**Ppp6c deficiency accelerates K-ras<sup>G12D</sup>-induced tongue carcinogenesis**

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**Abstract**

**Background:** Effective treatments for cancer harboring mutant RAS are lacking. In Drosophila, it was reported that PP6 suppresses tumorigenicity of mutant RAS. However, the information how PP6 regulates oncogenic RAS in mammals is limited.

**Methods:** We examined the effects of PP6 gene (Ppp6c) deficiency on tongue tumor development in K (K-rasG12D)- and KP (K-rasG12D + Trp53-deficient)-inducible mice.

**Results:** Mice of K and KP genotypes developed squamous cell carcinoma in situ in the tongue approximately 2 weeks after the induction of Ppp6c deficiency and was euthanized due to 20% loss of body weight. Transcriptome analysis revealed significantly different gene expressions between tissues of Ppp6c-deficient tongues and those of Ppp6c wild type, while Trp53 deficiency had a relatively smaller effect.
1 | INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) has a yearly incidence of 60,000 cases worldwide, with 40%–50% mortality. In HNSCCs that are HPV (−), EGFR/ERBB2, or EGFR1/3 alterations were most frequent among receptor tyrosine kinases. Kinase targets, such as HRAS, PI3CA, and PTEN, also showed mutations implying perturbed RTK/RAS/P3K signaling in HNSCC carcinogenesis. Moreover, the tumor suppressor Trp53 was mutant in 84% of HNSCCs that were HPV (−).1,2

Currently, effective treatments for cancers harboring mutations in RAS family genes (HRAS, NRAS, and KRAS) are lacking, partly because of an incomplete understanding of signaling within those tumors. A recent large-scale ethyl methanesulfonate (EMS)-induced genetic screen in Drosophila identified loss of protein phosphatase 6 (PP6) as cooperating with oncogenic Ras to induce tumor cell proliferation and invasion, suggesting that PP6 serves as a tumor-suppressor in RAS-related cancers. PP6 is a member of Ser/Thr protein phosphatases that binds to any one of the three regulatory proteins PP6R1, PP6R2, and PP6R3, which confer substrate specificity. Diverse phenotypes seen following siRNA-based Ppp6c knockdown in cultured mammalian cells suggest that PP6 regulates mitosis by dephosphorylating Aurora kinase A, activates DNA-PK to sensitize cells to ionizing radiation, and is required for homology-directed repair. There is also evidence that PP6 regulates NFκB signaling by blocking IkBe degradation in response to TNF and inactivating TAK1.

Accumulating pathological evidence suggests that Ppp6c may function as a tumor suppressor. The PP6 gene (Ppp6c) is reportedly mutated in 12% of human melanoma tissues11 and 15% of human skin basal cell carcinoma tissues, and PP6 expression is known to be repressed in some solid tumors. Some human breast cancers also show decreased protein levels of PP6, PP6R1, PP6R2, and PP6R3. Furthermore, there is evidence that Ppp6c is epigenetically regulated. For example, in psoriasis patients, miR-31,13 which is transcriptionally enhanced by NFκB, suppresses Ppp6c expression and promotes abnormal epidermal cell proliferation. Hepatocellular carcinoma cells also reportedly undergo hyperproliferation following Ppp6c suppression by upregulated miR-373 expression.14

We previously assessed Ppp6c function in a mouse model of skin carcinogenesis and found that Ppp6c loss in keratinocytes promoted 7,12-dimethylbenz[a]anthracene (DMBA)-induced papilloma formation and UVB-induced carcinogenesis. These findings support the idea that Ppp6c acts as a tumor suppressor in mouse skin cancers. Here, we analyzed PP6 function in HNSCCs by assessing mouse tongue carcinogenesis. To do so, we asked whether Ppp6c deficiency enhanced tongue carcinogenesis in K-ras G12D and Trp53-null mouse models.

2 | MATERIALS AND METHODS

2.1 | Generation of mice with inducible Kras G12D expression, Trp53 deletion, and Ppp6c deletion

The mouse strain Ppp6c−/− has been described. ROSA26-CreERT2 mice were obtained from Taconic Bioscience. K-rasLSL-G12D+/+ mice and Trp53lox/lox mice were obtained from the Jackson Laboratory.

