LETTERS

Tumour maintenance is mediated by eNOS

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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]. Although Ras activates several proteins to initiate human tumour growth, only PI3K, through activation of protein kinase B (PKB; also known as AKT), must remain activated by oncogenic Ras to maintain this growth[3]. Here we show that blocking phosphorylation of the AKT substrate, endothelial nitric oxide synthase (eNOS or NOS3), inhibits tumour initiation and maintenance. Moreover, eNOS enhances the nitrosylation and activation of endogenous wild-type Ras proteins, which are required throughout tumorigenesis. We suggest that activation of the PI3K–AKT–eNOS–(wild-type) 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 tumorigenic growth of otherwise non-tumorigenic 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 several proteins[4], we focused on BAD, FOXO, IKKα, TSC2 and eNOS, 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[5] (Supplementary Fig. 1). Non-tumorigenic PI3K-TtH^{LacZ} cells, derived from normal human kidney cells transformed by oncoproteins T/t-Ags and immortalized by hTERT (hereafter termed TtH cells), and which expressed p110-CAAX (to activate the PI3K-AKT pathway) and LacZ (to demark the cells in the tumour), had Bcl-X$_L$ short hairpin RNA (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 (Supplementary Fig. 1). Knockdown of Bcl-X$_L$ and eNOS, ectopic expression and nuclear localization of FOXO3a-A3 (ref. 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 (Supplementary Fig. 2). These five cell lines were mixed with tumorigenic 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 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; however, eNOS shRNA had the greatest effect on reducing the contribution of PI3K-TtH^{LacZ} (blue) cells in tumours (Fig. 1a).

To test if AKT phosphorylation, not just expression of eNOS, is required for tumour maintenance, AKT was validated to phosphorylate S_{1177} of eNOS[9–11]. This was 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 wild-type or S_{1177}A mutant configuration (Supplementary Fig. 3), and assayed for tumour maintenance by the aforementioned cell-mixing assay. Control PI3K-TtH^{LacZ} cells populated tumours, and this contribution was greatly reduced upon knockdown of eNOS, as evidenced by the reduction in blue staining. This loss was rescued by wild-type, 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[12], and catalyses the synthesis of nitric oxide. This can facilitate *S*-nitrosylation of the thiol group of cysteines in proteins[13], such as that of C_{118} of HRas, which enhances the dissociation of guanine nucleotides, thereby increasing GTP-bound HRas[14]. Wild-type Ras proteins can be required for activation of the MAPK pathway by oncogenic Ras[15] and membrane targeting of RasGAP, which inhibits wild-type but not oncogenic Ras, reverts oncogenic Ras transformation of NIH3T3 cells[16]. This suggests that wild-type 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 wild-type Ras through *S*-nitrosylation of C_{118}. To test this, activated AKT in PI3K-TtH was shown to foster HRas nitrosylation through eNOS. Specifically, most 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[14]) (Fig. 2b), or by knocking down eNOS (Fig. 2c). Conversely, HRas nitrosylation was elevated upon activation of AKT by p110-CAAX (Fig. 2c). Reduction of HRas nitrosylation by eNOS shRNA also reduced levels of active GTP-bound HRas (Fig. 2c). Because TtH cells express HRas and NRas, but not KRas (not shown), and C_{118} is conserved among 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 wild-type Ras proteins. Then, to assess the biological consequence of *S*-nitrosylation of wild-type HRas in tumour maintenance, we tested whether replacing endogenous wild-type 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 an shRNA-resistant

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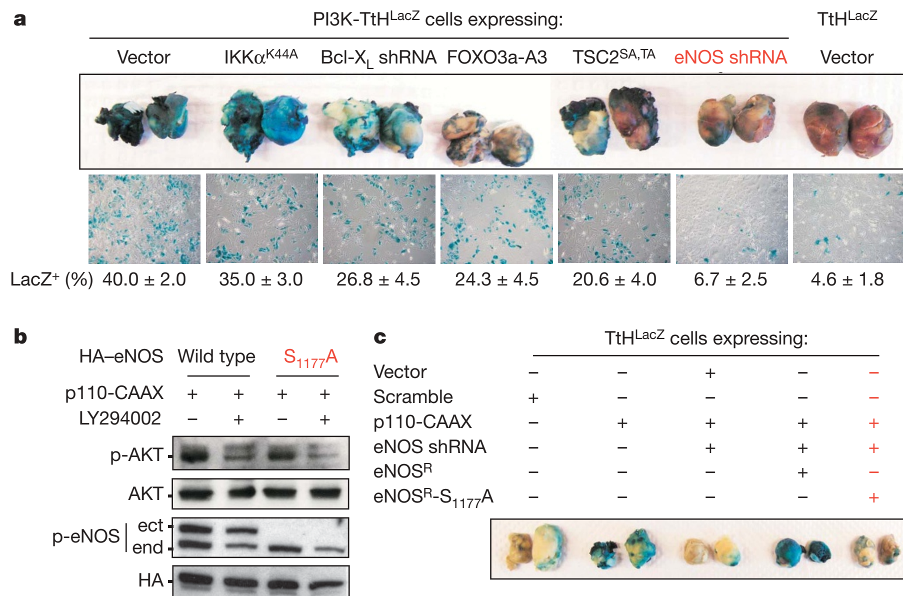

