Tumor suppressor NFκB2 p100 interacts with ERK2 and stabilizes *PTEN* mRNA *via* inhibition of miR-494

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Abstract

Emerging evidence from The Cancer Genome Atlas (TCGA) has revealed that *nfκb2* gene encoding p100 is genetically deleted or mutated in human cancers, implicating NFκB2 as a potential tumor suppressor. However, the molecular mechanism underlying the anti-tumorigenic action of p100 remains poorly understood. Here, we report that p100 inhibits cancer cell anchorage-independent growth, a hallmark of cellular malignancy, by stabilizing the tumor suppressor *PTEN* mRNA *via* a mechanism that is independent of p100's inhibitory role in NFκB activation. We further demonstrate that the regulatory effect of p100 on PTEN expression is mediated by its downregulation of miR-494 as a result of the inactivation of ERK2, in turn leading to inhibition of c-Jun/AP-1-dependent transcriptional activity. Furthermore, we identify that p100 specifically interacts with non-phosphorylated ERK2 and prevents ERK2 phosphorylation and nuclear translocation. Moreover, the death domain at C-terminal of p100 is identified as being crucial and sufficient for its interaction with ERK2. Taken together, our findings provide novel mechanistic insights into the understanding of the tumor suppressive role for NFκB2 p100.

Keywords

NFκB2 (p100/ p52); PTEN; ERK2; miR-494; c-Jun

Introduction

Transcription factors of the nuclear factor κB (NFκB) play critical roles in a variety of biological and pathological processes.[1] The NFκB family consists of five members, NFκB1 (p105/p50), NFκB2 (p100/p52), RelA (p65), RelB, and c-Rel.[1] Precursor protein NFκB2 p100 is encoded by *nfκb2* gene, and is well known as a fourth IκB protein that suppresses

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both canonical and noncanonical NFκB activation by preventing nuclear localization and DNA binding of NFκB dimers.[2] Genetic mutation or chromosomal rearrangements of the *nfκb2* gene have been previously observed in human lymphomas and common variable immunodeficiency (CVID).[3, 4] In addition, emerging evidence from The Cancer Genome Atlas (TCGA) has also revealed that *nfκb2* gene is genetically deleted or mutated in several human solid tumors including colorectal, gastric and prostate cancer, and that those colorectal cancer individuals with these alterations have poor clinical outcome,[5] suggesting that NFκB2 may play an inhibitory role in tumor development. Recently, the wild-type p100 has been reported to significantly inhibit tumor growth in severe combined immunodeficiency (SCID) mice,[6] implicating p100 as a potential tumor suppressor. Although tumor suppressive effects of p100 have been well documented, the molecular mechanism underlying the anti-tumorigenic action of p100 remains poorly understood.

PTEN (phosphatase and tensin homolog deleted on chromosome 10), a well-characterized tumor suppressor,[7] principally acts as a negative regulator of PI3K/Akt signaling by dephosphorylating phosphatidylinositol-3,4,5-trisphosphate (PIP3),[8] thus leading to inactivation of Akt and suppression of cell proliferation, cell survival and oncogenic cellular transformation.[7] Despite frequent mutation or deletion of *PTEN* gene in human cancers, there are still 25% of cancer patients showing a positive correlation between loss of *PTEN* mRNA and its protein expression,[9] indicating that the donwregulation of PTEN protein in those individuals could be attributed to the dysregulation of transcription factors involved in the regulation of *PTEN* transcripts such as early growth-response protein 1 (EGR1)[10] and c-Jun[11], as well as the non-coding RNAs that regulate the stability of *PTEN* mRNA including *PTEN* pseudogene 1 (*PTENP1*)[12] and microRNAs (miRNAs)[9]. NFκB subunits, p65 and p50, have been previously reported to repress *PTEN* transcription through direct or indirect mechanisms.[13, 14] However, as an inhibitory regulator of canonical and noncanonical NFκB signaling, whether NFκB2 has any regulatory roles in PTEN expression remains to be elucidated.

Here, we show that NFκB2 p100 modulates PTEN expression *via* a mechanism that is independent of p100's inhibitory role in NFκB signaling. Moreover, we identify that p100, but not p52, physically interacts with ERK2 and attenuates ERK2 phosphorylation, thereby leading to suppression of c-Jun/AP-1/miR-494 axis and stabilization of *PTEN* mRNA.

Results

NFκB2 deficiency promotes cancer cell anchorage-independent growth through PTEN inhibition

Although NFκB subunits, p65 and p50, have been reported to repress PTEN expression at transcriptional level,[13, 14] nothing is known about the roles of NFκB2, p100 and p52, in the regulation of PTEN expression. To determine the regulatory roles of NFκB2 in PTEN expression, we compared PTEN protein expression in NFκB2+/+ and NFκB2−/− immortalized murine embryonic fibroblasts (MEFs). Intriguingly, NFκB2 knockout led to a dramatic reduction of PTEN expression (Fig. 1A). Consistent with the alteration of PTEN protein, Akt phosphorylation at Thr308/Ser473, a well-characterized PTEN downstream substrate, was markedly upregulated in NFκB2−/− cells (Fig. 1A). To define whether these

observed effects are the direct consequence of NFκB2 deficiency, we used 2 sets of specific short hairpin RNAs (shRNAs) targeting NFκB2 to knockdown its expression in NFκB2+/+ cells. We then established stable transfectants NFκB2+/+(shNFκB2-1#), NFκB2+/+ (shNFκB2-2#), and their scramble control NFκB2+/+(Nonsense) (Fig. 1B). The results obtained from these stable transfectants consistently indicated that NFκB2 inhibition impaired PTEN expression accompanied by an increase in Akt phosphorylation at Thr308/Ser473 (Fig. 1B). Due to frequent genetic deletion or mutation of *nfκb2* gene in human malignancies,[3–5] we determined the biological roles of NFκB2 in cancer cells by using human colon cancer HCT116 cells with wild-type NFκB2 and wild-type PTEN.[15–17] Consistent with the observations in MEFs, knockdown of NFκB2 expression in HCT116 showed a similar effect on PTEN expression and Akt phosphorylation (Fig. 1C). More importantly, soft-agar assay confirmed that NFκB2 knockdown significantly promoted anchorage-independent growth of HCT116 cells (Fig. 1D), suggesting that NFκB2 plays a suppressive role in cancer cellular transformation. To further investigate the association between the altered PTEN expression and anchorage-independent growth in HCT116 cells upon NFκB2 depletion, GFP-PTEN was re-introduced into HCT116 NFκB2 knockdown cells. As shown in Figs. 1E and 1F, over-expression of GFP-PTEN successfully abolished aberrant Akt activation and further attenuated anchorage-independent growth of HCT116-shNFκB2 cells, revealing that PTEN serves as a crucial downstream mediator responsible for the growth inhibitory roles of NFκB2 in cancer cells.

