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Tumour maintenance is mediated by eNOS

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Abstract

Tumour cells become addicted to the expression of initiating oncogenes like Ras, such that loss of oncogene expression in established tumours leads to tumour regression[1]. HRas, NRas, or KRas are mutated to remain in the active GTP-bound oncogenic state in many cancers[2]. While oncogenic Ras activates a number of proteins to initiate human tumour growth, only PI3K, through activation of the kinase AKT (PKB), must remain activated by oncogenic Ras to maintain this growth[3]. Here we show that blocking phosphorylation of the AKT substrate, eNOS, inhibits tumour initiation and maintenance. Moreover, eNOS enhances the nitrosylation and activation of endogenous wildtype Ras proteins, which is required for throughout tumorigenesis. We suggest that activation of the PI3K-AKT-eNOS-(wildtype)Ras pathway by oncogenic Ras in cancer cells is required to initiate and maintain tumour growth.

The reduction of Ras oncogene dependence to activation of AKT appears to be a consequence of redundant signalling provided by the established tumour microenvironment. Indeed, activation of AKT fosters tumourigenic growth of otherwise non-tumourigenic cells, provided such cells are mixed with tumour cells to establish the tumour microenvironment[3]. We exploited this cell-mixing assay to interrogate the signalling pathway downstream of AKT required for tumour maintenance. Although AKT can phosphorylate a number of proteins[4], we focused on BAD, FOXO, IKKα, TSC2, and endothelial nitric oxide synthase (eNOS or NOS3), as the consequence of AKT phosphorylation of these proteins is not redundant with the functions of the oncoproteins expressed in cells used in the cell-mixing assay of tumour maintenance (Ref 3[-5], Supp. Fig. 1). Non-tumourigenic PI3K-TtH^{LacZ} cells, derived from normal human kidney cells transformed by oncoproteins T/t-Ags and immortalized by hTERT (hereafter termed TtH cells), expressing p110-CAAX (to activate the PI3K-AKT pathway) and LacZ (to demark the cells in the tumour), had Bcl-X_L shRNA, eNOS shRNA, or as reported by others[6-8], dominant-acting FOXO3a-A3 or TSC2^{SA,TA} (mutated at AKT phosphorylation sites), or IKKα^{K44A} (kinase-inactive) proteins expressed to suppress the effects of AKT on these individual pathways (Supp. Fig. 1). Knockdown of Bcl-X_L and eNOS, ectopic expression and nuclear localization of FOXO3a-A3[6], ectopic expression of TSC2^{SA,TA} leading to mTOR repression, as assessed by decreased S6k phosphorylation[7], and ectopic expression of IKKα^{K44A} leading to repression of NF-κB, as assessed by nuclear exclusion of p65[8], were validated (Supp. Fig. 2). These five cell lines were mixed with tumourigenic HRas^{G12V} transformed TtH cells (termed Ras^{G12V}-TtH cells) to establish a tumour microenvironment, injected into mice, and assayed for their contribution to the resultant tumour mass by treating tumours or derived tumour cells with X-gal to stain LacZ$^+$ cells blue. Positive control vector

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PI3K-TtH^{LacZ} cells extensively populated tumours, whereas negative control vector TtH^{LacZ} cells contributed little to the tumour mass, as evidenced by the prominent or weak blue staining, respectively. Expression of IKKα^{K44A} had little effect, Bcl-X_L shRNA, FOXO3a-A3, and TSC2^{SA,TA} had a mild effect, but eNOS shRNA had the greatest effect on reducing the contribution of PI3K-TtH^{LacZ} (blue) cells in tumours (Fig. 1a). To then test if AKT phosphorylation, and not just expression of eNOS, is required for tumour maintenance, AKT was validated to phosphorylate S_{1177} of eNOS[9], as evidenced by a loss of S_{1177} phosphorylation of endogenous eNOS upon pharmacological inhibition of AKT signalling with LY294002 or by mutating S_{1177} of ectopic eNOS to alanine in PI3K-TtH^{LacZ} cells (Fig. 1b). PI3K-TtH^{LacZ} cells in which eNOS was knocked down were then engineered to express eNOS^R in the wildtype or S_{1177}A mutant configuration (Supp. Fig. 3) and assayed for tumour maintenance by the aforementioned cell-mixing assay. Control PI3K-TtH^{LacZ} cells populated tumours, which was greatly reduced upon knockdown of eNOS, as evidenced by the reduction in blue staining. This loss was rescued by wildtype, but not S_{1177}A mutant eNOS^R (Fig. 1c). Thus, activation of the PI3K-AKT-eNOS pathway promotes tumour maintenance.