Rosa26-CreERT2 mice were crossed with K-rasLSL-G12D+/+ mice to generate ROSA26-CreERT2/K-rasLSL-G12D+/+ mice, which were then bred with Ppp6c−/− mice to generate ROSA26-CreERT2/K-rasLSL-G12D+/+Ppp6c−/− mice. These mice were further crossed with Ppp6c−/− to generate mice of the following three genotypes: ROSA26-CreERT2/K-rasLSL-G12D+/+Ppp6c−/− (designated K[F/F] mice), ROSA26-CreERT2/K-rasLSL-G12D+/Ppp6c−/− (designated
We also crossed ROSA26-CreER\textsuperscript{T2}/K-ras\textsuperscript{LSL-G12D+/−}/Ppp6c\textsuperscript{lox/flx+} mice with Trp53\textsuperscript{flx/flx} mice to obtain ROSA26-CreER\textsuperscript{T2}/K-ras\textsuperscript{LSL-G12D+/−}/Trp53\textsuperscript{flx/flx}/Ppp6c\textsuperscript{lox/flx+} mice. Following the crossing of ROSA26-CreER\textsuperscript{T2}/K-ras\textsuperscript{LSL-G12D+/−}/Trp53\textsuperscript{flx/flx}/Ppp6c\textsuperscript{lox/flx+} with Trp53\textsuperscript{flx/flx}/Ppp6c\textsuperscript{lox+} mice, we obtained mice of three genotypes: ROSA26-CreER\textsuperscript{T2}/K-ras\textsuperscript{LSL-G12D+/−}/Trp53\textsuperscript{flx/flx}/Ppp6c\textsuperscript{lox/flx+} (designated KP[F/F] mice), ROSA26-CreER\textsuperscript{T2}/K-ras\textsuperscript{LSL-G12D+/−}/Trp53\textsuperscript{flx/flx}/Ppp6c\textsuperscript{lox+} (designated KP[F/+] mice) and ROSA26-CreER\textsuperscript{T2}/K-ras\textsuperscript{LSL-G12D+/−}/Trp53\textsuperscript{flx/flx}/Ppp6c\textsuperscript{lox+} (designated KP[+/+] mice) (Figure 1A right). Littermates served as controls. All animal experiments were performed with approval of the Miyagi Cancer Center Research Institute Animal Care and Use committee (MCCAE-2020-1).
2.2 | 4-Hydroxytamoxifen treatment

4-Hydroxytamoxifen (4HT), purchased from Toronto Research Chemicals, was used to induce CreER<sup>T2</sup>-dependent recombination, as reported.<sup>18</sup> Entire upper surface of tongues of 8-week-old mice was painted with 4HT at 10 mg/ml in ethanol three times every other day for a week.

2.3 | PCR genotype analysis

To confirm exon 4 deletion from floxed Ppp6c, we used primers: (a) 5′-TATCACGAGGCGCTTTCG-3′; (b) 5′-TAGTGAACCTTCTCGAGG-3′ (Figure 1B). To confirm excision of the loxP-flanked transcription stop cassette from the LSL-K-ras<sup>G12D</sup> allele, we used primers; (c) 5′-GTCTTTCCCCAGACAGTGC-3′; (d) 5′-CTCTTGCTTACGCAAGTGC-3′; (e) 5′-AGCTAGCCACATGGCAGTAGTCGCC-3′ (Figure 1C). To confirm the deletion of exons 2–10 from the p53-floxed allele, we used primers; (f) 5′-GGTTAAACCCAGCTTGACCA-3′; (g) 5′-GGAGGAGGAGACAGTTGGAG-3′ (Figure 1D).

2.4 | Phosphoprotein microarray analysis

Phospho Explorer Antibody Microarray designed by Moon BioSystems, was used. For more information, see Doc S1.

2.5 | Histopathology and immunohistochemistry

Histopathology and immunohistochemistry were performed as previously described.<sup>15</sup> For immunohistochemistry, the following antibodies were used: anti-cytokeratin 5 (CK5) (#53121), anti-Ki-67 (#15580), anti-p-Erk1 (#28818) and anti-p-Akt (#38499), all from Abcam; and anti-γH2AX (#9718), anti-p-Erk1/2 (#4370), anti-p-4EBP1 (#2855), anti-MCM2 (#3619) and anti-NFκB p65 (RelA) (#8242), all from Cell Signaling Technology. e-Count2 software was used to count Ki-67- and MCM2-positive nuclei. Phosphorylation levels of ERK1/2 and AKT were evaluated by assessing the intensity of immunoreactivity in the cytoplasm on a 0 to 4+ scale. A pathologist (T.T.) determined the presence of SCCIS epithelial lesions based on the location of cancer cells in the whole epithelium but lack of invasivity.<sup>21</sup>

2.6 | RNA preparation and sequencing

Total RNA was extracted from fresh frozen tissue using RNeasy Plus Universal Mini Kit (QIAGEN). For more information, see Doc S1.

2.7 | Transcriptome analysis

To evaluate expression levels of differentially expressed genes between groups, transcriptome analysis was performed. For more information, see Doc S1.

2.8 | Statistical analysis

Kaplan–Meier survival curves and corresponding statistical analysis, as well as log-rank tests, were performed using Prism version 8 (GraphPad Software Inc.). Other assessment of statistical significance was performed using Student’s <i>t</i> test. <i>p</i> < 0.05 served as the cut-off for significance.

3 | RESULTS

3.1 | Ppp6c deficiency promotes significant tongue thickening and early death in K and KP mice

Starting with 4HT-inducible KRAS(G12D) expressing mice (K-ras<sup>LSL-G12D+/+</sup>; K mice), we generated three lines: mice also homozygous for the Ppp6c<sup>flox</sup> allele, heterozygous for that allele (Ppp6c<sup>flox+/+</sup>), and wild-type (Ppp6c<sup>+/+</sup>) mice and designated them K(F/F), K(F/+) and K(+/+) respectively (Figure 1A left). We also generated 4HT-inducible KRAS(G12D) expressing plus 4HT-inducible Trp53 deficient mice (K-ras<sup>LSL-G12D+/-</sup>/Trp53<sup>flox</sup>; KP mice), which have the same three Ppp6c<sup>flox</sup> genotypes, KP(F/F), KP(F/+) and KP(+/+) (Figure 1A right).