NFκB2 p100, but not p52, regulates PTEN expression independently of p100's inhibitory role in NFκB signaling

Since depletion of the *NFκB2* gene resulted in the deficiency of p100 and p52 protein expression, our subsequent efforts were directed to the identification of which, p100 or p52, is responsible for NFκB2-mediated regulation of PTEN expression. Protein levels of PTEN and phospho-Akt were determined in NFκB2−/− cells following transient transfection of p100 or p100 C, the mutant one which is a p100 with deletion of the C-terminal (443–900 aa) but is able to generate intact p52 subunit as described in previous studies.[18, 19] As illustrated in Fig. 2A, ectopic expression of wild-type p100 in NFκB2−/− cells restored PTEN expression and attenuated aberrant Akt activation, whereas p100 C did not had any observed effects on either PTEN protein expression or Akt activation. These results clearly indicate that p100, but not p52, is responsible for the upregulation of PTEN by NFκB2. This notion was further supported by the results obtained from human colon cancer HCT116 (Fig. 2B) and prostate cancer DU-145 cells (Fig. 2C). Importantly, reconstituted expression of p100 in NFκB2 knockdown cells reversed the rapid anchorage-independent growth of HCT116-shNFκB2 cells, while p100 C slightly augmented anchorage-independent growth of HCT116-shNFκB2 cells under the same experimental conditions (Fig. 2D).

It has been reported that p100 functions as an IκB-like suppressor in NFκB activation and inhibits the transactivation of their target genes by sequestering NFκB dimers in cytoplasm.[2] As expected, NFκB2−/− cells indeed exhibited much higher NFκB-dependent transcriptional activities than NFκB2+/+ cells (Supplementary Fig. S1). Consistent with the regulatory roles of p100 and p100 C in PTEN expression, reconstituted expression of p100, but not p100 C, abolished this activation in NFκB2−/− cells (Supplementary Fig. S1),

suggesting that the inhibitory role for p100 in NFκB signaling may mediate its regulation of PTEN expression. We further examined the effects of NFκB2 deficiency on the expression of other NFκB subunits, including NFκB1 (p105 and p50), p65 (RelA) and RelB. As shown in Fig. 2E, knockout of NFκB2 had no observable effects on the protein levels of NFκB1 (p105/p50) or p65, but led to a reduction of RelB expression, which is consistent with a previous report showing that NFκB2 p100 and p52 are indispensable for the stabilization of RelB protein.[20] Similar results were also obtained from HCT116 cells upon NFκB2 depletion (data not shown). To verify whether the downregulation of RelB mediates the regulatory effects of NFκB2 p100 on PTEN expression, we used two sets of shRNAs specifically targeting human RelB to knockdown its expression in HCT116, and found that RelB knockdown failed to mimic the effects of NFκB2 depletion on PTEN expression and Akt activation (Fig. 2F), suggesting the regulatory role of p100 in PTEN expression might not be attributed to the downreguation of RelB in NFκB2 knockout or knockdown cells. Moreover, p65 has been reported to inhibit PTEN expression at transcriptional level,[14, 15] and NFκB2 knockout would increase NFκB p65-DNA binding activity.[2] We, therefore, anticipated that increased p65-dependent transcriptional activity might be responsible for the reduction of PTEN expression in NFκB2−/− cells though p65 protein levels were unaltered in these cells (Fig. 2E). To verify this notion, we knocked down NFκB2 expression in p65−/− cells. Unexpectedly, NFκB2 knockdown still resulted in the reduction of PTEN and the activation of Akt in p65−/− cells (Figs. 2G and 2H), revealing that NFκB2 regulates PTEN expression independently of p65. Collectively, these results suggest that p100, but not p52, regulates PTEN expression in an NFκB transcriptional-independent manner.

MiR-494 inhibition mediates tumor suppressive effects of NFκB2 p100 *via* stabilizing *PTEN* mRNA

It has recently been reported that subtle downregulation of PTEN has a profound impact on tumorigenesis.[21] Therefore, PTEN expression is delicately regulated at multiple levels, including transcriptional, post-transcriptional, translational, and post-translational levels.[7] To uncover whether protein degradation is involved in p100 regulation of PTEN expression, we compared the PTEN protein degradation rates between NFκB2+/+ and NFκB2−/− cells in the presence of the protein synthesis inhibitor cycloheximide (CHX). The results showed that NFκB2 depletion increased PTEN protein stability (Fig. 3A), indicating that the defect of PTEN expression in NFκB2−/− cells does not occur at protein degradation level. Thus, we further compared *PTEN* mRNA levels between NFκB2+/+ and NFκB2−/− cells, and found that *PTEN* mRNA expression was profoundly downregulated in NFκB2−/− cells as compared to NFκB2+/+ cells (Fig. 3B). However, the activity of PTEN promoter-driven luciferase reporter was comparable between NFκB2+/+ and NFκB2−/− cells (Fig. 3C). Consistently, PTEN promoter activity was not affected by ectopic expression of either p100 or p100 C in NFκB2−/− cells (Fig. 3D). These results strongly indicate that the regulation of PTEN by NFκB2 occurs at post-transcriptional level. To ascertain this notion, NFκB2+/+ and NFκB2−/− cells were treated with the *de novo* mRNA synthesis inhibitor actinomycin D (Act D), and the decay rate of *PTEN* mRNA was assessed by RT-PCR. As expected, *PTEN* mRNA stability was dramatically reduced in NFκB2−/− cells in comparison to NFκB2+/+ cells (Fig. 3E).

Given that microRNAs (miRNAs), a class of 20~23 nucleotide evolutionarily conserved non-coding RNAs, are able to repress the mRNA stability or the translational efficiency of their target genes predominantly by binding to the 3′-untranslated region (3′-UTR),[22] we performed a bioinformatics search for putative miRNAs with the potential binding to 3′-UTR of *PTEN* mRNA (Supplementary Table S1), and determined the expression levels of those predicted miRNAs including miR-29a, miR-29c, miR-214, miR-380, miR-494, miR-495, and miR-543. As shown in Fig. 3F, among the tested miRNAs, miR-494 was the only one that was elevated in NFκB2−/− as compared to NFκB2+/+ cells. Consistently, miR-494 expression was also elevated in NFκB2+/+ cells stably transfected with NFκB2 shRNA (Fig 3G). Moreover, elevated levels of miR-494 in both NFκB2−/− and HCT116-shNFκB2 cells were reversed by overexpression of p100, but not by p100 C (Supplementary Fig. S2). These results demonstrate that p100 is able to specifically inhibit miR-494 expression. To validate whether miR-494 targets the 3′-UTR of PTEN, the luciferase reporter containing wild-type PTEN-3′-UTR or miR-494 binding site mutant PTEN-3′-UTR (Fig. 3H) were stably transfected into NFκB2+/+ and NFκB2−/− cells, respectively. The results showed that the wild-type PTEN-3′-UTR luciferase activity was greatly suppressed in NFκB2−/− cells as compared to NFκB2+/+ cells (Fig. 3I). In contrast, the mutant luciferase activity was nearly comparable between NFκB2+/+ and NFκB2−/− cells (Fig. 3I), suggesting that miR-494 plays an essential role in NFκB2-mediated regulation of PTEN *via* directly targeting PTEN-3′-UTR. To further verify the role of miR-494 in regulation of PTEN expression, miR-494 expression construct was stably transfected into both NFκB2+/+ and HCT116 cells. Overexpression of miR-494 significantly inhibited PTEN expression and increased Akt phosphorylation (Supplementary Fig. S3). Moreover, inhibition of miR-494 by using anti-miR-494 restored PTEN expression and Akt phosphorylation in NFκB2−/− and HCT116-shNFκB2 cells (Figs. 3J and K). Importantly, miR-494 inhibition dramatically attenuated the anchorage-independent growth of HCT116-shNFκB2 cells (Fig. 3L).

