Activation of Notch-1 signaling maintains the neoplastic phenotype in human Ras-transformed cells

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Truncated Notch receptors have transforming activity *in vitro* and *in vivo*. However, the role of wild-type Notch signaling in neoplastic transformation remains unclear. Ras signaling is deregulated in a large fraction of human malignancies and is a major target for the development of novel cancer treatments. We show that oncogenic Ras activates Notch signaling and that wild-type Notch-1 is necessary to maintain the neoplastic phenotype in Ras-transformed human cells *in vitro* and *in vivo*. Oncogenic Ras increases levels and activity of the intracellular form of wild-type Notch-1, and upregulates Notch ligand Delta-1 and also presenilin-1, a protein involved in Notch processing, through a p38-mediated pathway. These observations place Notch signaling among key downstream effectors of oncogenic Ras and suggest that it might be a novel therapeutic target.

Notch genes encode heterodimeric transmembrane receptors that regulate differentiation, proliferation and apoptosis[1,2]. Mammals have four known Notch genes and two families of Notch ligands designated 'Delta' and 'Jagged', respectively. Upon receptor-ligand interaction, Notch proteins are cleaved by a presenilin-1 (PS-1)-dependent γ-secretase activity[3]. This releases a cytoplasmic subunit (N^{IC}), which migrates to the nucleus. This 'active' form of Notch regulates the function of several transcription factors. The best-characterized Notch downstream effectors are known as CSL factors (CBF-1 in mammals, suppressor of hairless in *Drosophila* and LAG-1 in *Caenorhabditis elegans*). Several reports suggest that Notch signaling participates in neoplastic transformation. Extracellular deletions of Notch-1 have been implicated in T-cell acute lymphoblastic leukemia[4]. Similar truncated Notch receptors have transforming activity *in vitro*[5–7] and in animal models[8–10]. However, truncated Notch receptors are uncommon in human malignancies. Deregulated expression of wild-type Notch receptors, ligands and downstream targets has been described in cervical, lung, colon, head and neck and renal carcinomas[11–14], acute myeloid leukemia[15] as well as Hodgkin and large-cell lymphomas[16]. However, it is unknown whether wild-type Notch signaling has a role in spontaneous tumor progression and/or in maintaining the neoplastic phenotype.

Ras proto-oncogenes carry activating mutations in approximately 30% of human malignancies[17]. Overexpression of wild-type Ras proteins and activation of Ras signaling by other oncogenes further increase the number of malignancies in which Ras signaling is deregulated. The Ras signaling network is considered a prime target for the development of novel anti-neoplastic agents. Consequently, the identification of key Ras signaling mediators in human neoplastic cells is of considerable significance.

Here we show that Notch-1 is a downstream effector of oncogenic Ras, and that downregulation of Notch-1 in Ras-transformed human cells is sufficient to abolish key elements of the neoplastic phenotype *in vitro* and *in vivo*.

Notch-1 is upregulated in Ras-transformed cells

We investigated the role of Notch-1 in two human tumor models, human foreskin fibroblasts (BJ) and human embryonic kidney epithelial cells (HEK), expressing the human telomerase reverse transcriptase subunit (hTERT), SV40 oncoproteins and oncogenic H-RasV12[18]. Western blots with antibodies to intra-