When mice were 8 weeks old, we used a brush to apply 4HT to the entire upper surface of the tongues of K(F/F) and KP(F/F) mice and assessed genomic recombination in tongue tissue a week later. In all groups, we observed the recombination of the Ppp6c<sup>flox</sup> (Figure 1B), K-ras<sup>LSL-G12D</sup> (Figure 1C), and Trp53<sup>flox</sup> (Figure 1D) alleles. Mice of other genotypes, including K(F/+), K(+/+), KP(F/+) and KP(+/-), were similarly treated with 4HT, and we observed Ppp6c<sup>flox</sup>, K-ras<sup>LSL-G12D</sup> and Trp53<sup>flox</sup> recombination in tongue tissue in all cases (data not shown).

We then checked body weight every day up to 30 days after the first 4HT application to the tongue (Figure 2) and euthanized mice that had lost 20% of body weight. K(F/F) mice showed a statistically significant weight loss of ~20% at around day 13 after induction and were euthanized (Figure 2A), while the rate of weight loss in K(+/+) relative to K(F/F) mice was slower and differed in a statistically significant manner. Weight loss in K(F/+) mice occurred at a rate midway between that seen in K(F/F) and K(+/-) mice (Figure 2A). By 13 days after 4HT induction, the tongues of K(F/F) mice grossly showed thickening across the ventral and dorsal
sites (Figure 2B, Figure S1A). On the H&E stained tongue tissue from mice with Ppp6c homozygous deletion in the presence of K-ras<sup>G12D</sup>, the entire squamous epithelium was markedly thickened in comparison to that of mice expressing K-ras<sup>G12D</sup> alone which was indistinguishable from wild-type tongues (Figure 2C, Figure S1B).

In KP mice, effects of Ppp6c deficiency were similar to those seen in K mice: relative to Ppp6c (+/+) and (+/+) KP mice, Ppp6c (F/F) KP mice lost weight more rapidly and that difference was statistically significant (Figure 2D). Gross appearance and histopathological findings of the 4HT-treated KP(F/F) tongue (Figure 2E,F) were comparable to phenotypes seen in K(F/F) mice (Figure 2B,C).

Autopsy analysis performed to determine the cause of weight loss in 4HT-treated K(F/F) and KP(F/F) mice showed that their stomachs contained less food content than did stomachs of 4HT-treated K(+/+) and KP(+/+) mice. Macroscopic examination revealed no tumors in the esophagus, stomach, or intestine. These observations suggest that large tongue tumors interfere with feeding, causing mice to become asthenic.

### 3.2 Ppp6c deficiency induces squamous cell carcinoma in situ in tongues of K and KP mice

To assess morphological alterations and growth of tongue epithelial cells, we next performed immunohistochemistry analysis. As shown in Figure 3A, which are high power of views of Figure 2C, tumorous lesions were observed in the 4HT-treated
K(F/F) tongue tissue stained with H&E. In lesions found in 4HT-treated K(F/F) tongue, atypical cells throughout the epithelium showed loss of cell polarity, although we did not observe Stromal invasion of these cells (Figure 3A). Immunohistochemical staining using antibodies, two cell proliferation markers Ki-67 and MCM2, and the squamous cell marker CK5 on serial sections showed nuclear positive reactivities of Ki-67 and MCM2 and cytoplasmic positivity of CK5 throughout the tongue epithelium, counted using samples shown in (A) and from an immunohistogram obtained by staining sections of wild-type tongue (used in Figure 2C, bottom) with Ki-67 and MCM2 antibodies. In each sample, Ki67-positive cells in a ~1 mm wide region of the epidermis were counted. Data are means derived from six areas in independent samples ±SE. ****p < 0.0001

K(F/F) tongue tissue stained with H&E. In lesions found in 4HT-treated K(F/F) tongue, atypical cells throughout the epithelium showed loss of cell polarity, although we did not observe stromal invasion of these cells (Figure 3A). Immunohistochemical staining using antibodies, two cell proliferation markers Ki-67 and MCM2, and the squamous cell marker CK5 on serial sections showed nuclear positive reactivities of Ki-67 and MCM2 and cytoplasmic positivity of CK5 throughout the tongue epithelium of the 4HT-treated K(F/F) mice, suggesting squamous cell carcinoma in situ (SCC in situ/SCCIS). In addition, the numbers of Ki-67 and MCM2-positive cells of the K(F/F) tongue epithelium were significantly higher than those of the wild-type and K(+/+) tongues, both being comparable (Figure 3B). Similar tumorous lesions emerged in the 4HT-treated KP(F/F) tongue (Figure 4) and were also diagnosed as SCCIS. Immunohistochemical findings were seen in the 4HT-treated KP(F/F) tongue and those of K(F/F) were comparable (Figures 3a and 4a). Mild dysplasia developed in the tongue of F/F mice 1 week after 4HT application, and by 13 days, tongue SCCIS developed in all F/F mice that showed a decrease in body weight of 20% or more. Incidences of thickened squamous epithelium and SCCIS were 100% and 100%, respectively, by 13 days. Various degrees of squamous dysplasia occurred in all thickened squamous epithelium. We observed SCCIS in some areas of dysplastic lesions based on loss of cell polarity, severe nuclear atypia, and abnormal mitoses. These results indicate that Ppp6c loss promotes the development of SCCIS in squamous cells expressing K-rasG12D within 13 days and that Trp53 status has a minimal effect on the process.