eNOS has been detected in tumour cells[10], and catalyzes the synthesis of nitric oxide (NO), which can facilitate *S*-nitrosylation of the thiol group of cysteines in proteins[11], such as that of C_{118} of HRas, which enhances the dissociation of guanine nucleotides thereby increasing GTP-bound HRas[12]. Wildtype Ras proteins can be required for activation of the MAPK pathway by oncogenic Ras[13], and membrane-targeting of RasGAP, which inhibits wildtype but not oncogenic Ras, reverts oncogenic Ras transformation of NIH3T3 cells[14], suggesting that wildtype Ras proteins may facilitate oncogenic signalling. Collectively, we speculated that AKT activation of eNOS maintains tumour growth in the absence of oncogenic Ras by activating *wildtype* Ras through *S*-nitrosylation of C_{118}. To test this, activated AKT in PI3K-TtH was shown to foster HRas nitrosylation through eNOS, as the majority of HRas nitrosylation was lost by treatment with the PI3K inhibitor wortmannin (Fig. 2a), by mutating C_{118} in HRas to Serine, a minor change that exchanges the sulphur atom for oxygen but nevertheless blocks nitrosylation[12] (Fig 2b), or by knocking down eNOS (Fig. 2c), and as HRas nitrosylation was elevated upon activation of AKT (Fig. 2c). Reduction of HRas nitrosylation by eNOS shRNA also reduced levels of active GTP-bound HRas (Fig. 2c). Since TtH cells express HRas and NRas, but not KRas (not shown), and C_{118} is conserved amongst all Ras proteins, we tested and confirmed that activated AKT in PI3K-TtH cells also led to elevated levels of nitrosylated and GTP-bound endogenous NRas, which were reduced upon knockdown of eNOS (Fig. 2c). Thus, AKT activation of eNOS promotes nitrosylation and activation of wildtype Ras proteins. To then assess the biological consequence of *S*-nitrosylation of wildtype HRas in tumour maintenance, we tested whether *replacing* endogenous wildtype HRas with the nitrosylation-resistant C_{118}S mutant version reduced tumour maintenance. HRas was knocked down by shRNA in PI3K-TtH^{LacZ} cells and complemented with vector encoding HRas resistant to RNAi (HRas^R) in the wildtype configuration or the C_{118}S mutant configuration that reduced GTP loading, or as a control, no transgene (Supp. Fig. 4). These cell lines, or as controls cells expressing either a scramble control sequence or HRas shRNA alone, were mixed with Ras^{G12V}-TtH cells, injected into mice, and the resultant tumours assayed for the presence of blue LacZ$^+$ cells as a measure of tumour maintenance. Knockdown of wildtype HRas reduced the ability of PI3K-AKT signalling to foster tumour maintenance, as evidenced by a sixfold reduction of PI3K-TtH^{LacZ} (blue) cells in the tumours. This effect was reversed upon expressing wildtype, but less so with the C_{118}S mutant version of HRas^R (Fig. 2d). Thus, activation of the PI3K-AKT-eNOS pathway promotes tumour maintenance by *S*-nitrosylation and activation of wildtype Ras (Fig. 4g).

As oncogenic Ras must activate the PI3K-AKT pathway to both initiate and maintain tumour growth[3], we tested whether AKT-mediated activation of eNOS was also required for the

establishment of tumours. A scramble control or eNOS shRNA was introduced into tumourigenic Ras^{G12V}-TtH cells, and knockdown of eNOS complemented by RNAi-resistant eNOS ($eNOS^{R}$) in the wildtype configuration or the $S_{1177}A$ mutant configuration resistant to S_{1177} phosphorylation (Supp. Fig. 5). These four cell lines were injected into mice and tumour growth monitored over time. Scramble control cells rapidly formed tumours, whereas tumour growth was almost abolished upon knockdown of eNOS. This loss of tumour growth was rescued by the wildtype, but not the $S_{1177}A$ version of $eNOS^{R}$ (Fig. 3a, Supp. Fig. 6), indicating that S_{1177} phosphorylation of eNOS is required for tumour initiation and maintenance. These results were validated in a spontaneous cancer model. Chemical carcinogen DMBA followed by TPA were topically applied to $eNOS^{+/+}$ and $eNOS^{-/-}$ mice to induce skin papillomas characterized by *Ras* oncogenic mutations[15], with the end result being an approximate threefold drop in the number of tumours per $eNOS^{-/-}$ mouse (Fig. 3b, Supp. Fig. 7). Thus, independent models of cancer demonstrate eNOS is required for tumorigenesis.

