GFP expression, the cells were lysed in ice cold RIPA buffer supplemented with protease inhibitors and used for immunoblotting experiments.

siRNA transfection

nTERT immortalized primary human keratinocytes were maintained in EpiLife keratinocyte media supplemented with human keratinocyte growth supplement cocktail and 1000 U penicillin-streptomycin (Invitrogen, Carlsbad, CA). At around 60% confluency, cells were transfected with negative control or siRNA against PTEN (ON_TARGET plus siRNA, Dharmacon/ Fisher Scientific, Pittsburgh PA) using Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA) as per manufacturer's instructions. PTEN knockdown was analyzed by immunoblotting 72 hours post infection. siRNA transfection was performed at both basal and suprabasal stage of keratinocyte differentiation.

Fgf10 TOP-like sequence analysis

The entire 5'UTR sequence (675 bp) of *Fgf10* gene was amplified by PCR from mouse genomic DNA and inserted into the HindIII site within the pEGFP-N3 vector (Clontech, Mountain View, CA) to generate the wtTOP plasmid. The mutTOP plasmid contains the same 5'UTR sequence, except the six pyrimidines (CTTTCC) within the TOP-like sequence were mutated to GAAAGG. The primers used for cloning are: wtTOP Forward, GTTAAGCTTGGCTTTCCAAGGGACTTGGAG; mutTOP Forward, CGTTAAGCTTGGGAAAGGAGGGACTTGGAGGTGGAGAG; Reverse, ACCAAGCTTAATGTTTGGATCGTCATGGG. For functional analysis, pEGFP-N3, wtTOP or mutTOP plasmids were transfected into NIH-3T3 cells (60% confluency) using Turbofect reagent (Thermo scientific, Waltham, MA). After 24 hrs, cells were treated with mTOR inhibitors Torin 2 (150 nM; R&D systems, Minneapolis, MN) and Rapamycin (100 nM; Cell Signaling Technology, Beverly, MA) for another 24 hrs before lysed for immunoblotting analysis.

Quantitative Real Time PCR

Total RNA extracted from skin and keratinocyte samples was converted to cDNA using SuperScript®III RT kit (Invitrogen, Carlsbad, CA). qPCR analysis was performed in 10 μl reactions with the SYBR GREEN PCR Master Mix and analyzed on a StepOnePlus™ Real-Time PCR instrument (Invitrogen, Carlsbad, CA). Relative standard curves were generated by serial dilutions and all samples were run in triplicates. Primers used are: *Fgfr1* (GGACACCGAAGGGCTTTTAT and GGTTTTCTTCCAGCCTTTCC), *Fgf2* (GGCTGCTGGCTTCTAAGTCT and TTCCGTGACCGGTAAGTATTG), *Fgf7* (GGGAAATGTTCGTTGCCTTA and CCCTGCTGAATGAAACTGGT), *Fgf10* (CAATGGCAGGCAAATGTATG and GGAGGAAGTGAGCAGAGGTG), *Fgf22* (CGTGTGGACCTTGGTGG and ACACGGACAGAACGGATCTC) and *Gapdh* (AGGTCGGTGTGAACGGATTTG and TGTAGACCATGTAGTTGAGGTCA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Drs. Ruth Ashery-Padan, Michael N. Hall, David Ornitz and Markus A. Rüegg, Pamela Silver for mice and antibody, Raymond L Konger for advice, and members of the Zhang lab for discussions. The work was supported by grants from NIH (EY017061 and EY018868 to XZ). XZ is supported by Jules and Doris Stein Research to Prevent Blindness Professorship.

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HIGHLIGHTS

- *Pten* deletion induces Fgf10 expression in skin epidermis.
- Fgf10 is regulated at the translational level by mTORC1.
- Overexpression of Fgf10 reproduces skin papillomas.
- PTEN deficiency in human cutaneous SCC correlates with higher FGF10 levels.