Next, we examined gene expression in K(F/F) and KP(F/F) tumors (Figure 5). For these experiments, we treated 3 K(F/F) mice, 3 K(+/+) mice, 4 KP(F/F) mice, 4 KP(+/+) mice, plus three normal control C57BL/6 mice in the same manner described in Figure 1. Figure 5 shows a heat map representing gene expression in the epithelium of tongue tissue from each genotype, indicating that expression patterns of K and KP mice differed significantly between Ppp6c wild-type and -deficient mice. Again, the effect of Trp53 deficiency was relatively small compared to that of Ppp6c deficiency.
3.3 Identification of signaling pathways activated by Ppp6c deficiency in K and KP mice

To identify signaling pathways underlying SCCIS development over a 2-week timeframe, we characterized pathways commonly altered by Ppp6c loss in both K and KP mice. Table 1 shows signaling pathways altered by Ppp6c loss in the presence of K-ras<sup>G12D</sup> in both K and KP mice. Those significantly (p < 0.05) altered are shown in order of decreasing p value in K mice. Of particular interest were ‘Pathways in Cancer’ (ranked 16 in Table 1) and ‘Cytokine-cytokine receptor interaction’ (ranked 1 in Table 1), as these pathways are linked to RAS and PP6, respectively.

3.4 Ppp6c deletion activates ERK-ELK1-FOS and PI3K-AKT-CDK/cyclin pathways in the tongue of K and KP mice

ERK and AKT are major RAS effectors governing cell proliferation and growth. Thus, we screened for proteins that show increased phosphorylation in tongue epithelia of K(F/F) relative to K(+/+) mice on day 13 after 4HT administration using a phosphoprotein antibody array (Table S1). That analysis indicated significant phosphorylation of RAS/MAPK pathway proteins (Table S1A), with increased levels of phosphorylation of MEK1 (T286, T291, S298), ERK1/2 (T202), Elk1(S389), and p90<sup>RSK</sup> (T359/S363, T573) seen in 4HT-treated K(F/F) tongue tissue relative to that seen in K(+/+) animals. Immunohistochemistry analysis showed strong staining for phosphorylated ERK1/2 (T202/Y204) throughout the epithelium and phosphorylated Elk1 (S389) primarily in the nucleus in 4HT-treated K(F/F) tongue, while staining for both was weak in tongue tissues from K(+/+) mice (Figure 6A,B). Figure 6C compares the expression of MAPK signaling factors expressed in 4HT-treated K(F/F) versus the K(+/+) tongue and indicates a significant increase in expression of MKP factors (Dusp6, Dusp4, Dusp9, Dusp7, and Dusp5), which function in negative feed-back to block activated ERK signaling. We also observed an increase in Fos, a target of Elk1<sup>23</sup> (Figure 6C). In addition, the expression of an EGFR ligand, Areg (Amphiregulin), was also elevated (Figure 6C). Finally, in
KP mice, the effect of Ppp6c deletion on the phosphorylation of ERK and Elk1 (Figure S2A) was comparable to effects seen in K mice (Figure 6).

Relevant to the PI3K-AKT pathway, antibody array analysis of 4HT-treated K(F/F) mice showed the hyperphosphorylation of PDK1(S241), AKT1(T72, S124, T308, Y326, S473), TSC(S939), mTOR(S2481), and 4EBP1(S65) (Table S1B). Thus, we examined phosphorylation levels of AKT1 (T308) and 4EBP1(S65) in 4HT-treated K(F/F) and K (+/+) mice (Figure 7). AKT1 and 4EBP1 were highly phosphorylated in 4HT-treated K(F/F) relative to K(+/+) tongue (Figure 7A,B). It is of note that expression of Eif4ebp1 (4EBP1) itself was also upregulated in K(F/F) tongue (Figure 7C). These suggest protein synthesis in the K(F/F) tongue was potentially enhanced.24

Figure 7C also indicated an increase in Ccnd1 (CyclinD1) and a decrease in Cdkn1b (p27Kip1) in 4HT-treated K(F/F) tongue, expression of both is reportedly regulated...