To test whether eNOS mediates oncogenic Ras signalling in a cancer associated with the most commonly mutated Ras family member, *KRas*[2], the amount of activated (S_{1177} phosphorylated) eNOS was first assayed in cancer cell lines and tumour specimens isolated from patients diagnosed with pancreatic cancer. Compared to normal tissue specimens, CFPac-1, MIAPaCa-2, and Capan-1 cells exhibited the highest level of S_{1177} phosphorylation of eNOS (Fig. 4a). Activated KRas and S_{1177} phosphorylated eNOS were also elevated in the tumour specimens compared to matched and unmatched normal tissue controls (Fig. 4b and ref. [16]), with the caveat that biopsies also contain stromal tissue that could contribute to detected eNOS phosphorylation. Second, knockdown of eNOS was shown to reduce tumour growth by 50 fold in CFPac-1 cells and tumour size of MIAPaCa-2 cells by 80% (Fig. 4c, Supp. Figs. 8,9). As a control, knockdown of eNOS in cell lines AsPc-1 and SW1990 that exhibited poor eNOS activation had no obvious effect on tumour growth (not shown). Third, using a more direct assay for tumour maintenance, in vivo doxycycline (dox)-induced shRNA knockdown of eNOS in CFPac-1 and MIAPaCa-2 cells *after* tumours were established inhibited tumour growth, as evidenced by reduced size and/or gross necrosis of tumours excised at the termination of the experiment (Fig. 4d). In some mice, this eventually led to tumour regression (not shown). Thus, eNOS is required to both initiate and maintain tumour growth of these human pancreatic cancer cells. Fourth, to test if eNOS promoted tumour growth through nitrosylation of Ras in pancreatic cancer cells, we determined which Ras family members were inactivated by eNOS shRNA in CFPac-1 and MIAPaCa-2 cells. Not surprisingly, GTP-bound KRas was unchanged upon knockdown of eNOS (Fig. 4c, Supp. Fig. 9), as *K-Ras* is mutated to remain active in these two cell lines[17,18]. Consistent with this, oncogenic KRas harbouring the $C_{118}S$ mutation remained tumourigenic (Supp. Fig. 10), pointing towards wildtype Ras proteins as the target of eNOS signalling. GTP-bound endogenous wildtype HRas and NRas were reduced upon shRNA knockdown of eNOS, however the wildtype allele of *KRas* is deleted in the MIAPaCa-2 cells[18] and a number of oncogenic *KRas*-positive cell lines[18] and tumour tissues[19], suggesting that wildtype HRas and NRas, but not KRas, are the targets of eNOS signalling in pancreatic cancer cells (Fig. 4c, Supp. Fig. 9). To then test if activation of HRas or NRas by eNOS is required for pancreatic tumour growth, HRas or NRas were knocked down by shRNA in CFPac-1 and/or MIAPaCa-2 cells and complemented with an HRas or NRas engineered to be resistant to RNAi ($HRas^{R}$, $NRas^{R}$) in the wildtype or the $C_{118}S$ mutant configuration, and resultant cells assayed for tumour growth in mice. Positive control scramble treated CFPac-1 and/or MIAPaCa-2 cells readily formed tumours in mice, whereas this growth was reduced when endogenous HRas, and to a lesser degree NRas, was knocked down. This loss of tumour growth was rescued by expressing the appropriate wildtype HRas or NRas, but not the $C_{118}S$ nitrosylation mutants (Fig. 4e,f, Supp. Figs. 11-13). Similar results were found when the cells were assayed for transformed growth in vitro, suggestive of a tumour cell autonomous defect when wildtype Ras proteins cannot be nitrosylated (Supp. Fig. 14). Thus, oncogenic KRas-driven pancreatic

cancer tumour growth was mediated by eNOS nitrosylation of endogenous wildtype HRas and NRas (Fig. 4g).

In summary, we demonstrate that the continual need for PI3K-AKT signalling during initiation and maintenance of oncogenic Ras-driven tumour growth is due, at least in part, to activation of eNOS through phosphorylation of S_{1177}, which in turn leads to *S*-nitrosylation at C_{118} and correspondingly activation of the ***other*** wildtype Ras family members, perhaps as a means to ***diversify*** the Ras signal beyond that of oncogenic Ras (Fig. 4g). In agreement, the wildtype counterpart of oncogenic Ras is not required for tumorigenesis (Supp. Fig. 15), and is even deleted in some tumours[19], whereas wildtype HRas and NRas are required for oncogenic KRas-driven tumour growth, and appear to have non-redundant activies[20]. Effects of eNOS on tumourigenesis have been largely attributed to its activity in endothelial cells[10]. Our results now suggest a key role for tumour-expressed eNOS in the tumourigenic process. As eNOS plays multiple roles in tumourigenesis[10], and delivery of a peptide fragment of the protein cavtratin, which can inhibit eNOS, displays anti-tumour activity[21], we speculate that inhibition of eNOS, perhaps in combination with inhibition of wildtype Ras protein function or processing[2], could have therapeutic value in the treatment of oncogenic Ras-driven human cancers such as pancreatic.

METHODS SUMMARY

TtH and the pancreatic cancer cell lines[22] were stably infected retroviruses encoding the indicated shRNAs, transgenes, or no insert as described[23], and appropriate expression verified by immunoblot or RT-PCR. Detection of GTP-bound or nitrosylated Ras was performed as described[24]. One or a mixture of two cell lines were injected into the flanks of immunocompromised mice to assay for tumour growth, and where indicated, excised and assayed for LacZ-positive cells, as described[3]. Induction of shRNA in vivo by dox[3] and DMBA/TPA treatments[25] were performed as described. All animal work was approved by IACUC, all tissue samples were provided under an approved IRB protocol.

FULL METHODS

Plasmids

pBabepuro, neo, bleo, and hygro were used as control vectors[3]. HA-IKKα^{K44A} cDNA[26], FOXO3a-A3 cDNA[27] engineered with an N-terminal HA tag, FLAG-TSC2^{S393A,T1462A} cDNA[7] (termed here as TSC2^{SA,TA}), eNOS cDNA engineered with a C-terminal HA tag and to be resistant to shRNA by introducing the three silent mutations G_{1821}→A, T_{1827}→C and G_{1830}→A alone (eNOS^R) or in conjunction with the mutation A_{3519}GC→GCC that altered S_{1177} to A (S_{1177}A eNOS^R), and wildtype FLAG-epitope tagged HRas or NRas cDNAs engineered to be resistant to shRNA by introducing the silent mutations in the region targeted by RNAi (FLAG-HRas^R; FLAG-NRas^R) alone or in conjunction with the mutation T_{342}GT→TCT (C_{118}S FLAG-HRas^R, C_{118}S FLAG-NRas^R) that altered C_{118} to S, were subcloned into one of the aforementioned pBabe vectors. Bcl-X_L shRNA (5'-AGCGTAGACAAGGAGATGC), eNOS shRNA (5'-AAGAGTTATAAGATCCGCTTC), HRas shRNA (5'-GGCAAGAGTGCGCTGACCATC), NRas shRNA (5'-CAAGAAGAGTACAGTGCCATG) or an eNOS scramble control (5'-AAGCGTTAAAAGATCCGCTTC) sequences were cloned into pSUPER-PURO-RETRO (Oligoengine). The plasmid system for dox-inducible shRNA[3] was adapted to encode eNOS shRNA.