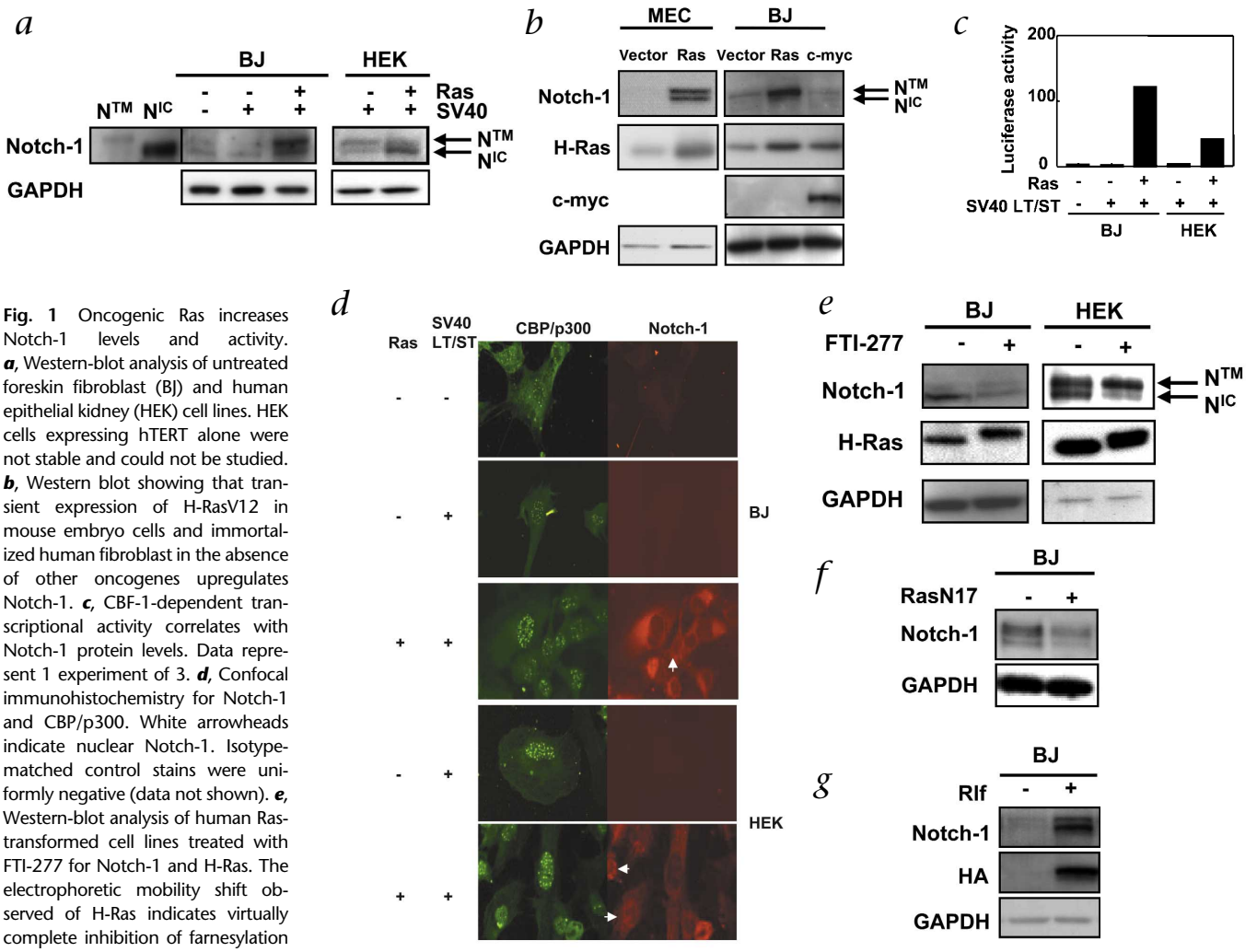

Fig. 1 Oncogenic Ras increases Notch-1 levels and activity. ***a***, Western-blot analysis of untreated foreskin fibroblast (BJ) and human epithelial kidney (HEK) cell lines. HEK cells expressing hTERT alone were not stable and could not be studied. ***b***, Western blot showing that transient expression of H-RasV12 in mouse embryo cells and immortalized human fibroblast in the absence of other oncogenes upregulates Notch-1. ***c***, CBF-1-dependent transcriptional activity correlates with Notch-1 protein levels. Data represent 1 experiment of 3. ***d***, Confocal immunohistochemistry for Notch-1 and CBP/p300. White arrowheads indicate nuclear Notch-1. Isotype-matched control stains were uniformly negative (data not shown). ***e***, Western-blot analysis of human Ras-transformed cell lines treated with FTI-277 for Notch-1 and H-Ras. The electrophoretic mobility shift observed of H-Ras indicates virtually complete inhibition of farnesylation under these conditions. ***f***, Western blot showing that Notch-1 protein levels were downregulated when Ras-transformed fibroblasts were stably transfected with H-RasN17. ***g***, Western blot showing that human fibroblasts transfected with Rlf-CAAX, a constitutively active downstream target of Ras, upregulated Notch-1 compared with vector-transfected cells.