Figure 1 | AKT promotes tumour maintenance by phosphorylation of eNOS. **a**, PI3K-TtH^{LacZ} or **c**, TtH^{LacZ} cells expressing indicated constructs, were mixed with Ras^{G12V}-TtH cells, injected into mice, and tumours or re-cultured tumour cells were stained with X-gal ($n = 5$, mean ± s.e.m.). **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 wild-type or S_{1177}A HA–eNOS treated with DMSO or LY294002.

HRas (HRas^{R}) in the wild-type or C_{118}S mutant configuration that reduced GTP loading, or, as a control, no transgene (Supplementary Fig. 4). These cell lines, or as control 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 wild-type 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 wild-type HRas^{R}, 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 wild-type Ras.

As oncogenic Ras must activate the PI3K-AKT pathway both to 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 tumorigenic Ras^{G12V}-TtH cells, and knockdown of eNOS complemented by RNAi-resistant eNOS (eNOS^{R}) in the wild-type or S_{1177}A mutant configuration resistant to S_{1177} phosphorylation (Supplementary 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 wild-type, but not the S_{1177}A version, of eNOS^{R} (Fig. 3a and Supplementary Fig. 6), indicating that S_{1177} phosphorylation of eNOS is required for tumour initiation and maintenance. These results were validated in a chemical carcinogen-induced spontaneous Ras-driven cancer model. DMBA (7,12-dimethylbenz(a)anthracene) followed by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were topically applied to *eNOS*$^{+/+}$ and *eNOS*$^{-/-}$ mice to induce skin papillomas characterized by *Ras* oncogenic mutations[17]. The result was an