Cell lines

TtH and the pancreatic cancer cell lines were previously described[22]. Derived lines were generated by stable infection with the indicated combinations of amphotrophic retroviruses generated from the aforementioned pBabe plasmids, as previously described[23].

Cell treatments

Cells were treated with LY294002 (Cell Signaling Technologies) or Wortmannin (Sigma) at a final concentration of 20 μM or 10 nM, respectively, for 1 hr prior to analysis.

Immunoblotting

HA-IKKα^{K44A}, FOXO3a-A3-HA, HA-eNOS or variants thereof, FLAG-TSC2^{SA,TA}, endogenous Bcl-X_L, p70 S6 kinase, T_{389} phosphorylated p70 S6 kinase, HRas, KRas or NRas, S_{1177} phosphorylated eNOS (both to detect activated eNOS and assess eNOS expression), S_{473} phosphorylated AKT, actin, p65, and tubulin were detected by immunoblotting with αHA (Roche), αFLAG (Sigma), αBcl-xL, αp70 S6 Kinase, αThr389 Phospho-p70 S6 Kinase, αSer1177 Phospho-eNOS, αSer473 Phospho-AKT (Cell Signalling Technology), αHRas, αKRas, αNRas, αactin (Santa Cruz), αp65 (Rockland), and αtubulin (Sigma) antibodies, respectively.

RT-PCR

eNOS and GAPDH mRNA was RT-PCR amplified with the primers 5'-CAGTGTCCAACATGCTGCTGGAAATTG and 5'-TAAAGGTCTTCTTCCTGGTGATGC, and the primers 5'-ACCACAGTCCATGCCATCAC and 5'-TCCACCACCCTGTTGCTGTA, respectively.

GTP and nitrosylated Ras

GTP-bound or nitrosylated Ras were captured as previously described[24] and immunoblotted with either an αFLAG (Sigma) or an αHRas, αKRas or αNRas (Santa Cruz) antibody to detect FLAG-H-Ras or endogenous H, N, or KRas proteins, respectively.

Soft agar

Soft agar assays were done in triplicate and twice independently as previously described[5].

Tumour growth

As previously described[3], the tested cell line (tumour initiation) or a mixture of two cell lines (cell mixing assay for tumour maintenance) were injected subcutaneously into 4 flanks of SCID/Beige mice. For tumour initiation experiments, tumours were removed and photographed when control tumours reached maximum volume. For cell mixing assays, the 4 tumours were removed when they reached maximum volume, human cells derived from the 2 tumours by re-culture in selective media (G418), and the 2 other whole tumours were treated with X-gal to stain $LacZ^+$ cells blue and photographed. CFPac-1 and MIAPaCa-2 cells engineered to contain a dox-inducible eNOS shRNA[3] were injected into both flanks of 5 SCID/Beige mice, tumours were permitted to reach a diameter of 0.6 cm, after which 3 mice were provided with doxycycline in their diet and 2 mice left untreated for 11 days (CFPac-1 cells) or 13 days (MIAPaCa-2 cells), after which tumours were removed and photographed. DMBA/TPA treatments were performed as previously described[25] on 15 $eNOS^{+/+}$ C57BL/6J and 15 $eNOS^{-/-}$ C57BL/6J (B6.129P2-*Nos3*^{tm1Unc}/J) mice[28] (Jackson Laboratory). All animal work was approved by DUMC IACUC.