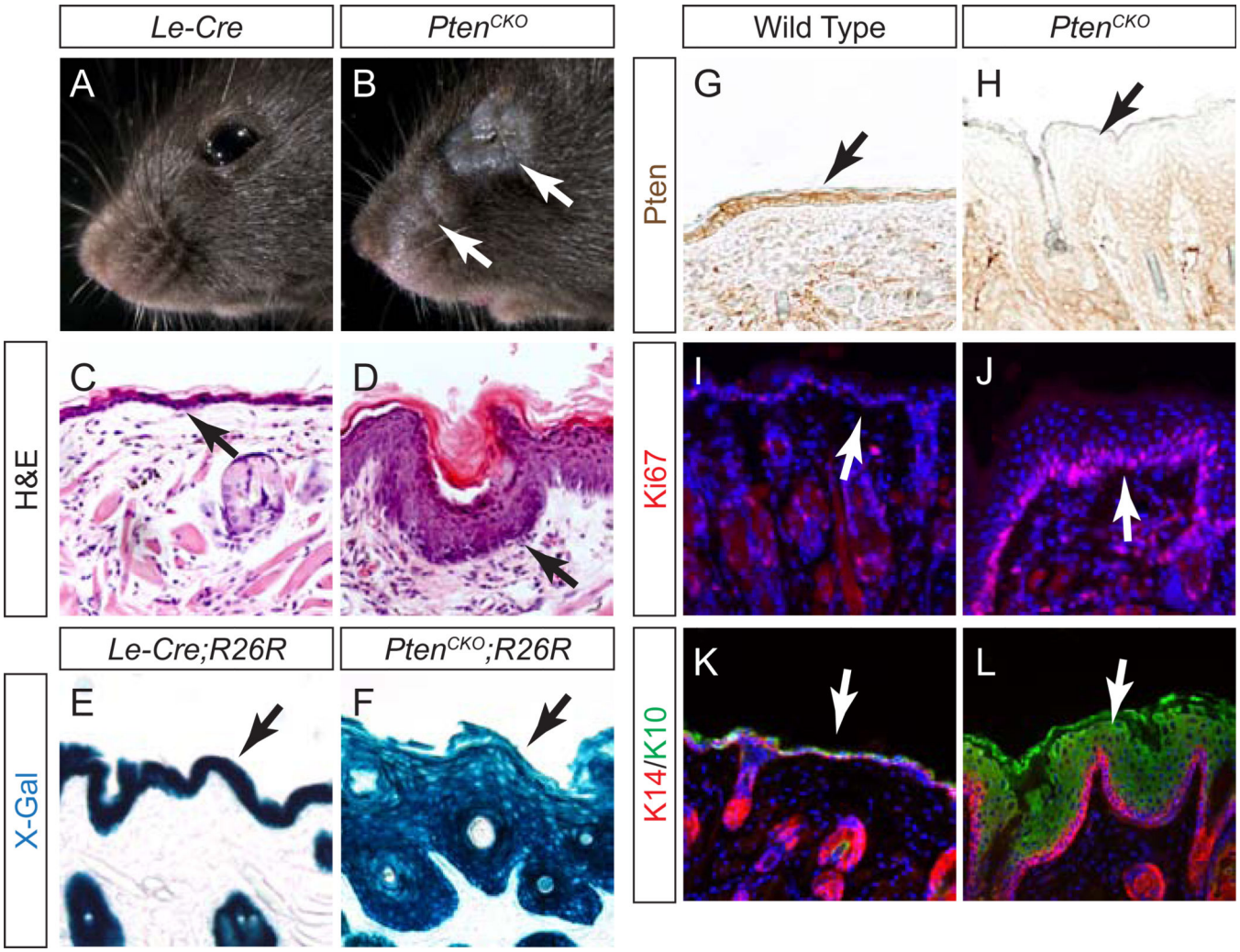

Figure 1. *Pten* loss initiates hyperkeratosis in the facial epidermis

(A and B) 4 week old *Le-Cre;Pten*$^{flox/flox}$ or *Pten*^{CKO} mice displayed hyperkeratosis in cheek and eyelid epidermis (arrows). (C and D) Paraffin sections of the skin revealed the expansion of epidermal layers in *Pten*^{CKO} mice (arrow). (E and F) X-gal staining indicated specific activity of *Le-Cre;R26R* in the epidermis (arrows). (G-L) *Pten*^{CKO} mice exhibited a loss of Pten from the epidermis (H, arrow), increased Ki67 staining denoting proliferation of the basal layer (J, arrow) and expansion of the suprabasal layer shown by K10 positive staining (L, arrow).