To examine the effects of miR-494 on *PTEN* mRNA levels, we compared the alterations of *PTEN* mRNA in both NFκB2+/+ and HCT116 cells with different levels of miR-494. Consistent with the role of miR-494 in PTEN protein expression, miR-494 overexpression dramatically suppressed *PTEN* mRNA levels in both types of cell (Figs. 4A and B). Conversely, inhibition of miR-494 using anti-miR-494 successfully restored *PTEN* mRNA expression in NFκB2−/− and HCT116-shNFκB2 cells (Figs. 4C and D). Furthermore, miR-494 inhibition also increased *PTEN* mRNA stability (Fig. 4E). Collectively, our results clearly indicate that miR-494 inhibits *PTEN* mRNA stability *via* directly binding to 3′-UTR of *PTEN* mRNA, which is distinctly different from the previous report showing that miR-494 inhibits PTEN protein expression without any apparent effects on *PTEN* mRNA levels in transient transfection system.[23] Taken together, our results demonstrate that p100 inhibits miR-494 expression, which results in the reduction of miR-494 binding to PTEN 3′-UTR and the subsequent stabilization of *PTEN* mRNA, thereby leading to the inhibition of anchorage-independent growth of cancer cells.

Suppression of c-Jun/AP-1 is essential for the regulatory roles of NFκB2 p100 in miR-494 and PTEN expression

Romano *et al.* have recently reported that miR-494 expression is regulated by transcription factor activator protein-1 (AP-1).[24] To identify the mechanism underlying the NFκB2 regulation of miR-494, we investigated whether NFκB2 regulated the expression of Jun family, including c-Jun, Jun B, and Jun D. NFκB2 deficiency led to a significant increase in c-Jun phosphorylation at Ser73, but not Jun B and Jun D expression (Figs. 5A and B). Furthermore, reconstituted expression of p100, but not p100 C, dramatically inhibited c-Jun phosphorylation (Fig. 5C), demonstrating that p100 is a negative regulator of c-Jun phosphorylation. Given the previous reports from others and us showing that PI3K/Akt functions as an upstream regulator of c-Jun/AP-1 activation in several cellular processes,[25–27] we sought to determine whether Akt was responsible for the regulation of c-Jun phosphorylation by NFκB2. As shown in Fig. 5D, forced expression of dominant negative mutant of Akt (T308A/S473A; DN-Akt) in NFκB2−/− cells did not alter c-Jun phosphorylation although it effectively suppressed Akt phosphorylation and its putative downstream target, GSK3β phosphorylation at Ser9. In addition, knockdown of NFκB2 in PTEN−/− cells failed to alter Akt phosphorylation, but significantly upregulated c-Jun phosphorylation at Ser73 (Figs. 5E and F), excluding the possibility that Akt is an essential player in the regulation of c-Jun phosphorylation by NFκB2. Next, to define the role of c-Jun in the regulation of miR-494 and PTEN expression by NFκB2, we stably transfected TAM67, a dominant negative mutant of c-Jun, into NFκB2−/− cells, and found that inhibition of c-Jun activation by TAM67 restored the expression of miR-494 and PTEN in NFκB2−/− cells (Figs. 5G and H). Taken together, our findings reveal that the alteration of c-Jun/AP-1 activity is required for the regulation of miR-494 and PTEN by NFκB2 p100.

Suppression of ERK2 phosphorylation is required for NFκB2 p100-mediated upregulation of PTEN expression

It is well know that c-Jun phosphorylation is regulated by the mitogen-activated protein kinases (MAPKs) family,[28] including ERK1/2, JNK1/2 and p38. To determine the mechanism responsible for NFκB2 p100-mediated regulation of c-Jun phosphorylation, we compared the phosphorylation levels of ERK1/2, JNK1/2, and p38 between NFκB2+/+ and NFκB2−/− cells. As shown in Figs. 6A and B, knockout or knockdown of NFκB2 expression specifically increased phosphorylation of ERK2, but not ERK1, while it slightly decreased phosphorylation of JNK1/2 and p38. Consistent with the regulatory roles of p100 and p52 in c-Jun phosphorylation and PTEN expression, ectopic expression of p100, but not p100 C, attenuated ERK2 phosphorylation in HCT116-shNFκB2 cells (Fig. 6C). Due to the increased c-Jun phosphorylation at Ser73 in NFκB2−/− cells (Fig. 5A), we anticipated that ERK2 might be the specific player responsible for p100-mediated regulation of c-Jun phosphorylation and PTEN expression. To test this notion, NFκB2−/− cells with higher levels of phospho-ERK2 were incubated for 3 h with U0126, an inhibitor of MEK1 (an upstream kinase for phosphorylating ERK1/2). ERK1/2 inhibition by U0126 blocked c-Jun phosphorylation at Ser73 and increased PTEN expression, accompanied by a reduction in Akt phosphorylation at Thr308/Ser473 (Fig. 6D). To further identify the specific role of ERK2 in this regulation, we found that only dominant negative mutant of ERK2 (DN-ERK2), but not DN-ERK1, increased PTEN protein expression and inactivated c-Jun and

Akt (Fig. 6E). Our results strongly confirm that ERK2 is a specific NFκB2 p100's downstream regulator that is responsible for p100-mediated regulation of c-Jun phosphorylation and PTEN expression.

NFκB2 p100 specifically interacts with ERK2 and prevents ERK2 nuclear translocation

To elucidate the mechanism of NFκB2 p100 regulation of ERK2 phosphorylation, we first tested whether NFκB2 targeted ERK upstream kinase cascade RAS/RAF/MEK. As shown in Fig. 7A, RAS/RAF/MEK axis was inactivated in NFκB2−/− cells in comparison to NFκB2+/+ cells, excluding the possibility that ERK2 activation in NFκB2−/− cells is mediated by activation of the RAS/RAF/MEK cascade. Similarly, Tpl-2 (MAP3K8), a kinase that positively regulates ERK2 phosphorylation,[29] was also excluded because it was downregulated in NFκB2−/− cells (Fig. 7A). Next, we examined the levels of the potential protein phosphatases PP2A and MKP-3 (DUSP6), which have been reported to be the negative regulators of ERK2 phosphorylation.[30, 31] These results showed that knockout of NFκB2 increased PP2A and MKP-3 expression (Fig. 7A), suggesting that these tested phosphatases might not be involved in the regulation of ERK2 phosphorylation by NFκB2. The protein-protein interaction has also been reported to participate in the modulation of ERK2 phosphorylation.[32–34] To test this possibility that p100 interacts with ERK2 to inhibit its phosphorylation, we therefore performed co-immunoprecipitation (Co-IP) assay by using HEK293T cells that expressed HA-ERK2 and Flag-p100. Intriguingly, HA-tagged ERK2 did present in the immunoprecipitates following anti-Flag antibodies pull down of Flag-tagged p100 (Fig. 7B). This physical interaction was further demonstrated in the immunoprecipitates using anti-HA antibodies pull down of HA-ERK2 (Fig. 7C). In addition, Flag-p52 was undetectable in the immunoprecipitates under the same experimental conditions (Fig. 7C), suggesting p100, but not p52, physically interacted with ERK2. Furthermore, endogenous ERK2, but not phospho-ERK2, was present in the immunoprecipitates pulled down with anti-NFκB2 antibodies by using NFκB2+/+ cells (Fig. 7D). Moreover, Immunodepletion assay indicated that, in comparison to anti-rabbit IgG, anti-NFκB2 antibodies depleted ~75% abundance of endogenous p100 from NFκB2+/+ cell extracts, and simultaneously pulled down ~46% of endogenous ERK2 without removing other tested proteins, including ERK1 and p-ERK1/2 (Fig. 7D). These results clearly revealed that p100 specifically interacted with the majority of non-phosphorylated ERK2. To further map the ERK2-binding region on p100, we co-transfected HA-tagged ERK2 along with a series of partial deletion mutants of p100's C-terminal, as described in Fig. 7E. Co-immunoprecipitation assays using anti-HA antibodies demonstrated that the death domain at the extreme C-terminal of p100 was not only essential, but also sufficient for its physical interaction with ERK2 (Figs. 7F and G). Moreover, the death domain was required for the regulatory effects of p100 on ERK2 phosphorylation and PTEN expression (Supplementary Fig. S4). This finding is consistent with previous reports that the members of death domain superfamily, PEA-15 and DAPK, interact with ERK2 and modulate ERK2 phosphorylation.[32–34] To further investigate the physiological consequence of this physical interaction between p100 and ERK2 in cells upon serum stimulation, we incubated NFκB2+/+ cells in 20% FBS for 30 min, and performed co-immunoprecipitation assay to pull down endogenous p100 from these cellular extracts by using anti-NFκB2 antibodies. The results showed that serum stimulation led to a substantial decrease in p100-