FIGURE 5  Heat map analysis of gene expression in the 4HT-treated tongue of K(F/F), K(+/+), KP(F/F), KP(+/+), and control mice. K(F/F) (n = 3), K(+/+) (n = 3), KP(F/F) (n = 4), and KP(+/+) (n = 4) mice plus normal (n = 3) control C57BL/6 (designated as [+/-]) mice were painted with 4HT at 10 mg/ml in ethanol on the tongue three times every other day for a week, and then 13 days later epithelial tongue tissue was harvested for mRNA preparation and transcriptomic analysis. The heat map was generated as described in Materials and Methods. A total of 15,921 genes expressed in at least one of the groups in each comparison were used for this analysis. The heat map shows differentially expressed mRNAs in K and KP mice with Ppp6c deficiency (F/F) or wild-type (+/+), and control (C57BL/6) mice. Red and green colors indicate high and low expression levels, respectively.
| Rank | KEGG-pathway                                  | K mice # genes (DE/ALL) | p-value | KP mice # genes (DE/ALL) | p-value |
|------|-----------------------------------------------|-------------------------|---------|--------------------------|---------|
| 1    | Cytokine-cytokine receptor interaction         | 68/165                  | 1.12E-08| 93/169                   | 1.05E-06|
| 2    | Protein digestion and absorption              | 32/58                   | 2.09E-08| 40/58                    | 1.30E-05|
| 3    | ECM-receptor interaction                      | 34/66                   | 1.10E-07| 43/66                    | 1.53E-04|
| 4    | Mucin type O-glycan biosynthesis              | 16/21                   | 1.34E-07| 13/21                    | 0.041   |
| 5    | Metabolic pathways                            | 302/1118                | 2.99E-06| 616/1115                 | 2.71E-24|
| 6    | Hypertrophic cardiomyopathy (HCM)             | 30/64                   | 5.44E-06| 46/64                    | 4.46E-07|
| 7    | Neuroactive ligand-receptor interaction       | 44/109                  | 1.82E-05| 72/116                   | 8.60E-08|
| 8    | Focal adhesion                                | 61/173                  | 2.63E-05| 87/173                   | 0.02    |
| 9    | Biosynthesis of amino acids                   | 26/57                   | 4.11E-05| 38/57                    | 6.88E-05|
| 10   | Arginine and proline metabolism              | 20/40                   | 6.33E-05| 31/40                    | 2.47E-06|
| 11   | Small cell lung cancer                        | 31/86                   | 7.20E-05| 47/86                    | 3.61E-04|
| 12   | Drug metabolism—cytochrome P450               | 19/38                   | 9.63E-05| 29/38                    | 9.03E-06|
| 13   | Dilated cardiomyopathy (DCM)                  | 31/69                   | 1.31E-04| 50/69                    | 7.08E-06|
| 14   | Arrhythmogenic right ventricular cardiomyopathy (ARVC) | 26/59                  | 1.34E-04| 40/59                    | 1.83E-04|
| 15   | Retinol metabolism                            | 16/32                   | 3.41E-04| 21/33                    | 0.007   |
| 16   | Pathways in cancer                            | 122/435                 | 6.32E-04| 206/441                  | 0.006   |
| 17   | Calcium signaling pathway                     | 46/126                  | 6.62E-04| 78/132                   | 9.22E-05|
| 18   | DNA replication                               | 16/34                   | 8.05E-04| 21/34                    | 0.011   |
| 19   | Glutathione metabolism                        | 20/47                   | 9.36E-04| 31/46                    | 2.37E-04|
| 20   | Metabolism of xenobiotics by cytochrome P450  | 16/36                   | 0.002   | 24/37                    | 0.003   |
| 21   | Alanine, aspartate and glutamate metabolism  | 13/27                   | 0.002   | 17/27                    | 0.017   |
| 22   | Glycolysis/glucoseoneogenesis                 | 21/53                   | 0.002   | 33/53                    | 0.001   |
| 23   | Purine metabolism                             | 35/103                  | 0.002   | 64/103                   | 9.43E-06|
| 24   | Drug metabolism—other enzymes                 | 20/50                   | 0.002   | 29/51                    | 0.015   |
| 25   | beta-Alanine metabolism                       | 13/28                   | 0.003   | 22/28                    | 5.23E-05|
| 26   | Chemical carcinogenesis                       | 17/41                   | 0.003   | 27/43                    | 0.003   |
| 27   | Regulation of actin cytoskeleton              | 51/175                  | 0.004   | 94/177                   | 0.002   |
| 28   | Pyrimidine metabolism                         | 18/46                   | 0.005   | 26/46                    | 0.022   |
| 29   | Hematopoietic cell lineage                    | 20/54                   | 0.007   | 36/55                    | 1.88E-04|
| 30   | Mineral absorption                            | 16/39                   | 0.007   | 24/39                    | 0.038   |
| 31   | Histidine metabolism                          | 9/18                    | 0.007   | 13/18                    | 0.007   |
| 32   | Malaria                                       | 16/35                   | 0.008   | 23/35                    | 0.003   |
| 33   | Glycine, serine and threonine metabolism      | 13/31                   | 0.008   | 20/31                    | 0.006   |
| 34   | Adrenergic signaling in cardiomyocytes        | 38/117                  | 0.009   | 64/117                   | 0.005   |
| 35   | Salivary secretion                            | 23/66                   | 0.01    | 45/66                    | 6.48E-05|
| 36   | Arachidonic acid metabolism                   | 18/49                   | 0.011   | 27/49                    | 0.03    |
| 37   | Cysteine and methionine metabolism            | 15/39                   | 0.012   | 25/40                    | 0.005   |
| 38   | cAMP signaling pathway                        | 45/143                  | 0.013   | 79/147                   | 0.003   |
| 39   | Cell adhesion molecules (CAMs)                | 28/90                   | 0.021   | 47/91                    | 0.023   |
| 40   | Pyruvate metabolism                           | 12/32                   | 0.029   | 24/32                    | 8.92E-05|

(Continues)
by AKT via FOXO. 24 We also found the upregulation of Ccne1 (CyclinE1) and Cdk6, both part of the CDK complex which is activated by AKT through the phosphorylation/inhibition of p21 and p27 24 (Figure 7C). These data suggest that AKT signaling is up-regulated and the cell cycle is likely accelerated in 4HT-treated K(F/F) relative to K(+/+) tongue tissues. Again, effects on the phosphorylation of AKT and 4EBP1 seen following Ppp6c deletion in KP mice (Figure S2B) were comparable to those seen in K mice (Figure 7).