cellular Notch-1 revealed two major bands with apparent molecular masses of 110 and 97 kD, corresponding to the uncleaved transmembrane subunit N^{TM} and its intracellular cleavage product N^{IC}, respectively[19] (Fig. 1*a*). Cells expressing hTERT, SV40 oncoproteins and H-RasV12 showed increased amounts of Notch-1 protein and a more prominent N^{IC} band compared with cells expressing hTERT and SV40 oncoproteins or hTERT alone. Notch-4 showed a similar increase as Notch-1. Notch-2 was undetectable and Notch-3 did not vary among these cell lines (data not shown). When we transiently transfected primary mouse embryonic cells and primary human fibroblasts with H-RasV12 or empty vector, H-RasV12 markedly upregulated both N^{IC} and N^{TM} subunits compared with empty vector. A different oncogene, c-myc, did not affect Notch-1 expression (Fig. 1*b*).

Upon ligand-induced cleavage of Notch-1, the N^{IC} subunit binds to ubiquitous repressor CBF-1, converting it into a transcriptional activator[20]. Thus, activation of CBF-1-dependent transcription is an indicator of Notch-1 activity. CBF-1 reporter activity in Ras-transformed cells was directly correlated with Notch-1 protein levels (Fig. 1*c*), showing approximately 100-fold and 50-fold increases compared with controls in BJ and HEK cells, respectively. These differences did not result from variations in CBF-1 protein expression (data not shown).

Nuclear localization of N^{IC} occurs after Notch activation[3,21] and is necessary for the transforming activity of intracellular Notch-1[6]. We determined the cellular distribution of Notch-1 by confocal immunofluorescence (Fig. 1*d*). In cells lacking H-RasV12, Notch-1 immunoreactivity was nearly undetectable. H-RasV12-expressing cells showed strong Notch-1 immunoreactivity with a coarse, mostly cytoplasmic punctate pattern. Punctate nuclear signal was weakly visible in most but not all cells. This signal did not colocalize with CBP/p300 protein, which distributed to nuclear bodies. Previous studies have shown that minute amounts of N^{IC} are sufficient for nuclear Notch signaling[21–23].

Oncogenic Ras is upstream of Notch-1

We explored the relationship between Ras and Notch-1 using pharmacological and genetic approaches. Ras-transformed cells were treated with a farnesyltransferase inhibitor (FTI-277), blocking post-translational maturation of Ras family proteins.

Notch-1 protein levels, particularly of the active subunit N^{IC}, were significantly reduced in FTI-277-treated Ras-transformed cells (Fig. 1*e*). FTI-277 did not affect Notch-1 in cells lacking H-RasV12 (data not shown). We confirmed these results by stably introducing a dominant-negative form of H-Ras (N17) into human fibroblasts (Fig. 1*f*). Expression of H-RasN17 downregulated Notch-1. We then transfected human fibroblasts with constitutively activated Rlf[24], a downstream Ras mediator that can activate Ras effector Ral and c-*fos* promoter activity. Rlf significantly upregulated Notch-1 (Fig. 1*g*). Taken together, these data strongly indicate that Notch-1 is a downstream target of oncogenic H-Ras.