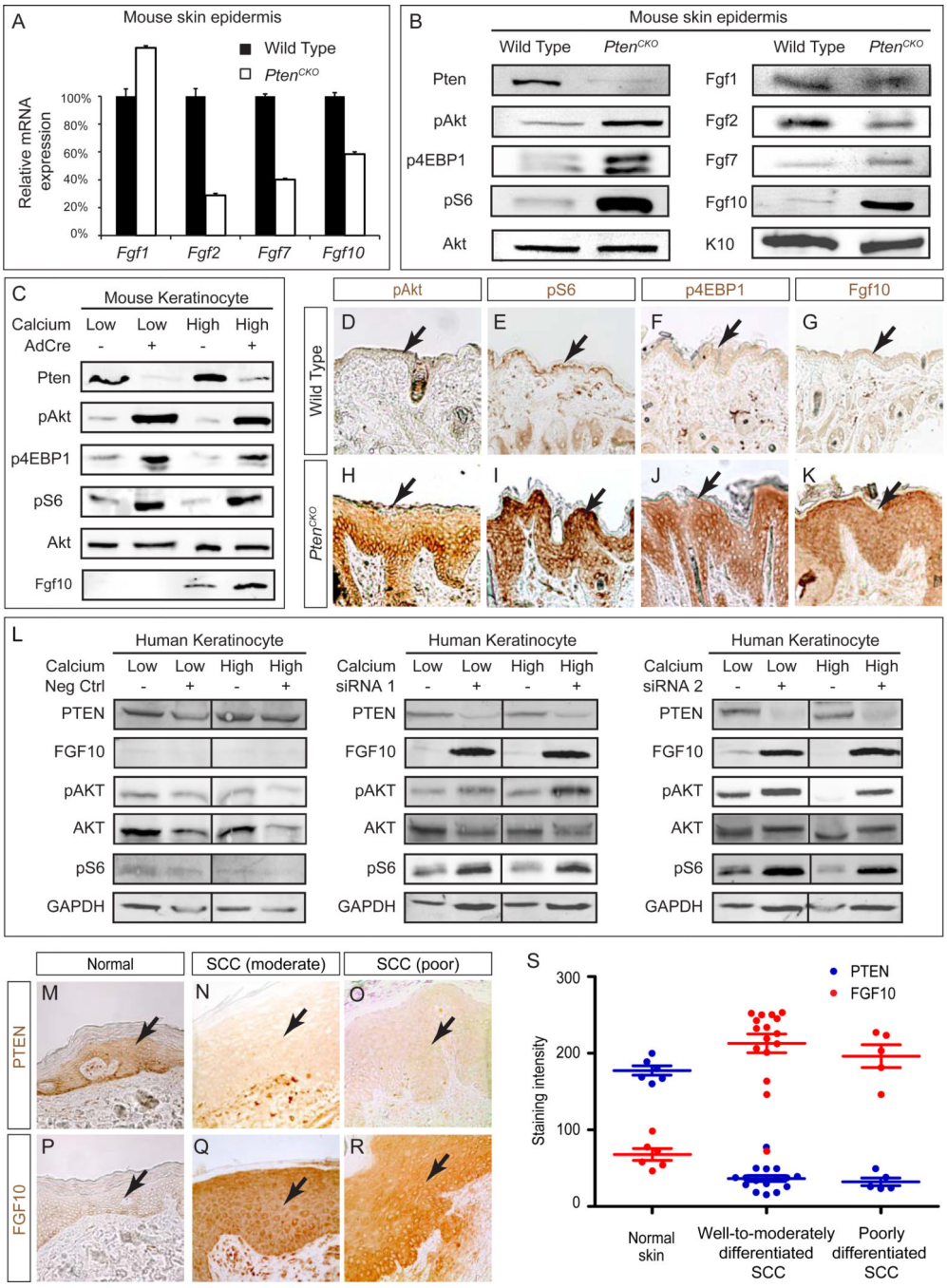

Figure 2. Loss of *Pten* induces Fgf10 protein but not mRNA expression

(A) qPCR analysis of *Fgf* mRNA expressions in the mouse epidermis. (B) Immunoblots of *Pten*^{CKO} mice facial and cheek epidermis showed a reduction in *Pten* levels and up-regulation of pAKT, p4EBP1 and pS6. Although there were little changes in Fgf1, 2 and 7 expression, Fgf10 was strongly up regulated in *Pten*^{CKO} mice. (C) *Pten* depleted keratinocytes switched from low to high Ca^{2+} media (basal to suprabasal state) showed increased levels of pAkt, p4EBP1, pS6 and Fgf10. (D-K) Immunohistochemistry confirmed strong pAKT, pS6, p4EBP1 and Fgf10 staining in the *Pten*^{CKO} facial skin. (L) siRNA

mediated PTEN depletion resulted in significant increase of pAKT, pS6 and FGF10 levels in human primary keratinocytes. (M-S) Both well-to-moderately and poorly differentiated human SCC stained weakly for PTEN but strongly for FGF10 in the epidermal layer. Arrows point to epidermis. Data are presented as mean ± s.e.m.