3.5 | Ppp6c deficiency promotes the accumulation of DNA damage and NFκB pathway activation in the tongue tissue of K and KP mice

We next assessed the effects of Ppp6c deficiency on DNA repair, as PP6 reportedly functions in DNA-PK-mediated repair of non-homologous end-joining (NHEJ). 8 Antibody array data relevant to phospho-proteins associated with DNA repair pathways are shown in Table S1C. Levels of

### TABLE 1 (Continued)

| Rank | KEGG-pathway | K mice | KP mice |
|------|--------------|--------|--------|
| 41   | Influenza A  | 39/123 | 63/121 |
| 42   | Steroid hormone biosynthesis | 9/22 | 17/24 |
| 43   | Tryptophan metabolism | 11/29 | 20/28 |
| 44   | Type I diabetes mellitus | 8/26 | 14/25 |
| 45   | Insulin secretion | 22/63 | 39/63 |
| 46   | Cell cycle | 34/114 | 62/114 |

Abbreviations: ALL, total proteins in each pathway; DE, differentially-expressed proteins. p < 0.05 values are shown in order of decreasing value.

**FIGURE 6** Ppp6c deletion activates the ERK-ELK, AKT-4EBP1, and CDK/cyclin axes in the 4HT-treated tongue tissue of K mice. Activation of the ERK-ELK axis. (A, B) Microscopic analysis of the 4HT-treated tongue of K(F/F) and K(+/+) mice, from tissues shown in Figure 2C. Immunohistochemistry was performed using anti-phospho ERK1/2 (A) and anti-phospho Elk1 (B) antibodies. Scale bar: 100 μm. The insets with a scale bar in immunohistochemistry images for K(F/F) tongues is enlarged (×3) one corresponding to the squares in the main image panels. Scale bar: 33.3 μm. (C) Transcripts of components of the MAPK signaling pathway (KEGG 4010) are affected by Ppp6c deletion in the tongue tissue of 4HT-treated K-mice. mRNA was extracted from tongue tissues of 4HT-treated K(F/F) and K(+/+) mice, and RNA-seq performed as described in Methods. Figure was generated using iPathwayGuide (Advaita Bioinformatics) software. Log FC: log of fold-change in gene expression. Box and whisker plot: box ends are upper and lower quartiles and the span represents the interquartile range. Horizontal line inside the box is the median, and whiskers indicate highest and lowest observations. The level of intensity corresponds to the level of upregulation (red) or downregulation (blue) of the differential genes in the 4HT-treated K(F/F) tongue versus those of K(+/+). Black arrows indicate negative regulators of the ERK pathway. Red arrow indicates Fos, a target of Elk1. Red arrowhead indicates Areg (Amphiregulin).
phosphorylated DNA-PK (T2638), Histone H2AX (S139), BRCA1 (S1457, S1524) ATRIP (S68/72), Chk1 (S286), and Chk2 (T68, T383) increased in 4HT-treated K(F/F) relative of K(+/-) tongue, suggesting enhanced DNA damage and activation of DNA repair pathways. As confirmation, we performed immunohistochemical analysis and observed significant accumulation of γH2AX in 4HT-treated K(F/F) tongue, greater than that seen in K(+/-) tissue (Figure 8A,B). KRAS(G12D) reportedly promotes DNA breaks by inducing reactive oxygen species (ROS); however, the effect of KRAS(G12D) on γH2AX positive number in 4HT-treated K(+/-) tongue compared to those of wild type was marginal (Figure 8B), suggesting that Ppp6c deficiency induces significant DNA damage in tongue tissue expressing K-rasG12D. Similar effects of Ppp6c deletion were seen when we conducted this analysis in KP mice (data not shown).

Relevant to B signaling, previous studies show that PP6 specifically dephosphorylates IκBε. Our phosphoprotein analysis showed that IκBε (S22) phosphorylation was particularly up-regulated in 4HT-treated K(F/F) compared to K(+/-) tongue tissue (Table S1D). Since IκBε phosphorylation promotes its degradation and allows RelA to enter the nucleus, we undertook the immunohistological analysis of tongue tissues for the presence of RelA (Figure 8C). RelA was much more abundant than in 4HT-treated K(F/F) compared to K(+/-) tissue, and its localization was primarily nuclear and seen in cells mainly in the upper part of the epithelium (Figure 8C). Figure 8D shows the ratio of gene expression associated with the NFκB pathway in 4HT-treated K(F/F) versus K(+/-) tongue tissue.

It is noteworthy that expression of the RelA targets IL-1β, PtgS2(COX2) and TNF was markedly enhanced in 4HT-treated K(F/F) tongue relative to K (+/+) controls (Figure 8D). Finally, KP mice showed comparable effects of Ppp6c deletion on RelA localization and target gene expression (data not shown).