Ras and Notch-1 expression correlate in breast carcinomas

Notch-1[25] and Notch-4[9] have been linked to murine mammary carcinogenesis. Ras signaling is commonly activated in breast carcinomas due to increased expression of wild-type H-Ras and/or overexpression or activation of epidermal growth factor–receptor (EGFR) family receptors or downstream targets[26]. H-Ras is thought to have a key and initial role in mammary carcinogenesis in humans[27]. Therefore, we studied H-Ras and Notch-1 expression by immunohistochemistry in seven cases of human primary breast ductal carcinoma (Fig. 2*a*). Consistent with previous studies[27], Ras was detectable in 4 of 7 cases (Fig. 2*a*). All breast cancers were positive for Notch-1 (Fig. 2*b*). However, Ras-positive tumors showed stronger and diffuse Notch-1 staining, whereas Ras-negative tumors showed weaker staining with scattered individual cells more intensely stained. In cancer cells, as in Ras-transformed fibroblasts and epithelial kidney cells, Notch-1 immunostaining appeared punctate and primarily cytoplasmic, with some but not all cells showing nuclear signal. Normal breast ducts and lobules at the margins of the tumor sections showed absent or barely detectable Notch-1 staining. We verified that overexpression of wild-type H-Ras in hTERT/SV40-immortalized fibroblasts upregulated Notch-1 (Fig. 2*c*). Larger numbers of cases and other tumor types will have to be systematically studied to gain a clear picture of Notch expression in human malignancies. However, these observations suggest that a common epithelial cancer in which Ras signaling has a key role shows elevated Notch-1 expression.

Notch-1 downregulation inhibits Ras-transformed cells

We explored the role of Notch-1 in Ras-transformed cells using genetic and pharmacological strategies. Ras-transformed fibroblasts were transfected with human antisense Notch-1 (ASN) under the control of a doxycycline-inducible cytomegalovirus (CMV) promoter. ASN-expressing cells clearly downregulated Notch-1 expression (Fig. 3*a*). This did not result in increased cell death in these cells. ASN-expressing cells showed a significant reduction ($P<0.0001$) in proliferation compared with vector-transfected controls (Fig. 3*b*). This was confirmed when fibroblasts were treated with a γ-secretase inhibitor (GSI)[28], which blocks Notch cleavage and activation (Fig. 3*c*). Untransformed, SV40-expressing fibroblasts were not significantly affected by Notch inhibition, whereas proliferation of Ras-transformed fibroblasts was reduced by 80% ($P < 0.0001$) (Fig. 3*c*). Additionally, ASN-transfected cells showed a statistically significant reduction ($P = 0.0014$) in anchorage-independent growth compared with vector-transfected cells seven days after plating (Fig. 3*d* and Supplementary Fig. A online). GSI treatment of Ras-transformed fibroblasts also significantly inhibited anchorage-independent growth ($P = 0.0184$) (Fig. 3*e*).

To establish whether Notch-1 downregulation has anti-neoplastic effects *in vivo*, immunodeficient SCID/NOD mice were injected with ASN-transfected or vector-transfected Ras-transformed fibroblasts. ASN expression was induced by daily injection with doxycycline beginning the day of tumor injection. Tumor growth could be detected in all mice that received vector-transfected, but not ASN-transfected cells, 10 days after tumor injection (Fig. 3*f*). Vector-transfected tumors grew exponentially until day 39. In contrast, tumor growth of ASN-transfected cells was negligible during the first 35 days, after which it increased until day 65.

To determine whether established tumors were affected by ASN, SCID/NOD mice were injected with ASN- or vector-transfected Ras transformed fibroblasts and ASN expression was in-