Tumour and normal human specimens

Flash frozen tissue samples were provided devoid of all identifying information under a DUMC approved IRB protocol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Giuriato S, et al. Conditional animal models: a strategy to define when oncogenes will be effective targets to treat cancer. Semin. Cancer Biol 2004;14:3–11. [PubMed: 14757531]
2. Downward J. Targeting RAS signalling pathways in cancer therapy. Nat. Rev. Cancer 2003;3:11–22. [PubMed: 12509763]
3. Lim KH, Counter CM. Reduction in the requirement of oncogenic Ras signaling to activation of PI3K/AKT pathway during tumor maintenance. Cancer Cell 2005;8:381–392. [PubMed: 16286246]
4. Luo J, Manning BD, Cantley LC. Targeting the PI3K-Akt pathway in human cancer: rationale and promise. Cancer Cell 2003;4:257–262. [PubMed: 14585353]
5. Ali SH, DeCaprio JA. Cellular transformation by SV40 large T antigen: interaction with host proteins. Semin. Cancer Biol 2001;11:15–23. [PubMed: 11243895]Yeh E, et al. A signalling pathway controlling Myc degredation that impacts oncogenic transformation of human cells. Nat. Cell Biol 2004;6:308–318. [PubMed: 15048125]
6. Brunet A, et al. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 1999;96:857–868. [PubMed: 10102273]
7. Manning BD, et al. Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Mol. Cell 2002;10:151–162. [PubMed: 12150915]
8. Regnier CH, et al. Identification and characterization of an IkappaB kinase. Cell 1997;90:373–383. [PubMed: 9244310]
9. Michell BJ, et al. The Akt kinase signals directly to endothelial nitric oxide synthase. Curr. Biol 1999;9:845–848. [PubMed: 10469573]Fulton D, et al. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature 1999;399:597–601. [PubMed: 10376602]Dimmeler S, et al. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature 1999;399:601–605. [PubMed: 10376603]
10. Fukumura D, Kashiwagi S, Jain RK. The role of nitric oxide in tumour progression. Nat. Rev. Cancer 2006;6:521–534. [PubMed: 16794635]
11. Hess DT, et al. Protein S-nitrosylation: purview and parameters. Nat. Rev. Mol. Cell Biol 2005;6:150–166. [PubMed: 15688001]
12. Lander HM, et al. Redox regulation of cell signalling. Nature 1996;381:380–381. [PubMed: 8632794]
13. Hamilton M, Wolfman A. Ha-ras and N-ras regulate MAPK activity by distinct mechanisms in vivo. Oncogene 1998;16:1417–1428. [PubMed: 9525741]
14. Huang DC, Marshall CJ, Hancock JF. Plasma membrane-targeted ras GTPase-activating protein is a potent suppressor of p21ras function. Mol. Cell. Biol 1993;13:2420–2431. [PubMed: 8455619]
15. Quintanilla M, et al. Carcinogen-specific mutation and amplification of Ha-ras during mouse skin carcinogenesis. Nature 1986;322:78–80. [PubMed: 3014349]

16. Lim KH, et al. Divergent roles for RalA and RalB in malignant growth of human pancreatic carcinoma cells. Curr. Biol 2006;16:2385–2394. [PubMed: 17174914]

17. Moore PS, et al. Genetic profile of 22 pancreatic carcinoma cell lines. Analysis of K-ras, p53, p16 and DPC4/Smad4. Virchows Arch 2001;439:798–802. [PubMed: 11787853]

18. Kita K, et al. Growth inhibition of human pancreatic cancer cell lines by anti-sense oligonucleotides specific to mutated K-ras genes. Int. J. Cancer 1999;80:553–558. [PubMed: 9935156]

19. Wan J, et al. Loss of heterozygosity of Kras2 gene on 12p12−13 in Chinese colon carcinoma patients. World J. Gastroenterol 2006;12:1033–1037. [PubMed: 16534842]Li J, et al. LOH of chromosome 12p correlates with Kras2 mutation in non-small cell lung cancer. Oncogene 2003;22:1243–1246. [PubMed: 12606951]

20. Parikh C, Subrahmanyam R, Ren R. Oncogenic NRAS, KRAS, and HRAS Exhibit Different Leukemogenic Potentials in Mice. Cancer Res 2007;67:7139–7146. [PubMed: 17671181]Esteban LM, et al. Targeted genomic disruption of H-ras and N-ras, individually or in combination, reveals the dispensability of both loci for mouse growth and development. Mol. Cell. Biol 2001;21:1444–1452. [PubMed: 11238881]Johnson L, et al. K-ras is an essential gene in the mouse with partial functional overlap with N-ras. Genes Dev 1997;11:2468–2481. [PubMed: 9334313]Fotiadou PP, et al. Wild-Type NRas and KRas Perform Distinct Functions during Transformation. Mol. Cell. Biol 2007;27:6742–6755. [PubMed: 17636015]

21. Gratton JP, et al. Selective inhibition of tumor microvascular permeability by cavtratin blocks tumor progression in mice. Cancer Cell 2003;4:31–39. [PubMed: 12892711]

22. Lim KH, et al. Activation of RalA is critical for Ras-induced tumorigenesis of human cells. Cancer Cell 2005;7:533–545. [PubMed: 15950903]

23. O'Hayer KM, Counter CM. A genetically defined normal somatic human cell system to study ras oncogenesis in vitro and in vivo. Methods Enzymol 2006;407:637–647. [PubMed: 16757358]

24. de Rooij J, Bos JL. Minimal Ras-binding domain of Raf1 can be used as an activation-specific probe for Ras. Oncogene 1997;14:623–625. [PubMed: 9053862]Jaffrey SR, et al. Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. Nat. Cell Biol 2001;3:193–197. [PubMed: 11175752]

25. Ancrile B, Lim KH, Counter CM. Oncogenic Ras-induced secretion of IL6 is required for tumorigenesis. Genes Dev 2007;21:1714–1719. [PubMed: 17639077]

26. Woronicz JD, et al. IkappaB kinase-beta: NF-kappaB activation and complex formation with IkappaB kinase-alpha and NIK. Science 1997;278:866–869. [PubMed: 9346485]

27. Hu MC, et al. IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. Cell 2004;117:225–237. [PubMed: 15084260]

28. Shesely EG, et al. Elevated blood pressures in mice lacking endothelial nitric oxide synthase. Proc. Natl. Acad. Sci. U.S.A 1996;93:13176–13181. [PubMed: 8917564]

1. Lim KH, Counter CM. Reduction in the requirement of oncogenic Ras signaling to activation of PI3K/AKT pathway during tumor maintenance. Cancer Cell 2005;8:381–392. [PubMed: 16286246]

2. Woronicz JD, et al. IκB kinase-β: NF-κB activation and complex formation with IκB kinase-α and NIK. Science 1997;278:866–869. [PubMed: 9346485]