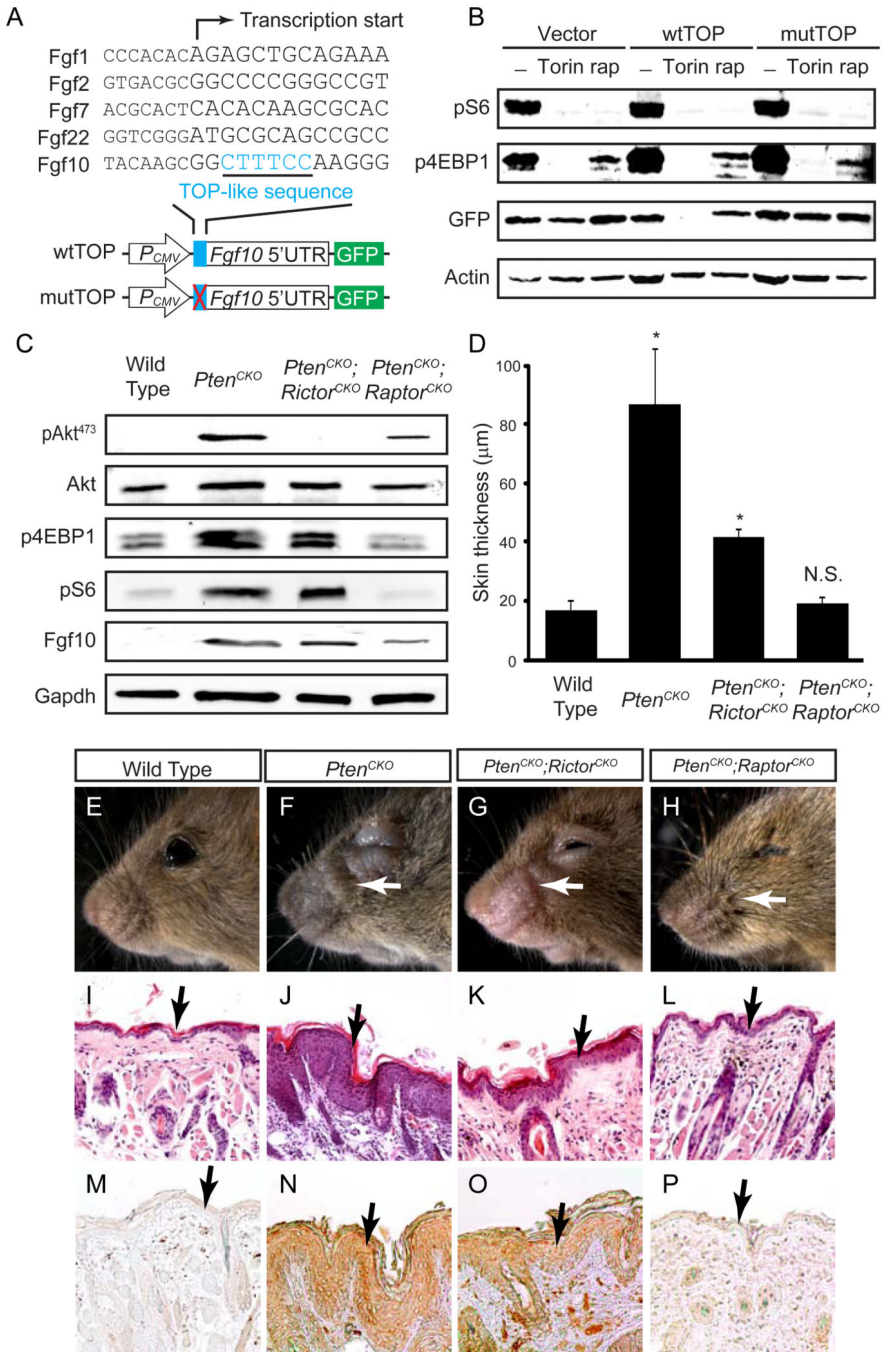

Figure 3. mTOR signaling is required for up regulation of Fgf10

(A) Alignment of RefSeq gene sequences with mouse genome shows that *Fgf10* mRNA has a TOP-like sequence motif, defined as containing at least five pyrimidines within four nucleotides of the transcription start site. The *Fgf10* 5'UTR sequences carrying either wild type TOP-like sequence (wtTOP) or 6 bp transversion mutations (mutTOP) were cloned into GFP reporter vectors. (B) In transfected NIH-3T3 cells, Torin suppresses phosphorylation of S6 and 4EBP1, and expression of GFP in wild type but not mutant *Fgf10* reporters. Rapamycin (rap) treatment, however, abolished S6 phosphorylation, but left 4EBP1

phosphorylation partially reduced and GFP expressions unchanged. (C) A reduction in levels of mTORC1 downstream effectors (p4EBP1 and pS6) and Fgf10 was seen in lysates from $Pten^{CKO}$;$Raptor^{CKO}$ mice. (D-P) Phenotypic comparison of facial skin in wild type (n=9), $Pten^{CKO}$ (n=9), $Pten^{CKO}$;$Rictor^{CKO}$ (n=9) and $Pten^{CKO}$;$Raptor^{CKO}$ (n=3) mice; Rescue of the hyperkeratosis (L) and loss of Fgf10 (P) were observed in $Pten^{CKO}$;$Raptor^{CKO}$ mice. One-way ANOVA test: * $P < 0.001$; N.S., not significant. Data are presented as mean ± s.e.m. White and black arrows point to facial skin (E-H) and epidermis (I-P), respectively.