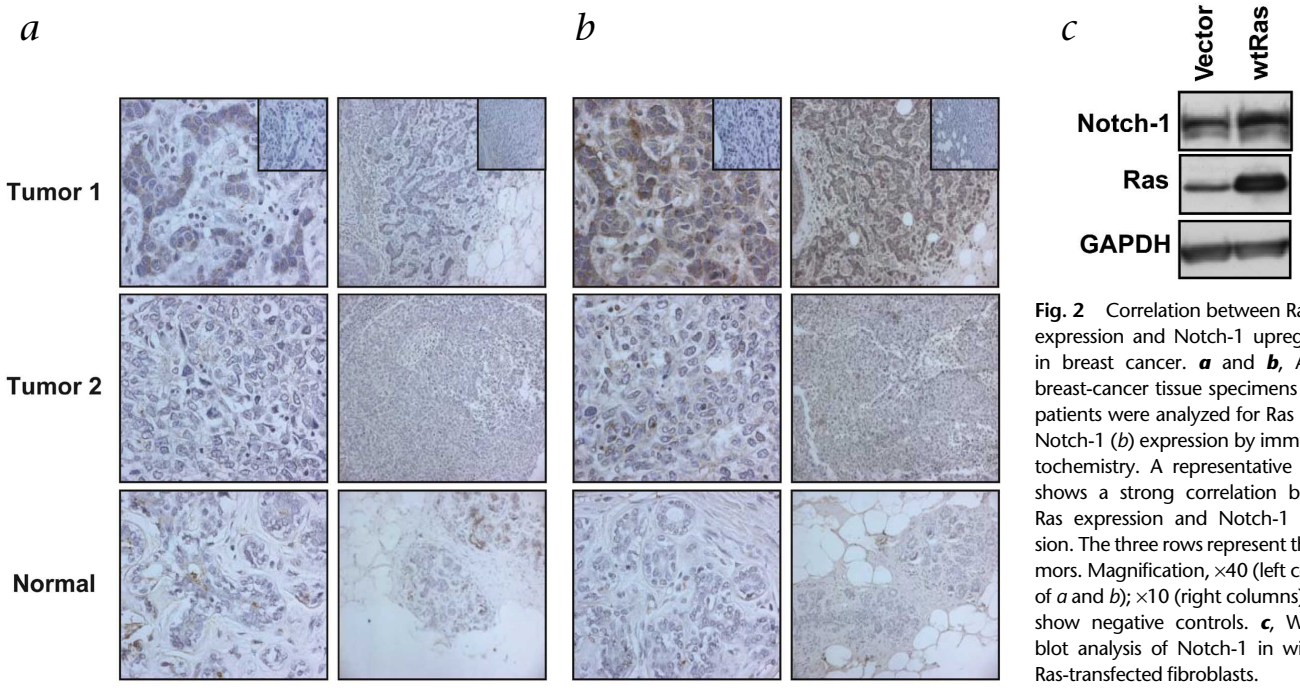

Fig. 2 Correlation between Ras overexpression and Notch-1 upregulation in breast cancer. ***a*** and ***b***, Archival breast-cancer tissue specimens from 7 patients were analyzed for Ras (*a*) and Notch-1 (*b*) expression by immunohistochemistry. A representative sample shows a strong correlation between Ras expression and Notch-1 expression. The three rows represent three tumors. Magnification, ×40 (left columns of *a* and *b*); ×10 (right columns). Insets show negative controls. ***c***, Western-blot analysis of Notch-1 in wild-type Ras-transfected fibroblasts.