3. Hu MC, et al. IκB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. Cell 2004;117:225–237. [PubMed: 15084260]

4. Manning BD, et al. Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Mol. Cell 2002;10:151–162. [PubMed: 12150915]

5. Lim KH, et al. Activation of RalA is critical for Ras-induced tumorigenesis of human cells. Cancer Cell 2005;7:533–545. [PubMed: 15950903]

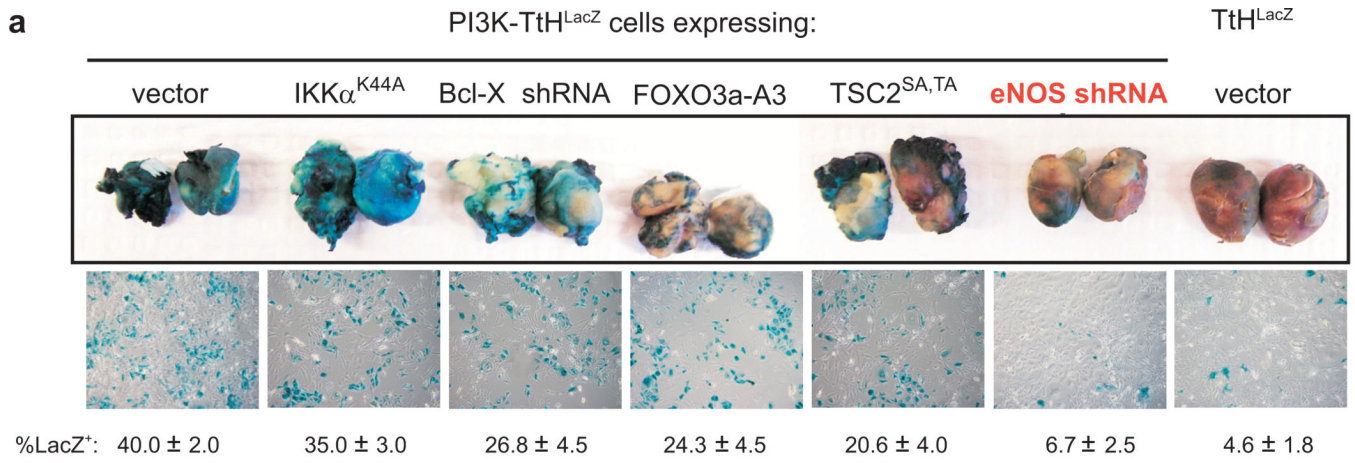
6. O'Hayer KM, Counter CM. A genetically defined normal somatic human cell system to study ras oncogenesis in vitro and in vivo. Methods Enzymol 2006;407:637–647. [PubMed: 16757358]

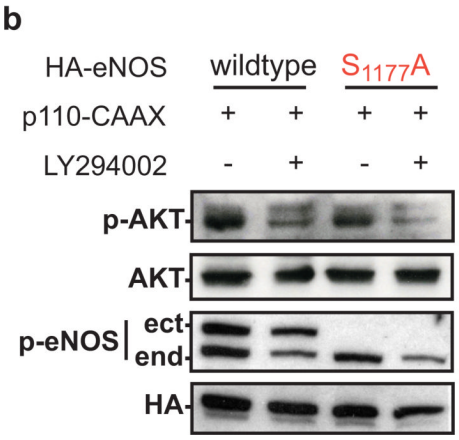
7. de Rooij J, Bos JL. Minimal Ras-binding domain of Raf1 can be used as an activation-specific probe for Ras. Oncogene 1997;14:623–625. [PubMed: 9053862]

8. Jaffrey SR, et al. Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. Nat. Cell Biol 2001;3:193–197. [PubMed: 11175752]

9. Ancrile B, Lim KH, Counter CM. Oncogenic Ras-induced secretion of IL6 is required for tumorigenesis. Genes Dev 2007;21:1714–1719. [PubMed: 17639077]

10. Shesely EG, et al. Elevated blood pressures in mice lacking endothelial nitric oxide synthase. Proc. Natl. Acad. Sci. USA 1996;93:13176–13181. [PubMed: 8917564]





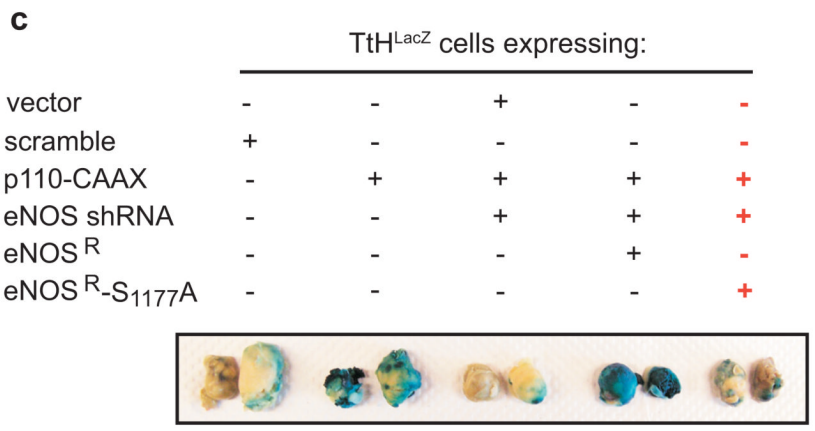


Figure 1.