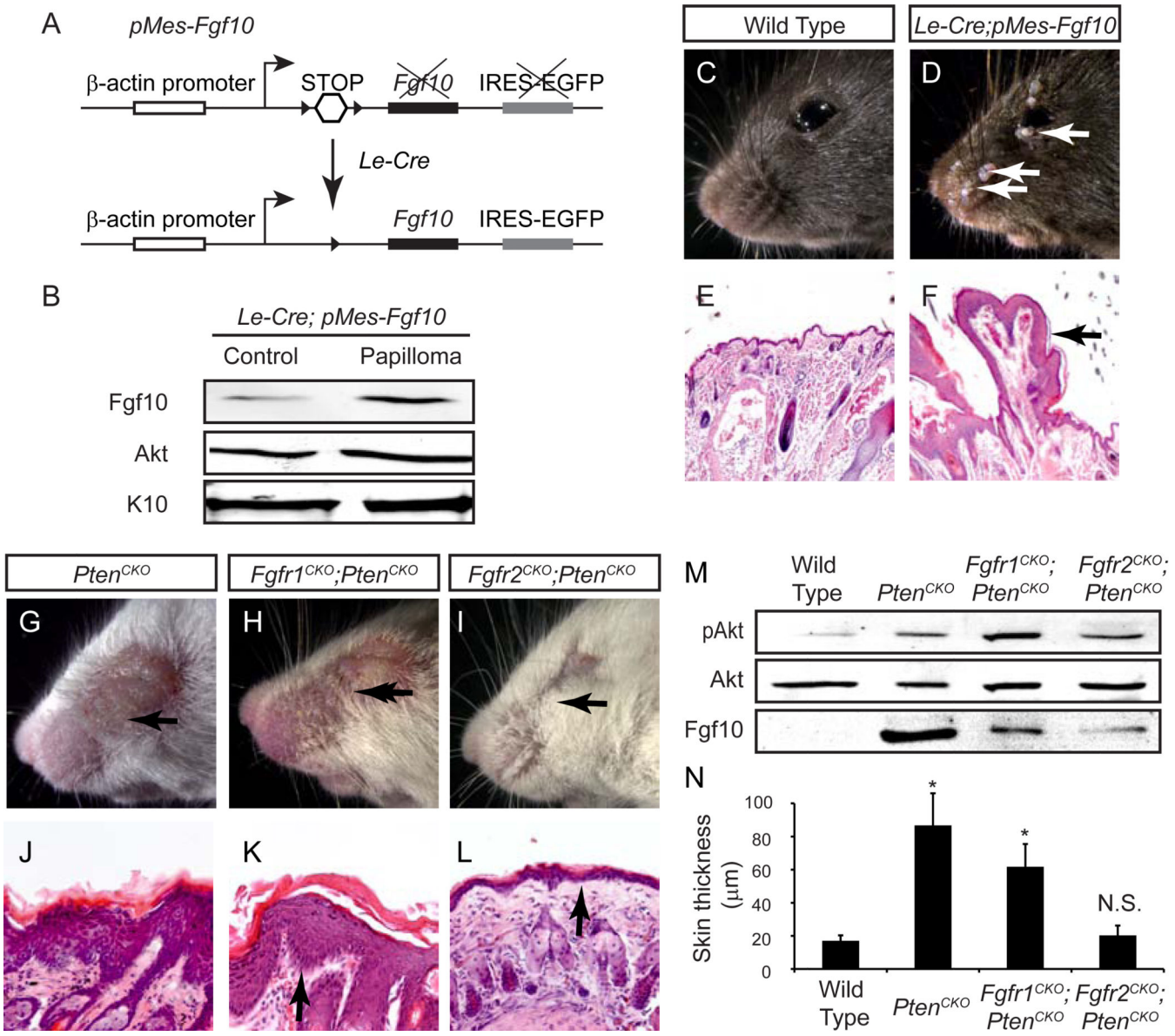

Figure 4. Fgf10-Fgfr2 signaling potentiates Pten-deletion induced SCC

(A) Schematics of *pMef-Fgf10* transgene. After crossing with *Le-Cre* driver, excision of STOP cassette in *pMef-Fgf10* transgene permits constitutive expression of Fgf10 in skin epidermis. (B) Fgf10 expression increased in papilloma obtained from *Le-Cre;pMef-Fgf10* mice. (C-F) Papillomas appeared in *Le-Cre;pMef-Fgf10* cheek and eyelid regions (arrows). (G-L) Epidermal hyperplasia phenotype seen in $Pten^{CKO}$ animals were modestly reduced in $Pten^{CKO};Fgfr1^{CKO}$, but abolished in $Pten^{CKO};Fgfr2^{CKO}$ mice (arrows). (M) Immunoblots showed reduced pAKT and Fgf10 levels in $Pten^{CKO};Fgfr2^{CKO}$ epidermal lysates. (N) Measurements of epidermal thickness in wild type (n=9), $Pten^{CKO}$ (n=9), $Pten^{CKO};Fgfr1^{CKO}$ (n=3) and $Pten^{CKO};Fgfr2^{CKO}$ (n=9) mice are shown as mean ± s.e.m.. One-way ANOVA test: * $P < 0.001$; N.S., not significant.