associated ERK2 protein in NFκB2+/+ cells, whereas anti-NFκB2 antibodies failed to capture endogenous ERK2 in NFκB2−/− cells under the same experimental conditions (Fig. 7H), suggesting that p100 specifically interacted with ERK2. Moreover, NFκB2 p100 is predominantly localized in the cytoplasm,[6] and therefore we anticipated that the dissociation of ERK2 from p100 would result in an increase in the nuclear translocation of ERK2 following serum stimulation. To test this notion, cytoplasmic and nuclear fractions from NFκB2+/+ and NFκB2−/− cells upon serum stimulation were isolated and further subjected to immunoblotting analysis. As shown in Fig. 7I (*left panel*), the majority of ERK2 protein was sequestered in the cytoplasm of both NFκB2+/+ and NFκB2−/− cells following 24h of serum deprivation, while it shuttled from cytoplasm to nucleus after 30 min of serum stimulation (Fig. 7I; *right panel*). Furthermore, ERK2 shuttled faster in NFκB2−/− than NFκB2+/+ cells upon serum stimulation (Fig. 7I), suggesting that p100 may play an inhibitory role in ERK2 nuclear translocation through physically interacting with ERK2. Taken together, our findings demonstrate that the death domain of p100 is essential and sufficient for specific interaction with non-phosphorylated ERK2; this physical interaction might sequester ERK2 in the cytoplasm and inhibit ERK2 phosphorylation, thereby preventing ERK2 nuclear translocation.

Discussion

Noncanonical NFκB pathway is well known for its regulatory roles in a variety of immunological processes.[35] In 1998, Caamano *et al.* established the physiological roles for NFκB2 in secondary lymphoid organogenesis and architecture organization and B-cell maturation by using *NFκB2*−/− (*p100*−/−/*p52*−/−) mice.[36] Precursor protein NFκB2 p100 has been reported to principally act as a fourth IκB protein that suppresses both canonical and noncanonical NFκB activation by sequestering NFκB dimers in cytoplasm.[2] Although also defective in hematopoietic tissues such as spleen and thymus, 90% of *p100*−/− mice die by 4 week of age due to remarkable gastric hyperplasia,[37] supporting an inhibitory or regulatory role for p100 in epithelial cell growth. The tumor suppressive function of NFκB2 p100 was originally linked to its inhibitory role in NFκB activation.[35] However, to the best of our knowledge, we are the first to report a novel function for p100 in modulating PTEN expression through a miR-494-mediated mechanism that is independent of p100's inhibitory role in NFκB transactivation (Fig. 7J). Our finding establishes a novel link between PTEN alteration and the suppressive role for NFκB2 p100 in cell growth, which may help us better understand the occurrence of gastric hyperplasia in *p100*−/− mice. It has recently been reported that subtle alteration of PTEN expression has a profound impact on tumorigenesis.[21] It is, therefore, reasonable to anticipate that p100 would play a role in regulation of tumor initiation, promotion and/or progression. However, due to short life-span in *p100*−/− mice,[37] the precise role of p100 in these settings is not clear and needs further investigation by using conditional p100 KO mice in future study.

The ERK1/2 pathway mediates mitogenic and/or non-mitogenic signaling, which might be essential for the control of cell proliferation, differentiation, and transformation.[38] Due to the frequent dysregulation of this cascade in various types of human cancer, considerable efforts have been made to develop pharmacological inhibitors that target the RAF/MEK/ERK pathway.[39] We reported here that NFκB2 p100 might act as a promising natural inhibitor of

ERK2 through specifically interacting with ERK2 and preventing its nuclear translocation. Moreover, we found that the majority of ERK2 was bound to endogenous p100 in unstimulated cells, but dissociated from p100 and shuttled from cytoplasm to nucleus in cells following serum stimulation. Recently, Yilmaz *et al.* has reported that LTβR pathway triggers interdependent processing of p100 and NFκB1 p105, and LTβR-induced p105 proteolysis requires the C-Terminal Destruction Box of p100.[40] This necessitates the further investigation of the regulatory effects of LTβR-induced p100/p105 proteolysis on the physical interaction or dissociation between p100 and ERK2 proteins, though our preliminary result showed the regulation of ERK2 phosphorylation by p100 might be independent of the processing of p100 under serum-stimulated conditions (Supplementary Fig. S5). Additionally, the molecular mechanism that mediates the dissociation of ERK2 from p100 in cells upon serum stimulation also merits further investigation, and the results from which will help us better understand the physiological relevance of the physical interaction between p100 and ERK2.

In agreement with our finding about the inhibitory role for p100 in cancer cell anchorage-independent growth, p100 has been previously observed to dramatically suppress cellular transformation driven by oncogenic H-Ras[G12V].[18] The oncogenic role of H-Ras[G12V] in cellular transformation has been well demonstrated to depend on the activation of RAF/MEK/ERK and PI3K/Akt pathways.[41, 42] Here, we found that p100 not only specifically interacted with non-phosphorylated ERK2 and suppressed ERK2 phosphorylation; it also repressed PI3K/Akt signaling through upregulating PTEN expression. Therefore, our findings might provide direct molecular evidence for better understanding of suppressive role for p100 in H-Ras[G12V]-mediated cellular transformation.

It has previously been reported that miR-494 acts as an oncomir in gastrointestinal stromal tumors (GISTs) *via* targeting proto-oncogene KIT[43] and that it inhibits ischemia/reperfusion-induced cardiac injury by targeting pro-apoptotic proteins (PTEN, ROCK1, and CaMKIIδ) and anti-apoptotic proteins (FGFR2 and LIF) [44]. In the present study, we found that miR-494 played an essential role in NFκB2 p100 regulation of PTEN expression *via* binding to 3′-UTR of *PTEN* mRNA. Consistent with its suppressive role in PTEN protein expression, we found that miR-494 significantly inhibited *PTEN* mRNA level by destabilizing *PTEN* mRNA. Furthermore, we found that p100 suppressed miR-494 expression through attenuating c-Jun phosphorylation. C-Jun, one important member of AP-1 family,[45] is highly phosphorylated in many cancers.[46] It has been reported to promote cellular survival by regulating the expression of PTEN through a poorly characterized mechanism, in which c-Jun suppresses the mRNA and protein expression of PTEN without any significant effects on PTEN promoter activity.[11] However, our findings suggest that miR-494 probably mediates this previously undefined suppression of PTEN by c-Jun.