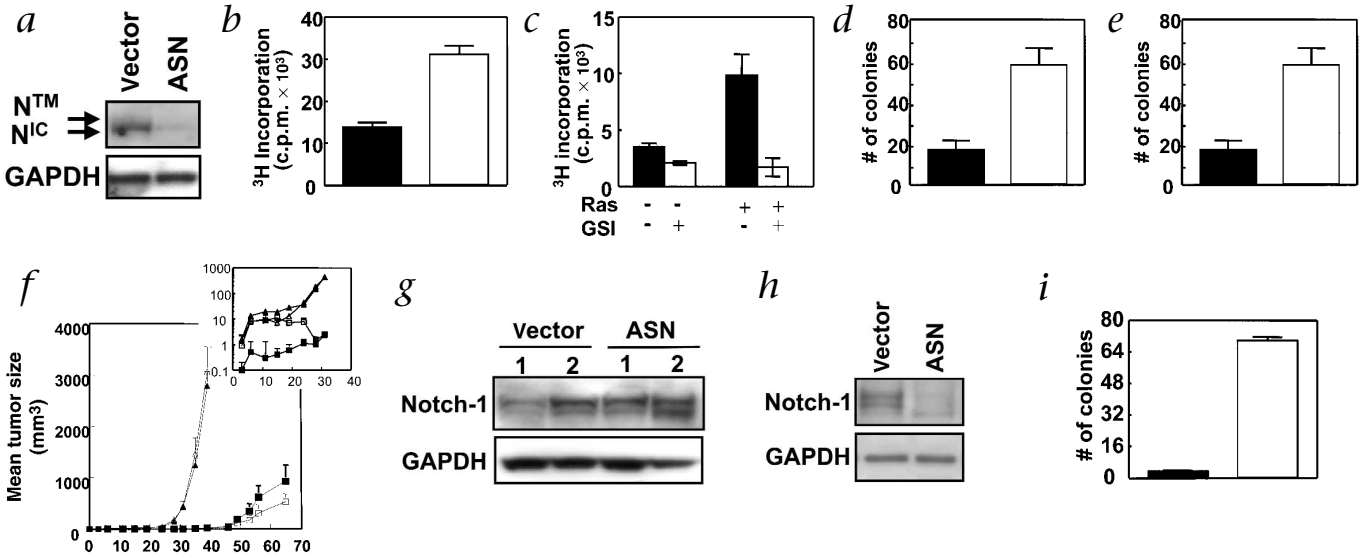

Fig. 3 Inhibition of Notch-1 expression or activation in Ras-transformed cells inhibits the transformed phenotype *in vitro* and causes loss of tumorigenicity *in vivo*. ***a***, Western-blot analysis of Notch-1 in doxycycline treated ASN- or vector-transfected Ras-transformed fibroblasts. ***b***, In a proliferation assay, a significant difference ($P < 0.0001$) between ASN- (■) and vector- (□) transfected Ras-transformed cells was observed using an unpaired *t*-test. ***c***, Ras-transformed fibroblasts treated with GSI showed a strong anti-proliferative effect ($P < 0.0001$), whereas untransformed, SV40-expressing fibroblasts were only modestly affected. This concentration of GSI did not induce toxicity as determined by Trypan blue exclusion assay. ***d***, In a soft-agar assay, a significant difference ($P = 0.0014$) was observed between ASN- (■) and vector- (□) transfected Ras-transformed cells using an unpaired *t*-test. ***e***, Treatment of human Ras-expressing fibroblasts with GSI (■) resulted in a significant decrease ($P = 0.0184$) in colony formation in soft agar using an unpaired *t*-test. □, DMSO. ***f***, Induction of expression of ASN in Ras-transformed cells from the day of tumor injection (■) significantly delayed and reduced tumor formation in SCID/NOD mice ($n = 10$) compared with vector-transfected cells (▲). When doxycycline induction was delayed by 2 wk, both ASN- and vector-transfected cells formed tumors. Induction of ASN (□) caused a striking decrease in tumor size compared with vector-transfected cells (△). Inset shows tumor growth during the first 30 days. Decrease in tumor size in doxycycline-induced ASN-transfected established tumors is evident. ***g***, Notch-1 expression was determined by western blot in ASN- and vector-transfected tumors that were excised 55 d after tumor injection. ***h***, Transfection of ASN in CaSki cervical cancer cells decreased Notch-1 expression. ***i***, Expression of ASN (■) versus vector (□) in CaSki cells nearly abolished anchorage independent growth in soft agar ($P < 0.0000001$). Pictures of representative soft agar plates are shown in Supplementary Fig. A online.

duced two weeks after tumor injection, when both ASN- and vector-transfected tumors were palpable (~10 mm^3). Once doxycycline injection was started, ASN-transfected tumors regressed and remained small (1 mm^3) (Fig. 3*f*, inset). Vector-transfected cells continued to grow exponentially. Similar to mice that had received doxycycline from day 1, tumor growth of ASN-transfected tumors began to increase after 35 days. To determine whether this delayed tumor growth resulted from loss of ASN effect, tumors from vector-transfected and ASN-transfected mice were excised, cultured *in vitro* for 1 week, incubated with doxycycline for 48 hours and analyzed for Notch-1 (Fig. 3*g*). Notch-1 expression in ASN-transfected tumors had significantly increased and was comparable with that of vector-transfected tumors. This was likely due to selection of tumor cells with low ASN expression.