4 | DISCUSSION

Here, we examined the effect of Ppp6c deficiency on tumorigenesis in mice with tongue-specific expression of K-rasG12D (K-mice) as well as in K-rasG12D mice deficient in Trp53 (KP-mice). Both K and KP mice developed intraepithelial carcinoma of the tongue and had to be killed approximately 2 weeks after the induction of K-rasG12D, Trp53, and Ppp6c mutations. Histopathological analyses performed 2 weeks after induction indicated that Ppp6c deficiency more strongly drove K-rasG12D-initiated tumorigenesis than did Trp53 deficiency.

Signaling pathways significantly (p < 0.05) deregulated by Ppp6c deletion in the presence of K-rasG12D are shown in Table 1. Among them, ‘Pathways in Cancer’ includes intrinsic factors underlying tumorigenesis and associated with: (a) sustained angiogenesis; (b) evasion of apoptosis; and (c) proliferation. As shown in Figure S3, all three activities are upregulated by transcription factors (c-myc and c-fos), cell cycle regulators (CDK/cyclin and E2F), and COX2 in different ways but all depend on ERK/AKT/NFκB signaling, which was activated by Ppp6c deletion in the K(F/F) tongue. It is noteworthy that DNA replication was
upregulated (ranked 18 among KEGG pathways) and that 16 proteins associated with DNA replication were upregulated (Figure S4A). Cell cycle was also activated (ranked 46) as shown in Figure S4B. It is also notable that upregulated DNA replication requires a large supply of nucleotides and proteins. Overall, metabolic changes associated with nucleotides and amino acids (ranked 5) were characteristic of 
Ppp6c
deficiency. Relevant to nucleotides, purine (ranked 23) and pyrimidine (ranked 28) metabolism was upregulated. Relevant to protein, protein digestion and absorption (ranked 2), biosynthesis of amino acids (ranked 9), and several categories related to amino acid metabolism (ranked 10, 21, 25, 31, 33, 38 and 43) were upregulated. Enhanced amino acid metabolism may reflect enhanced protein synthesis driven by activated 4EBP1 (Figure 7B).

Enhanced DNA replication may be enabled by 
Ppp6c
deletion in the presence of 
K-rasG12D
, which supports cell proliferation by facilitating activity of cell machineries required for rapid cell division.



![Figure 8](https://example.com/figure8.png)

**Figure 8**  
Ppp6c deletion promotes the accumulation of DNA damage and NFκB activation in the tongue tissue of K mice. (A) Microscopic analysis of the 4HT-treated tongue of K(F/F) and K(+/+) mice, as shown in Figure 2C, stained with anti-γH2AX antibody. Scale bar: 100 μm. The insets with a scale bar in immunohistochemistry images for K(F/F) & K(+/+) tongues are enlarged (×3) one corresponding to the squares in the main image panels. Scale bar: 33.3 μm. (B) Quantification of the number of γH2AX-positive cells in an area ~1 mm wide in tongue epithelium, counted using samples shown in (A). Data are means derived from six areas in independent samples ±SE. ****p < 0.0001. (C) Microscopic analysis as described in (a) stained with an anti-RelA antibody. Scale bar: 100 μm. The insets were as described in (A). (D) Expression of genes functioning in NF-kappa B signaling pathway (KEGG 4064) in the 4HT-treated tongue of K(F/F) relative to expression seen in K(+/+) mice. mRNA was extracted and figure was generated as described in Figure 6C. Arrows indicate targets of RelA.

As shown in Figure S5, the expression level of several cytokines such as 
Ccl3, Cxcl3, Cxcl1, Cxcl5, Cc12
is highly elevated (Figure S5). It is likely due to the enhancement of the TNF signaling pathway regulated by NFκB signaling (Figure S6A). In 4HT-K(F/F) tongue, TNF signaling is positively regulated by TNF (Figure S6A) and leading to an explosion of cytokines such as 
Ccl3, Cxcl1, and Cc12
but also proinflammatory cytokine genes 
TNF, IL-1β
, and extracellular remodeling factor such as 
Mmp9, and Mmp3,
and 
COX2
(Figure S6B). These factors together may lead to chronic and excessive inflammatory states. As shown in Figures 3 (HE) and 4 (HE), greater infiltration by inflammatory cells was seen in the stroma of K(F/F) relative to K(+/+) mice. Chronic inflammation reportedly promotes tumor progression in mice and humans, and our findings strongly suggest that inflammation driven by 
Ppp6c
deficiency in the presence of oncogenic RAS is a factor in tongue carcinoma.

To examine the effects of p53 loss on signaling, we compared the transcriptome of 4HT-treated KP(F/F) tongue to that of K(F/F) animals (Table S2). ‘The Pathway in Cancer’ is identified in Table S2. We found that sustained angiogenesis, evasion of apoptosis, and proliferation were enhanced in the
KP(F/F) relative to the K(F/F) tongue. (Figure S7), indicating that by 13 days, KRAS tumorigenicity caused by Ppp6c deficiency is further enhanced by p53. This finding also suggests that if animals remain viable beyond 2 weeks, 4HT-treated KP(F/F) tongues may be more malignant than those of K(F/F).

Recently, however, some have proposed a different mechanism for PP6 protein downregulation. Fujiwara et al. showed that PP6 associates with the autophagic adaptor protein p62/SQSTM1 and is degraded in a p62-dependent manner. Nonetheless, whatever the mechanism, our results strongly suggest that in the presence of oncogenic RAS, PP6 downregulation promotes Ras-initiated tumorigenesis.