In summary, our studies have revealed a new ERK2/AP-1/miR-494/PTEN pathway that is responsible for the tumor suppressive role of NFκB2 p100 in cellular transformation. We show a novel link between p100 and PTEN expression through a mechanism that is independent of p100's inhibitory role in NFκB signaling. More importantly, we identify a physical interaction between p100 and ERK2, and further point out that the death domain at C-terminal of p100 is essential and sufficient for its interaction with ERK2. Taken together,

our findings provide a novel molecular evidence for an improved understanding of the tumor suppressive role of p100 in cancer cells, suggesting that p100 could potentially be used as a therapeutic target in the future cancer therapy.

Methods and Materials

Cell lines, constructs, reagents and antibodies

These details were described in Supplementary Information.

Soft agar assay

Soft agar assay was performed to assess the ability of anchorage-independent growth of cancer cells according to the protocol described previously.[47]

Luciferase reporter assay

Luciferase reporter assays were performed as previously described.[48]

Jeverse transcription-PCR (RT-PCR) and Quantitative RT-PCR

RT-PCR and quantitative RT-PCR assay were performed to examine the expression level of *PTEN* mRNA and mature miRNAs, respectively, as described previously.[49] The primers used in this study were listed in Supplementary Table S2.

Immunoblotting assay

Immunoblotting assay was performed as described previously.[50] Cellular cytoplasmic and nuclear fractions were isolated according to the protocol of the Nuclear/Cytosol Fractionation Kit (BioVison, CA). The density of bands was quantified relative to that of loading control by using Quantity One software.

Co-immnunoprecipitation and Immunodepletion assay

Twenty-four hours post-transfection with constructs indicated, HEK293T cells were cultured in 0.1% serum containing medium for 24–28 h. Cells were then collected and lysed in 1× Cell Lysis Buffer (Cell Signaling Technology) containing protease inhibitors (Roche, Branchburg, NJ) followed by brief sonication. For pulldown of HA- or FLAG-tagged proteins, cell extracts were incubated with Anti-HA agarose beads (Vector Laboratories, Burlingame, CA) or Anti-FLAG M2 magnetic beads (Sigma-Aldrich) overnight at 4 °C. For pulldown of endogenous NFκB2/p100 protein complex, cell extracts were incubated with protein A/G magnetic beads and anti-NFκB2 antibody (1:50 dilution; Cell Signaling Technology; #4882) or anti-rabbit IgG (Cell Signaling Technology; #7074) overnight at 4 °C. Following a brief centrifuge, the supernatant was saved as depleted product for immunoblotting analysis (named as Immunodepletion Assay). The beads were washed three times with 1× Cell Lysis Buffer, and the bound proteins were eluted by boiling in 3×SDS sample buffer (Cell Signaling Technology) and subjected to immunoblotting assay.

Statistical analysis

The student's *t-test* was used to determine significant differences. P< 0.05 is considered significantly. The results are expressed as the mean±SD from at least three independent experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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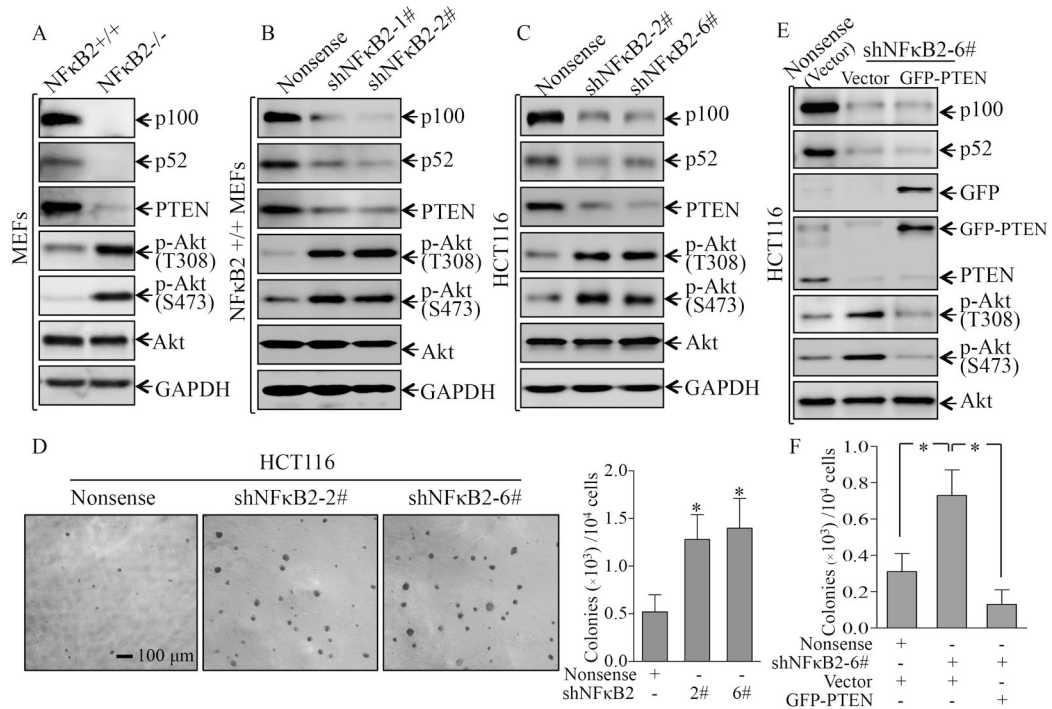
Figure 1. NFκB2 deficiency promotes cancer cell anchorage-independent growth through PTEN inhibition