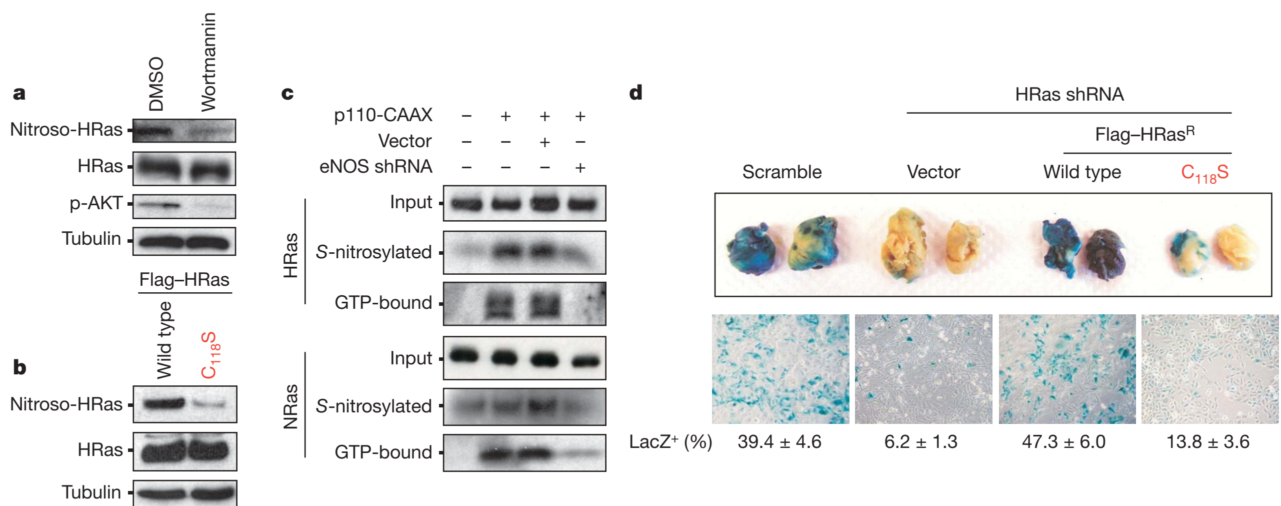

Figure 2 | eNOS activates wild-type HRas to promote tumour maintenance. **a–c**, Protein levels of *S*-nitrosylated (nitroso), GTP-bound, total or input HRas or NRas, phosphorylated AKT (p-AKT), or as a loading control tubulin in PI3K-TtH^{LacZ} cells treated with DMSO or wortmannin (**a**), PI3K-TtH^{LacZ} cells transfected with wild-type or C_{118}S HRas (**b**), or TtH^{LacZ} cells expressing the indicated combinations of p110-CAAX, vector or eNOS shRNA (**c**). **d**, Ras^{G12V}-TtH cells were mixed with PI3K-TtH^{LacZ} cells expressing the indicated constructs, injected into mice, and tumours or re-cultured tumour cells were stained with X-gal to visualize PI3K-TtH^{LacZ} cells $n = 5$, mean ± s.e.m.).

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approximate threefold drop in the number of tumours per $eNOS^{-/-}$ mouse (Fig. 3b and Supplementary 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 with 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 with matched and unmatched normal tissue controls (Fig. 4b and ref. 18), with the caveat that biopsies also contained stromal tissue that could have contributed to detected eNOS phosphorylation.

Next, we tested whether eNOS was required for pancreatic tumour growth. Knockdown of eNOS reduced tumour growth by 50-fold in CFPac-1 cells, and tumour size of MIAPaCa-2 cells by 80% (Fig. 4c, and Supplementary Figs 8 and 9). As a control, knockdown of eNOS in cell lines AsPc-1 and SW1990, which exhibited poor eNOS activation, had no obvious effect on tumour growth (data not shown). 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 (data not shown). Thus, eNOS is required both to initiate and maintain tumour growth of these human pancreatic cancer cells.

To test whether 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 on knockdown of eNOS (Fig. 4c and Supplementary Fig. 9), as *KRas* is

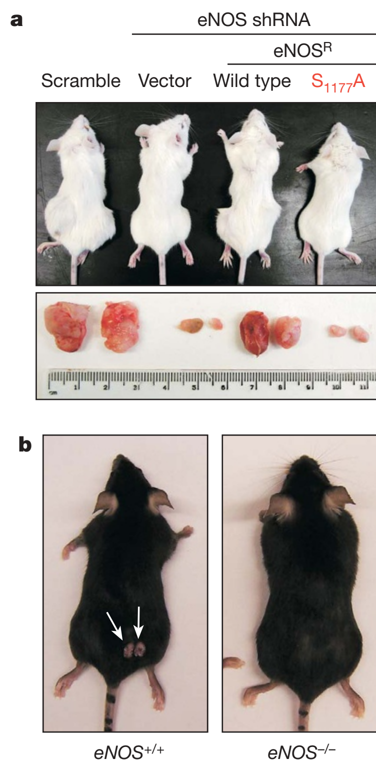

Figure 3 | eNOS activation is required for tumour growth. Representative mice and/or tumours: **a**, after injection with Ras^{G12V}-TtH cells expressing indicated constructs; or **b**, after treatment with DMBA/TPA to induce skin tumours on mice of indicated genotype (week 20).