Numerous anti-Ras reagents have been proposed as a treatment for RAS-initiated cancers, but successful therapies remain elusive. Here, we found that PP6 functions as a tumor suppressor by suppressing the activity of ERK, AKT, and NfκB. We conclude that PP6 activators could provide a novel therapeutic strategy to repress cancer pathways and cytokine–cytokine receptor interactions.

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CONFLICT OF INTEREST
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.

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REFERENCES
1. Leemans CR, Snijders PJF, Brakenhoff RH. The molecular landscape of head and neck cancer. Nat Rev Cancer. 2018;18(5):269-282.
2. The Cancer Genome Atlas Network. Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature. 2015;517(7536):576-582.
3. Dang CV, Reddy EP, Shokat KM, Soucek L. Drugging the ‘undruggable’ cancer targets. Nat Rev Cancer. 2017;17(8):502-508.
4. Ma X, Lu J-Y, Dong Y, Li D, Malagon JN, Xu T. PP6 disruption synergizes with oncogenic ras to promote JNK-dependent tumor growth and invasion. Cell Rep. 2017;19(13):2657-2664.
5. Ohama T. The multiple functions of protein phosphatase 6. Biochim Biophys Acta Mol Cell Res. 2019;1866(1):74-82.
6. Zeng K, Bastos RN, Barr FA, Gruneberg U. Protein phosphatase 6 regulates mitotic spindle formation by controlling the T-loop phosphorylation state of Aurora A bound to its activator TPX2. J Cell Biol. 2010;191(7):1315-1332.
7. Mi J, Dziegielewski J, Bolesta E, Brautigan DL, Larner JM. Activation of DNA-PK by ionizing radiation is mediated by protein phosphatase 6. PLoS One. 2009;4(2):e3495.
8. Zhong J, Liao JI, Liu X, et al. Protein phosphatase P6P is required for homology-directed repair of DNA double-strand breaks. Cell Cycle. 2011;10(9):1411-1419.
9. Stefansson B, Brautigan DL. Protein phosphatase 6 subunit with conserved Sit4-associated protein domain targets IscBe. J Biol Chem. 2006;281(32):22624-22634.
10. Kajino T, Ren H, Iemura S-I, et al. Protein phosphatase 6 downregulates TAK1 kinase activation in the IL-1 signaling pathway. J Biol Chem. 2006;281(52):39891-39896.
11. Krauthammer M, Kong Y, Ha BH, et al. Exome sequencing identifies recurrent somatic RAC1 mutations in melanoma. Nat Genet. 2012;44(9):1006-1014.
12. Bonilla X, Parmentier L, King B, et al. Genomic analysis identifies new drivers and progression pathways in skin basal cell carcinoma. Nat Genet. 2016;48(4):398-406.
13. Yan S, Xu Z, Lou F, et al. NF-κB-induced microRNA-31 promotes epidermal hyperplasia by repressing protein phosphatase 6 in psoriasis. Nat Commun. 2015;6:7652.
14. Wu N, Liu X, Xu X, et al. MicroRNA-373, a new regulator of protein phosphatase 6, functions as an oncogene in hepatocellular carcinoma. FEBS J. 2011;278(12):2044-2054.
15. Hayashi K, Momoi Y, Tanuma N, et al. Abrogation of protein phosphatase 6 promotes skin carcinogenesis induced by DMBA. Oncogene. 2015;34(35):4647-4655.
16. Kato H, Kurosawa K, Inoue Y, et al. Loss of protein phosphatase 6 in mouse keratinocytes increases susceptibility to ultraviolet-B-induced carcinogenesis. Cancer Lett. 2015;365(2):223-228.
17. Ogoh H, Tanuma N, Matsui Y, et al. The protein phosphatase 6 catalytic subunit (Ppp6c) is indispensable for proper post-implantation embryogenesis. Mech Dev. 2016;139:1-9.
18. Seibler J, Zevnik B, Kütter-Luks B, et al. Rapid generation of inducible mouse mutants. Nucleic Acids Res. 2003;31(4):e12.
19. Jackson EL, Willis N, Mercer K, et al. Analysis of lung tumor initiation and progression using conditional expression of oncogenic K- ras. Genes Dev. 2003;17(4):324-3248.
20. Marino S, Vooijs M, HvD G, Jonkers J, Berns A. Induction of meiotically deficient mouse mutants. Cell Cycle. 2006;281(32):22624-22634.
24. Manning BD, Toker A. AKT/PKB signaling: navigating the network. *Cell*. 2017;169(3):381-405.

25. DiDonato JA, Mercurio F, Karin M. NF-κB and the link between inflammation and cancer. *Immunol Rev*. 2012;246(1):379-400.

26. Varfolomeev E, Vucic D. Intracellular regulation of TNF activity in health and disease. *Cytokine*. 2018;101:26-32.

27. Goradel NH, Najafi M, Salehi E, Farhood B, Mortezaee K. Cyclooxygenase-2 in cancer: a review. *J Cell Physiol*. 2019;234(5):5683-5699.

28. Fujiwara N, Shibutani S, Sakai Y, et al. Autophagy regulates levels of tumor suppressor enzyme protein phosphatase 6. *Cancer Sci*. 2020;111(12):4371-4380.

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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