(**A** & **B**) Immunoblotting analysis of whole-cell lysates derived from NFκB2+/+ and NFκB2–/– MEFs (A) or NFκB2+/+ stably transfected with shRNA NFκB2 (shNFκB2) (B). (**C**) Immunoblotting analysis of whole-cell lysates obtained from stable HCT116 NFκB2 knockdown cells. (**D**) Anchorage-independent cell growth of HCT116 stably transfected with shRNA NFκB2 was determined by soft agar assay. The colony formation was observed and captured under an inverted microscope (40× magnification; *left panel*) and numbers of colonies were scored on day 15, and presented as colonies ($\times 10^3$)/10^4 cells *(right panel)*. The symbol (*) indicates a significant difference in comparison to that of control transfectants ($P < 0.05$). (**E**) HCT116 cells stably transfected with nonsense or shNFκB2-6# were transiently transfected with the constructs expressing GFP-PTEN or vector, respectively. Twenty-four hours post transfection, transfectants were extracted for immunoblotting analysis following synchronization with McCoy's 5A medium containing 0.1% FBS overnight and stimulation with 10% FBS for another 3 h. (**F**) Following the transient transfection described in "**E**", soft agar assay was performed, and the number of colonies was scored on day 12. The symbol (*) indicates a significant difference ($P < 0.05$).
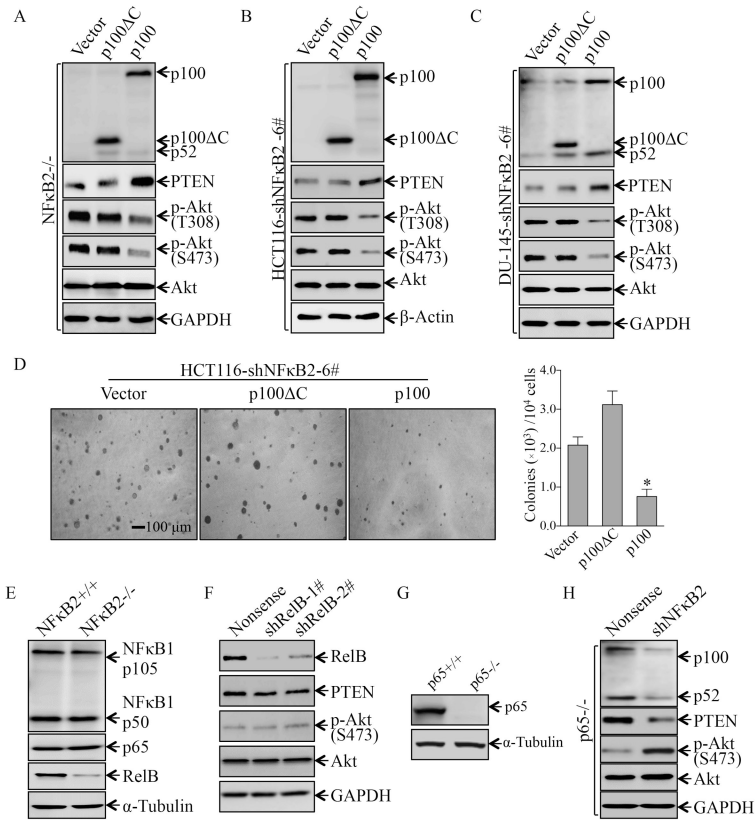
Figure 2. NFκB2 p100, but not p52, regulates PTEN expression independently of its inhibitory role in NFκB signaling

(**A**) Following twenty-four hours of transfection with pcDNA3.1 (vector), pcDNA3.1-p100 C, or pcDNA3.1-p100, NFκB2–/– cells were synchronized with 0.1% FBS DMEM overnight and stimulated with 10% FBS for another 3 h, and were then collected for immunoblotting analysis using indicated antibodies. GAPDH was used as a loading control. (**B** & **C**) Stable HCT116-shNFκB2-6# (**B**) and DU-145-shNFκB2-6# cells (**C**) were stably transfected with constructs described in "**A**". Following synchronization with 0.1% FBS overnight, stable transfectants were stimulated with 10% FBS for 6 h and then extracted for immunoblotting analysis using indicated antibodies. GAPDH or β-Actin was used as a loading control. (**D**) Soft agar assay was performed using HCT116-shNFκB2 cells stably transfected with vector, p100 C or p100. The colony formation was observed and captured under an inverted microscope (40× magnification; *left panel*). Numbers of colonies were scored on day 15 and presented as colonies (×10^3)/10^4 cells *(right panel)*. The symbol (*) indicates a significant difference in comparison to that of control transfectants (P< 0.05). (**E** & **F**) Immunoblotting analysis of whole-cell lysates derived from NFκB2+/+ and NFκB2–/– cells (E) or HCT116 cells stably transfected with shRNA RelB (shRelB) (F). (**G** & **H**) MEFs p65+/+ and p65–/– cell extracts were subjected to the immunoblotting analysis for identification of p65 (G). Following synchronization, p65–/– cells stably transfected with shRNA NFκB2 were stimulated with 10% FBS for 3 h and then extracted for immunoblotting analysis (H).
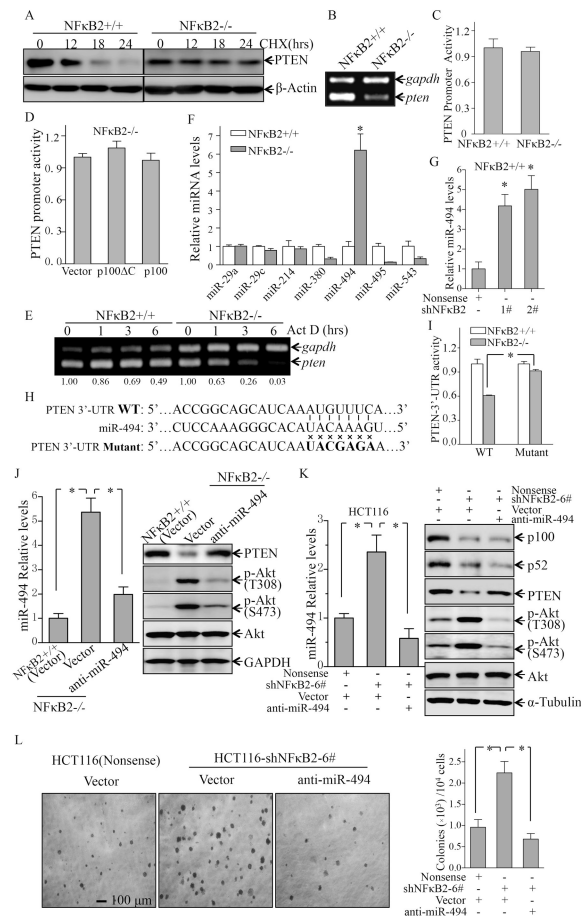
Figure 3. MiR-494 inhibition is crucial for NFκB2 p100-mediated PTEN expression and anchorage-independent growth of cancer cells

(**A**) NFκB2+/+ and NFκB2−/− cells were treated with 50 μg/ml cycloheximide (CHX) for the indicated time and the cell extracts were subjected to immunoblotting analysis of PTEN protein expression. β-Actin was used as protein loading control. (**B**) Total RNA was isolated and was then subjected to RT-PCR analysis of *pten* mRNA expression in NFκB2+/+ and NFκB2−/− cells. The *gapdh* mRNA was used as a loading control. (**C**) Cells were co-transfected with PTEN promoter-driven luciferase reporter and pRL-TK. pRL-TK was used as an internal control to normalize the transfection efficiency. Twenty-four hours post transfection, cells were synchronized overnight and stimulated with 10% FBS medium for 3h, and the luciferase reporter assay was performed. The value was expressed as mean±SD from three independent experiments. (**D**) NFκB2−/− cells were co-transfected with PTEN promoter-driven luciferase reporter, pRL-TK, and constructs encoding vector, p100, or p100 C, as indicated. The luciferase reporter activity was evaluated and normalized to that of NFκB2−/− cells transfected with vector. (**E**) After synchronization, NFκB2+/+ and NFκB2−/− cells were treated with 5 μg/ml Actinomycin D (Act D) for the indicated time, then total RNA was isolated and subjected to RT-PCR analysis for mRNA levels of *pten* and *gapdh*. Densitometric quantification of *pten* is shown. (**F**) The levels of indicated microRNAs in NFκB2+/+ and NFκB2−/− cells were evaluated as described in "***Materials and methods***". The value was presented as mean±SD from three independent experiments.