AKT promotes tumour maintenance by phosphorylation of eNOS. **a**, PI3K-TtH^{LacZ} or **c**, TtH^{LacZ} cells expressing indicated constructs were mixed with Ras^{G12V}-TtH cells, injected into mice, and tumours or recultured tumour cells were stained with X-gal. n=5, mean±s.e.m. **b**, Protein levels of phosphorylated AKT (p-AKT), phopshorylated eNOS (p-eNOS, ect: ectopic, end: endogenous), HA-eNOS (HA), and AKT in PI3K-TtH^{LacZ} cells expressing wildtype or S$_{1177}$A HA-eNOS treated with DMSO or LY294002.

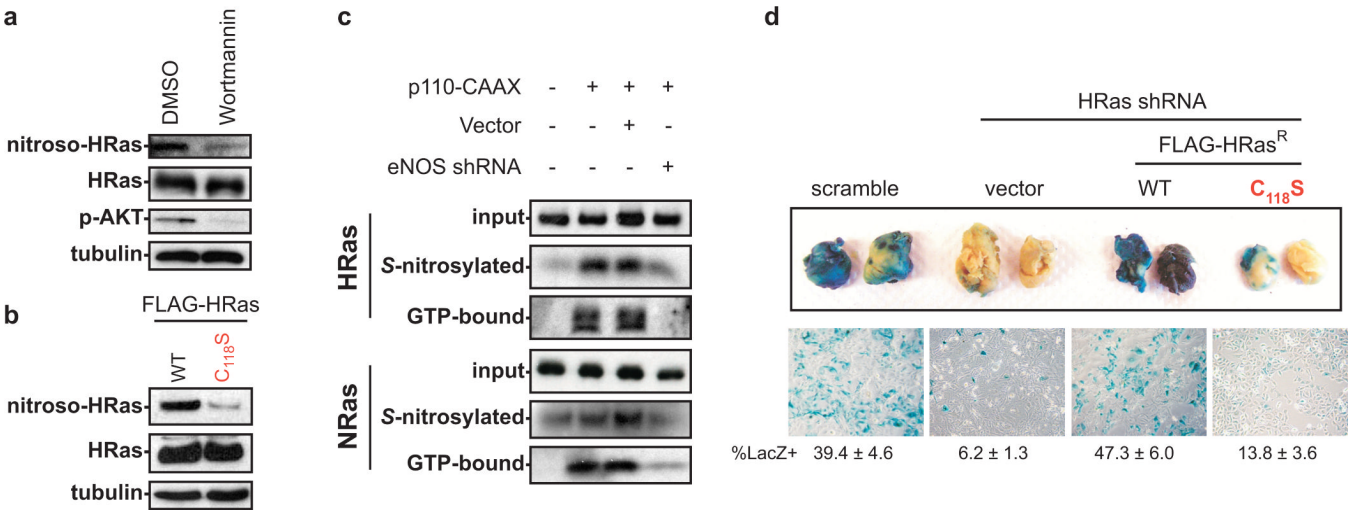


Figure 2.

eNOS activates wildtype HRas to promote tumour maintenance. Protein levels of *S*-nitrosylated (nitroso), GTP-bound, total, or input HRas or NRas, phosphorylated AKT (p-AKT), or as a loading control tubulin in **a**, PI3K-TtH^{LacZ} cells treated with DMSO or wortmannin, **b**, PI3K-TtH^{LacZ} cells transfected with wildtype or C_{118}S HRas or **c**, TtH^{LacZ} cells expressing the indicated combinations of p110-CAAX, vector or eNOS shRNA. **d**, Ras^{G12V}-TtH cells were mixed with PI3K-TtH^{LacZ} cells expressing the indicated constructs, injected into mice, and tumours or recultured tumour cells were stained with X-gal to visualize PI3K-TtH^{LacZ} cells. n=5, mean±s.e.m.