Cervical carcinomas spontaneously overexpress Notch-1 (refs. 11,12). Therefore, we transiently transfected CaSki, an HPV-16-positive cervical carcinoma line, with doxycycline-inducible ASN or empty vector (Fig. 3*h*). Stable transfection of ASN, but not empty vector, was consistently lethal to CaSki cells. In the presence of doxycycline, Notch-1 expression was markedly inhibited and anchorage-independent growth was virtually abolished ($P < 0.0000001$, Fig. 3*i*). These results suggest that Notch-1 is essential for the maintenance of the transformed phenotype in HPV-positive cervical cancer cells.

Oncogenic Ras upregulates presenilin-1 expression

We next examined the mechanism(s) through which Ras affects Notch-1. Real-Time RT-PCR showed that H-RasV12 only slightly increased (~2-fold) Notch-1 mRNA levels relative to hTERT/SV40 immortalized cells (data not shown), suggesting that Ras affects Notch-1 protein levels predominantly through post-transcriptional mechanisms. ^{35}S-labeling pulse-chase immunoprecipitation experiments (Fig. 4*a*) showed that Notch-1 protein processing was markedly affected in Ras-transformed cells. Band identity was confirmed using transfected constructs encoding N^{TM} or N^{IC}. In hTERT/SV40-immortalized cells Notch-1 appeared as a cluster of bands with apparent molecular masses of >200 kD, which were most likely incompletely reduced Notch-1 pre-protein and/or maturation intermediates. This pattern was observed until eight hours after chase. Bands co-migrating with authentic N^{TM} or N^{IC} were nearly undetectable, appearing as extremely faint shadows at 45, 60 and 90 minutes. Conversely, in Ras-transformed cells a 110-kD band, comigrating with authentic N^{TM}, and a 97-kD band, comigrating with authentic N^{IC}, appeared immediately and were detectable until at least 120 minutes. These observations indicate that, compared with hTERT/SV40-immortalized cells, Ras-transformed cells mature and activate Notch-1 with far more rapid kinetics. This most likely accounts for the higher steady state levels of N^{TM} and especially N^{IC} that we observed in Ras-transformed cells.

To determine the effects of blocking Notch activation pharmacologically, we treated BJ fibroblasts and HEK epithelial cells with GSI and analyzed them for Notch-1 protein (Fig. 4*b*). Band identity was confirmed using transfected N^{TM} and N^{IC} constructs. Inhibition of γ-secretase blocked Notch-1 processing, with rela-