The symbol (*) indicates a significant difference in comparison to that of NFκB2+/+ (P< 0.05). (**G**) The levels of miR-494 in NFκB2+/+ stably transfected with nonsense or NFκB2 shRNA were evaluated. The symbol (*) indicates a significant difference in comparison to control (P< 0.05). (**H**) Wild-type (WT) PTEN 3′-UTR containing miR-494 binding sites was aligned with the seed regions of miR-494. The predicted sites of PTEN 3′-UTR for miR-494 binding were mutated and were shown in bold. (**I**) Wild-type or mutant PTEN 3′-UTR luciferase reporters were co-transfected with pRL-TK into NFκB2+/+ and NFκB2−/− cells, respectively. Thirty-six hours post transfection, cells were synchronized overnight and stimulated with 10% FBS medium for 3 h, and the luciferase activity was measured. The value was expressed as mean±SD from three independent experiments. The symbol (*) indicates a significant difference (P< 0.05). (**J & K**) The indicated cells stably transfected with construct of anti-miR-494 were synchronized overnight and then stimulated in 10% FBS medium for 3 h. The miR-494 levels were then determined using qRT-PCR assay and expressed as mean±SD from three independent experiments. The symbol (*) indicates a significant difference in comparison to that in cells transfected with control vector (P< 0.05). The cell lysates were subjected to immunoblotting with the antibodies as indicated. GAPDH or α-Tubulin was used a protein loading control. These experiments were performed at least three times. (**L**) Soft agar assay was performed using HCT116-shNFκB2 cells stably transfected with anti-miR-494. The colony formation was observed and captured under an inverted microscope (40× magnification; *left panel*). Numbers of colonies were scored on day 14 and presented as colonies ($\times 10^3$)/10^4 cells *(right panel)*. The symbol (*) indicates P< 0.05.
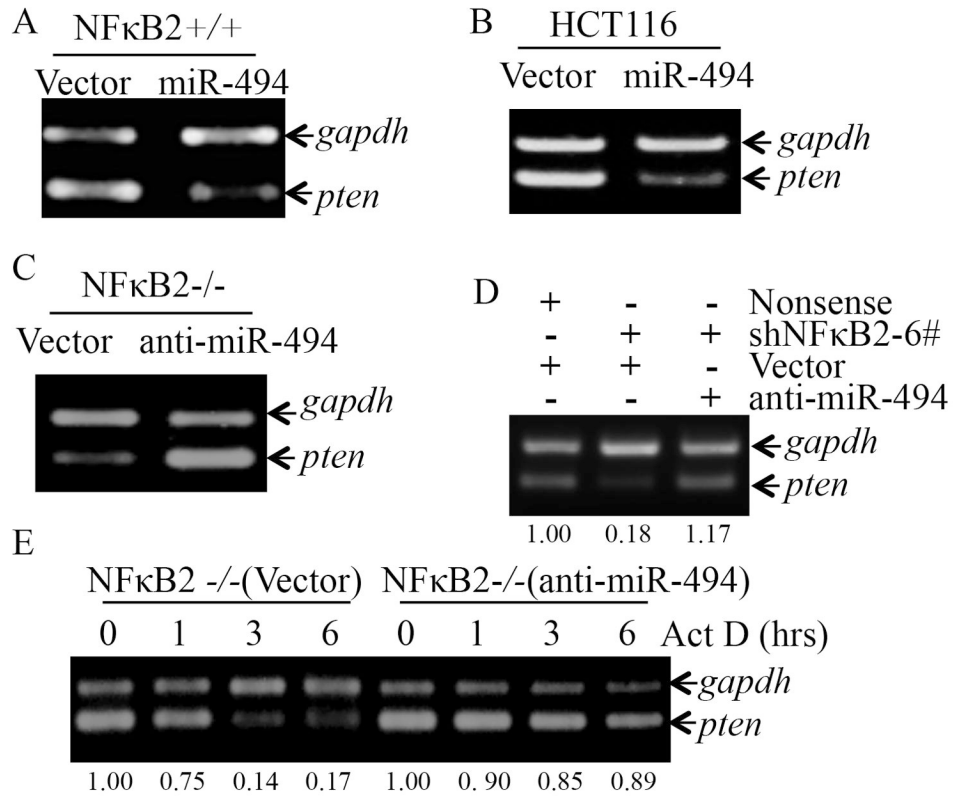
Figure 4. MiR-494 destabilizes *PTEN* mRNA

(**A** & **B**) Total RNAs were isolated from cells ectopically expressing miR-494 and were subjected to RT-PCR for determination of *PTEN* mRNA. The *gapdh* was used as a loading control. (**C** & **D**) Total RNAs were isolated from stable transfectants expressing anti-miR-494 and were subjected to RT-PCR for determination of *pten* mRNA. The *gapdh* was used as a loading control. Densitometric quantification of *pten* is shown. (**E**) Stable transfectants were treated with 5 μg/ml Actinomycin D (Act D) for the indicated time, then total RNAs were isolated and subjected to RT-PCR analysis of *pten* mRNA levels and *gapdh* was used as a loading control. Densitometric quantification of *pten* is shown.
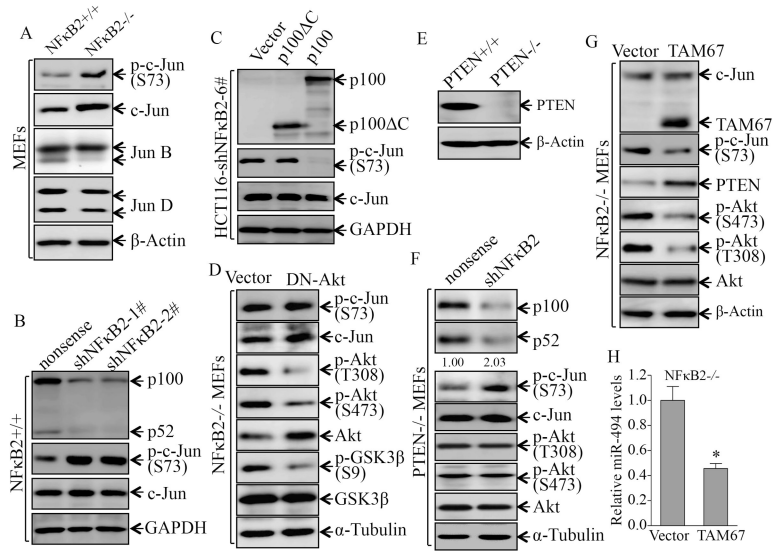
Figure 5. NFκB2 p100 inhibits miR-494 expression *via* downregulation of c-Jun/AP-1 cascade
(**A** & **B**) Immunoblotting analysis of whole-cell lysates from NFκB2+/+ and NFκB2−/−
cells (A) or NFκB2+/+ stably transfected with NFκB2 shRNA (shNFκB2) (B). (**C**)
Immunoblotting analysis of whole-cell lysates from HCT116-shNFκB2 cells stably
transfected with p100 or p100 C. (**D**) Immunoblotting analysis of whole-cell lysates from
NFκB2−/− cells transiently transfected with dominant negative Akt (DN-Akt). (**E** & **F**)
Immunoblotting analysis of whole-cell lysates from PTEN−/− cells (E) and their stable
transfectants with NFκB2 shRNA (shNFκB2) (F). Densitometric quantification of p-c-Jun
(S73) (relative to c-Jun) is shown. (**G**) Immunoblotting analysis of whole-cell lysates from
NFκB2−/− cells stably tranfected with dominant negative form of c-Jun (TAM67). (**H**)
Quantitative RT-PCR was performed for determination of miR-494 levels in NFκB2−/− cells
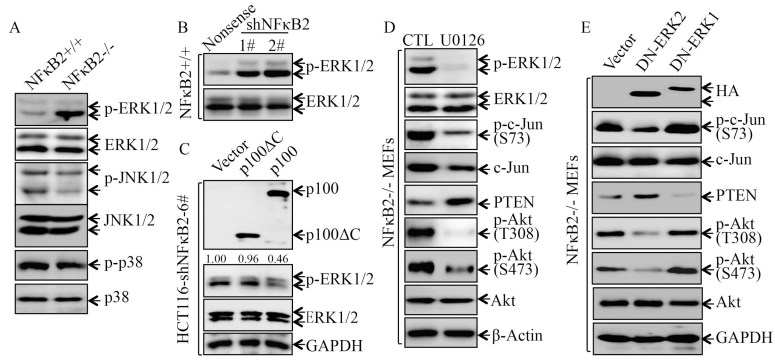stably tranfected with TAM67.