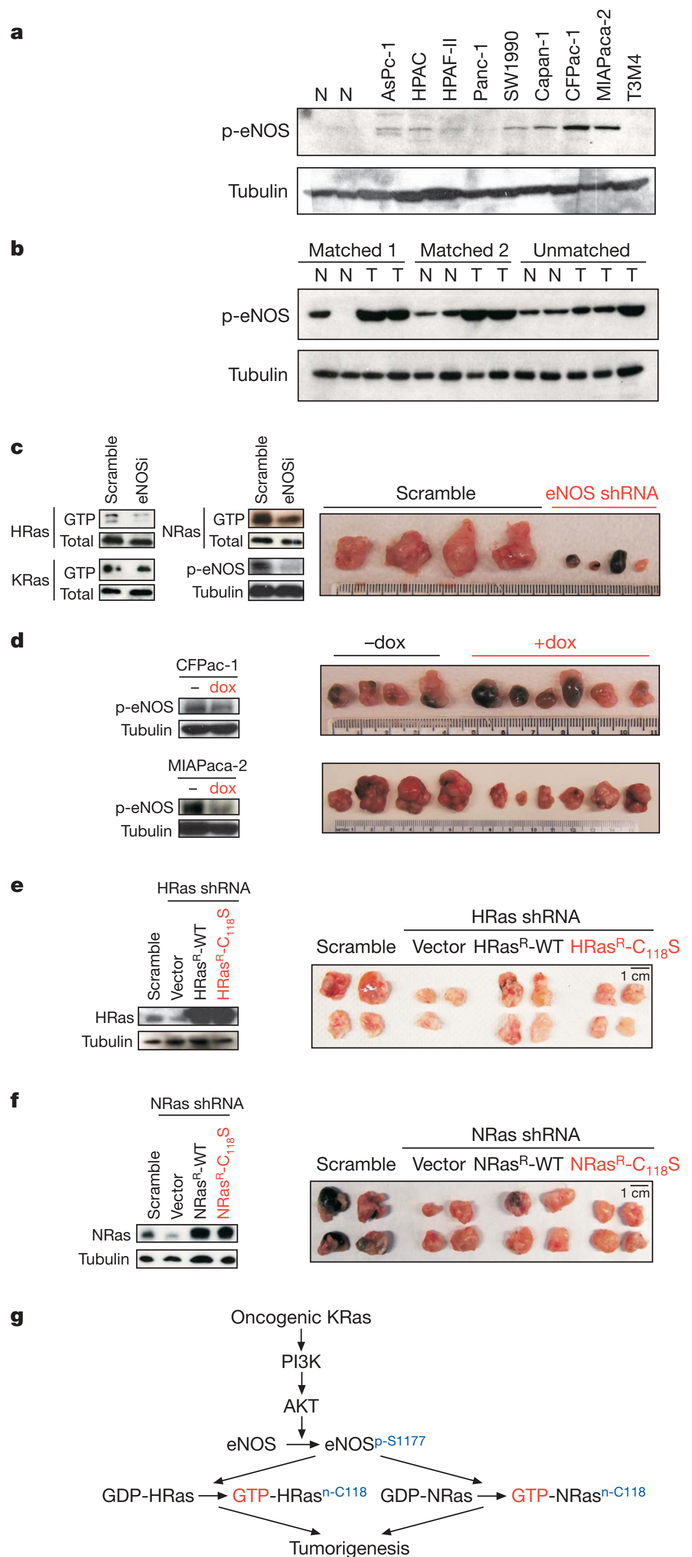

Figure 4 | eNOS activation fosters cancer cell growth through activation of endogenous Ras. **a**, **b** Protein levels of phosphorylated eNOS (p-eNOS) or as a loading control, tubulin, in pancreatic cancer lines (**a**), tumour, and normal tissue (**b**). **c**–**f**, Protein levels of GTP-bound or total HRas, NRas or KRas, p-eNOS or tubulin, and excised tumours of CFPac-1 cells expressing eNOS or scramble shRNA (**c**), CFPac-1 and MIAPaCa-2 cells expressing dox-inducible shRNA +/− dox (**d**), and CFPac-1 cells expressing HRas (**e**) or NRas shRNA (**f**) plus a vector or an RNAi-resistant wild-type or $C_{118}S$ HRas or NRas, or a scramble sequence. **g**, Proposed signalling.

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mutated to remain active in these two cell lines[19,20]. Consistent with this, oncogenic KRas harbouring the $C_{118}S$ mutation remained tumorigenic (Supplementary Fig. 10), pointing towards wild-type Ras proteins as the target of eNOS signalling. Indeed, GTP-bound endogenous wild-type HRas and NRas were reduced upon shRNA knockdown of eNOS. Moreover, as the wild-type allele of *KRas* is deleted in MIAPaCa-2 cells[20], several oncogenic *KRas*-positive cell lines[20] and tumour tissues[21], wild-type HRas and NRas, but not KRas, appear to be the targets of eNOS signalling in pancreatic cancer cells (Fig. 4c and Supplementary Fig. 9).