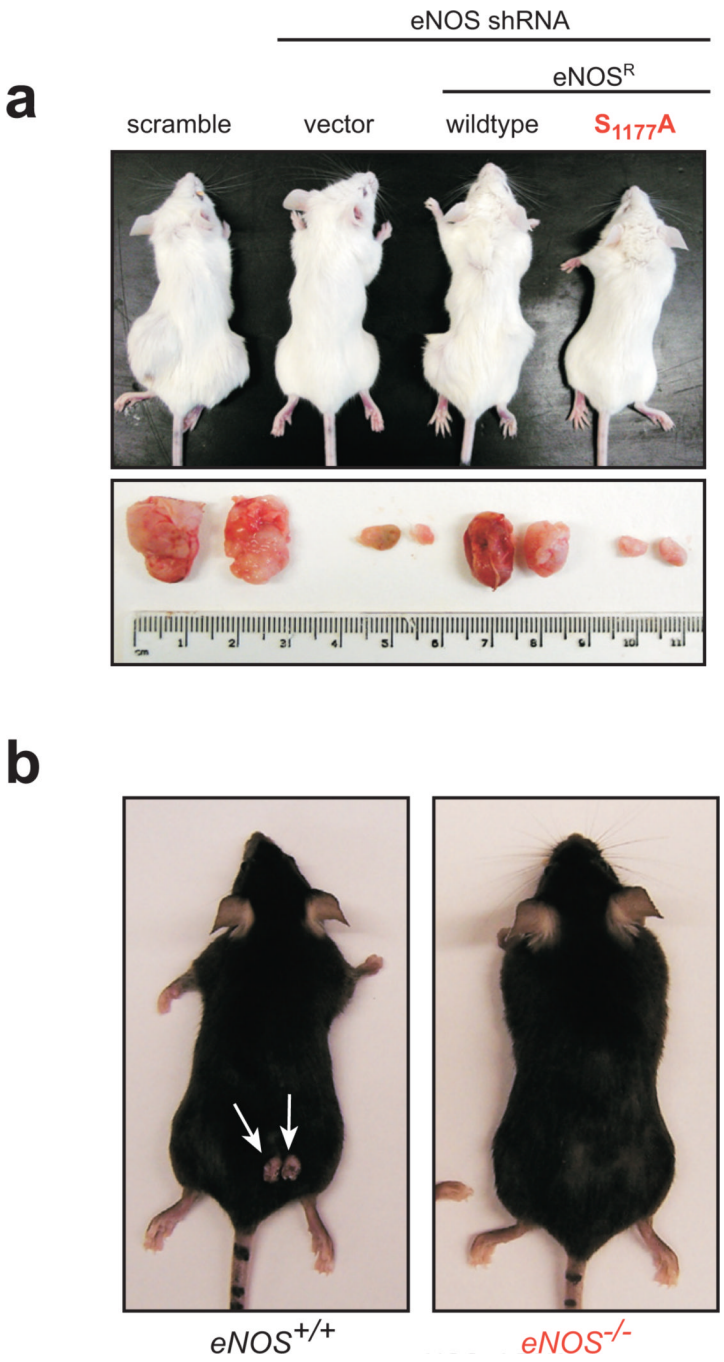


Figure 3.
eNOS activation is required for tumour growth. Representative mice and/or tumours **a**, following injection with Ras^{G12V}-TtH cells expressing indicated constructs or **b**, after treatment with DMBA/TPA to induce skin tumours on mice of indicated genotype (week 20).

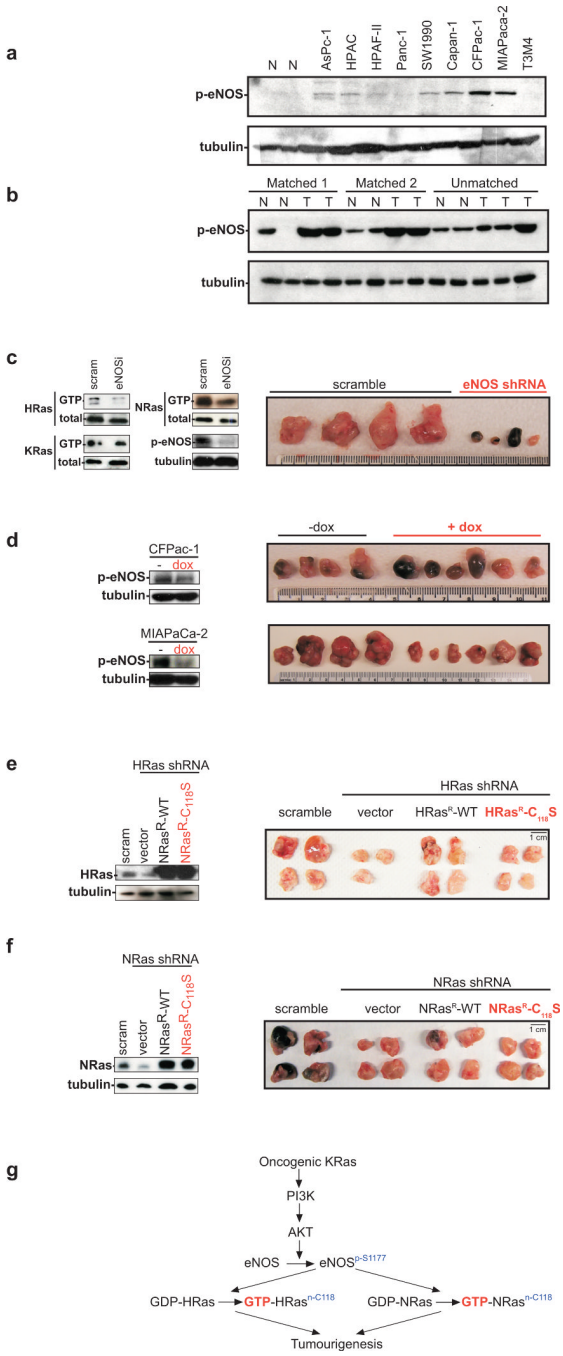


Figure 4.

eNOS activation fosters cancer cell growth through activation of endogenous Ras. Protein levels of phosphorylated eNOS (p-eNOS) or as a loading control, tubulin, in pancreatic **a**, cancer lines, **b**, tumour and normal tissue. Protein levels of GTP-bound or total HRas, NRas, or KRas, p-eNOS or tubulin, and excised tumours of **c**, CFPac-1 cells expressing eNOS or scramble shRNA, **d**, CFPac-1 and MIAPaCa-2 cells expressing dox-inducible shRNA +/− dox, and CFPac-1 cells expressing **e**, HRas or **f**, NRas shRNA plus a vector or an RNAi-resistant wildtype or $C_{118}S$ NRas or HRas, or a scramble sequence. **g**, Proposed signalling.