Figure 6. Suppression of ERK2 phosphorylation is required for NFκB2 p100-mediated upregulation of PTEN expression

(**A** & **B**) Immunoblotting analysis of whole-cell lysates from NFκB2+/+ and NFκB2−/− MEFs (A) or NFκB2+/+ stably transfected with NFκB2 shRNA (shNFκB2) (B). (**C**) Immunoblotting analysis of whole-cell lysates from HCT116-shNFκB2 cells stably transfected with pcDNA3.1 (vector), pcDNA3.1-p100, or p100 C. Densitometric quantification of p-ERK2 (relative to GAPDH) is shown. (**D**) Immunoblotting analysis of whole-cell lysates from NFκB2−/− cells following the treatment of 10 μM U0126 for 3 h. (**E**) Immunoblotting analysis of whole-cell lysates from NFκB2−/− cells stably transfected with HA-tagged dominant negative ERK1 (K71R; DN-ERK1) or ERK2 (K52R; DN-ERK2).
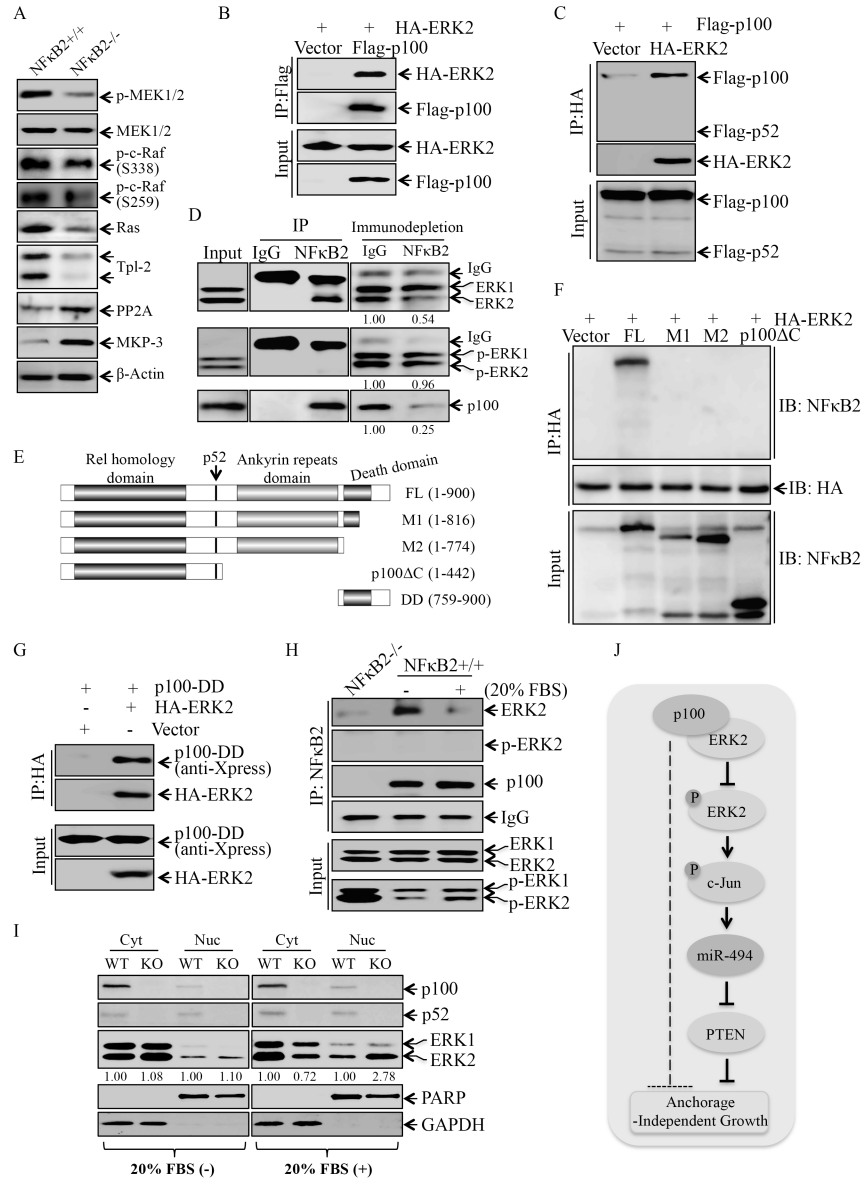
Figure 7. NFκB2 p100 specifically interacts with ERK2 and prevents ERK2 nuclear translocation

(**A**) Immunoblotting analysis of whole-cell lysates from NFκB2+/+ and NFκB2−/− cells with specific antibodies as indicated. (**B**) Immunoblotting analysis of whole-cell lysates (Input) and FLAG-immunoprecipitates (IP) obtained from HEK293T cells transfected with HA-ERK2 alone or in combination with FLAG-p100. (**C**) Immunoblotting analysis of whole-cell lysates (Input) and HA-immunoprecipitates (IP) obtained from HEK293T cells transfected with FLAG-p100 alone or in combination with HA-ERK2. (**D**) Endogenous p100 was immunoprecipitated from NFκB2+/+ cells using anti-NFκB2 antibody (anti-N-terminal). The immunoprecipitated complex (IP) and the supernatant (Immunodepletion) were collected for immunoblotting analysis with antibodies against NFκB2, p-ERK1/2 and ERK1/2. Densitometric quantification of ERK2 (relative to ERK1), p-ERK2 (relative to p-

ERK1) and p100 (relative to ERK1) are shown under each blot. (**E**) Schematic representation of full-length p100 (FL) and its several deletions in C-terminal. (**F**) Immunoblotting analysis of whole-cell lysates (Input) and HA-immunoprecipitates (IP) obtained from HEK293T cells transfected with HA-ERK2 alone or in combination with various p100 deletions as shown in "**E**". (**G**) Immunoblotting analysis of whole-cell lysates (Input) and HA-immunoprecipitates (IP) obtained from HEK293T cells transfected with p100-DD (759–900) alone or in combination with wild-type HA-ERK2. (**H**) Immunoblotting analysis of whole-cell lysates and anti-NFκB2-immunoprecipitates (IP) obtained from NFκB2+/+ cells following synchronization overnight in 0.1% FBS medium and further stimulation with 20% FBS medium for 30 min. (**I**) Immunoblotting analysis of cytoplasmic (Cyt) and nuclear (Nuc) fractions of NFκB2+/+ (WT) and NFκB2−/− (KO) following 24h of serum deprivation (*left panel*) and further stimulation with 20% FBS for 30 min (*right panel*). GAPDH and poly-(ADP-ribose) polymerase (PARP) are cytoplasmic and nuclear markers, respectively. Densitometric quantification of ERK2 expression is shown. (**J**) A novel molecular mechanism responsible for tumor suppressive effects of NFκB2 p100 on modulation of cancer cell anchorage-independent growth.