To 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 that was engineered to be resistant to RNAi ($HRas^R$, $NRas^R$) in the wild-type or $C_{118}S$ mutant configuration. Resultant cells were then 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 wild-type HRas or NRas, but not the $C_{118}S$ nitrosylation mutants (Fig. 4e, f and Supplementary 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 wild-type Ras proteins cannot be nitrosylated (Supplementary Fig. 14). Thus, oncogenic KRas-driven pancreatic cancer tumour growth was mediated by eNOS nitrosylation of endogenous wild-type 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}. This in turn leads to *S*-nitrosylation at C_{118} and correspondingly activation of the other wild-type Ras family members, perhaps as a means to diversify the Ras signal beyond that of oncogenic Ras (Fig. 4g). In agreement, the wild-type counterpart of oncogenic Ras is not required for tumorigenesis (Supplementary Fig. 15), and is even deleted in some tumours[21,22]; whereas wild-type HRas and NRas are required for oncogenic KRas-driven tumour growth, and appear to have non-redundant activies[23–26]. Effects of eNOS on tumorigenesis have been largely attributed to its activity in endothelial cells[12]. Our results now suggest a key role for tumour-expressed eNOS in the tumorigenic process. Because eNOS plays multiple roles in tumorigenesis[12], and delivery of a peptide fragment of the protein cavtratin, which can inhibit eNOS, displays anti-tumour activity[27], we speculate that inhibition of eNOS, perhaps in combination with inhibition of wild-type Ras protein function or processing[2], could have therapeutic value in the treatment of oncogenic Ras-driven human cancers such as those of the pancreas.

METHODS SUMMARY

TtH and the pancreatic cancer cell lines were stably infected retroviruses encoding the indicated shRNAs, transgenes or no insert as described[5], and appropriate expression verified by immunoblot or polymerase chain reaction with reverse transcription (RT–PCR). Detection of GTP-bound or nitrosylated Ras was performed as described in Methods. One or a mixture of two cell lines were injected into the flanks of immunocompromised mice to assay for tumour growth; where indicated, they were excised and assayed for LacZ-positive cells, as described[3]. Induction of shRNA *in vivo* by dox[3] and DMBA/TPA treatments[28] were performed as described.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions K.-H.L., B.B.A. and D.F.K. performed the experiments. All authors conceived and designed experiments and participated in the writing of the paper.

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METHODS

Plasmids. pBabepuro, neo, bleo and hygro were used as control vectors[3]. The following complementary DNAs (cDNAs) were subcloned into one of the aforementioned pBabe vectors: HA–IKKα^{K44A} cDNA[29], FOXO3a-A3 cDNA[30] engineered with an amino (N)-terminal HA tag, Flag–TSC2^{S393A,T1462A} cDNA[7] (termed here as TSC2^{SA,TA}), eNOS cDNA engineered with a carboxy (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 wild-type 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. Bcl-X_L shRNA (5′-AGCGTAGACAAGGAGATGC), eNOS shRNA (5′-AAGAGTTATAAGATCCGCTTC), HRas shRNA (5′-GGCAAGAGTG-CGCTGACCATC), NRas shRNA (5′-CAAGAAGAGTACAGTGCCATG) or 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[31]. Derived lines were generated by stable infection with the indicated combinations of amphotrophic retroviruses generated from the aforementioned pBabe plasmids, as previously described[5].

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 h before 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 anti-HA (Roche), anti-Flag (Sigma), anti-Bcl-xL, anti-p70 S6 Kinase, anti-Thr389 Phospho-p70 S6 Kinase, anti-Ser1177 Phospho-eNOS, anti-Ser473 Phospho-AKT (Cell Signaling Technology), anti-HRas, anti-KRas, anti-NRas, anti-actin (Santa Cruz), anti-p65 (Rockland) and anti-tubulin (Sigma) antibodies, respectively.

RT–PCR. eNOS and GAPDH mRNA was RT–PCR amplified with the primers 5′-CAGTGTCCAACATGCTGCTGGAAATTG and 5′-TAAAGGTCTTCTT-CCTGGTGATGC, and the primers 5′-ACCACAGTCCATGCCATCAC and 5′-TCCACCACCCTGTTGCTGTA, respectively.

GTP and nitrosylated Ras. GTP-bound or nitrosylated Ras were captured as previously described[32,33] and immunoblotted with either an anti-Flag (Sigma) or an anti-HRas, anti-KRas or anti-NRas (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 four 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 four tumours were removed when they reached maximum volume, human cells derived from the two tumours by re-culture in selective media (G418), and the two 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 five SCID/Beige mice. Tumours were allowed to reach a diameter of 0.6 cm, after which three mice were provided with doxycycline in their diet and two 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[28] on 15 eNOS$^{+/+}$ C57BL/6J and 15 eNOS$^{-/-}$ C57BL/6J (B6.129P2-*Nos3*^{tm1Unc}/J) mice[34] (Jackson Laboratory). All animal work was approved by the Duke University Medical Center Institutional Animal Care and Use Committee.

Tumour and normal human specimens. Flash-frozen tissue samples were provided devoid of all identifying information under a Duke University Medical Center approved Institutional Review Board protocol.

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