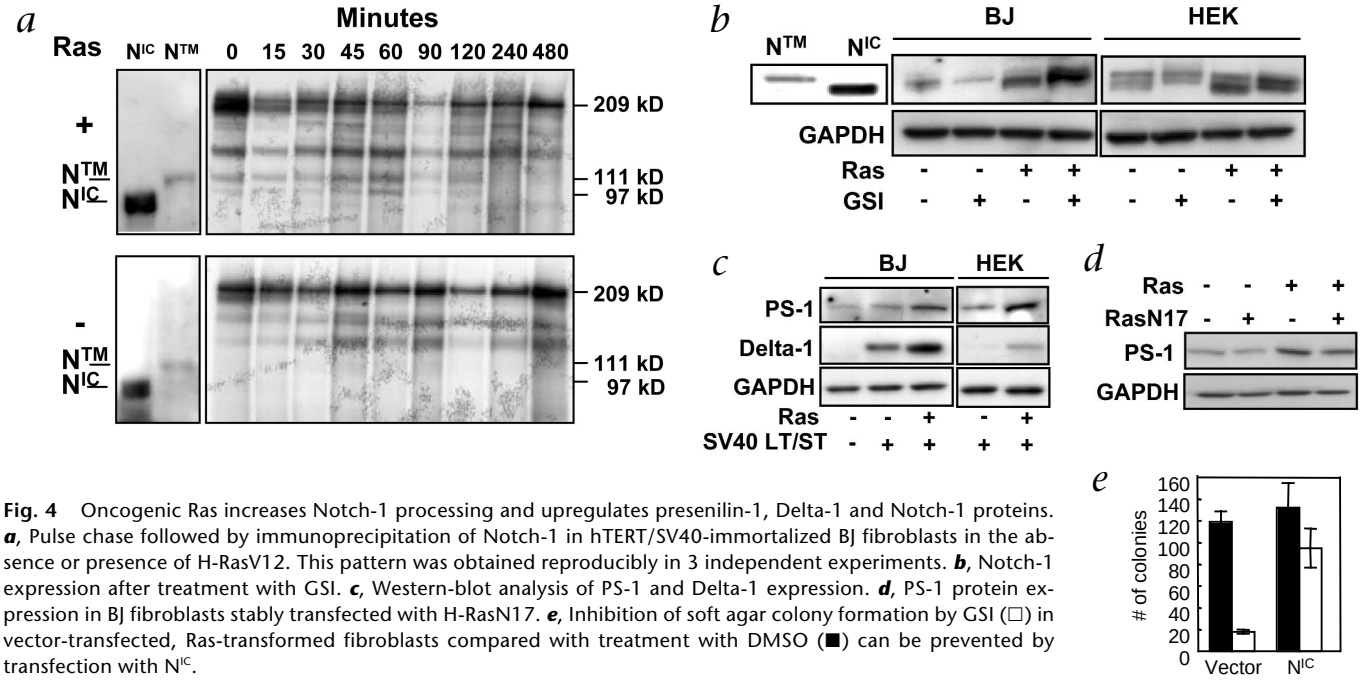

Fig. 4 Oncogenic Ras increases Notch-1 processing and upregulates presenilin-1, Delta-1 and Notch-1 proteins. ***a***, Pulse chase followed by immunoprecipitation of Notch-1 in hTERT/SV40-immortalized BJ fibroblasts in the absence or presence of H-RasV12. This pattern was obtained reproducibly in 3 independent experiments. ***b***, Notch-1 expression after treatment with GSI. ***c***, Western-blot analysis of PS-1 and Delta-1 expression. ***d***, PS-1 protein expression in BJ fibroblasts stably transfected with H-RasN17. ***e***, Inhibition of soft agar colony formation by GSI (□) in vector-transfected, Ras-transformed fibroblasts compared with treatment with DMSO (■) can be prevented by transfection with N^{IC}.

tive accumulation of N^{TM}. In GSI-treated cells, the N^{TM} band appeared as a doublet. This may be due to accumulation of the S2-cleavage intermediate, which is generated by an ADAM-like protease upon ligand binding before γ-secretase cleavage[29]. In light of the role of PS-1 in γ-secretase-mediated Notch activation, we investigated PS-1 expression in Ras-transformed cells. PS-1 protein expression was clearly increased in the presence of SV40 oncoproteins and even more so with H-RasV12 (Fig. 4*c*). Transfection with dominant-negative H-RasN17 downregulated PS-1 (Fig. 4*d*). Real-Time RT-PCR indicated that these effects are predominantly post-transcriptional (data not shown).

GSI treatment nearly abolished anchorage-independent growth in Ras-transformed cells compared with vehicle (P = 0.0007) (Fig. 4*e*). Transfection of N^{IC}, that is, intracellular Notch-1 that does not require γ-secretase for activation, restored anchorage independent growth in GSI-treated cells (Fig. 4*e*).

Notch-1 ligand Delta-1 was strongly upregulated in SV40-expressing cells and even more so in H-RasV12-transformed cells (Fig. 4*c*). Jagged-1 was present but unchanged (data not shown). Thus, Ras-transformed cells upregulate the expression of at least one Notch-1 ligand, Delta, and a key mediator of Notch-1 activation, PS-1, while greatly increasing the rate of maturation and activation of Notch-1.

Ras influences Notch-1 signaling through p38

We investigated which of the main Ras-activated pathways were

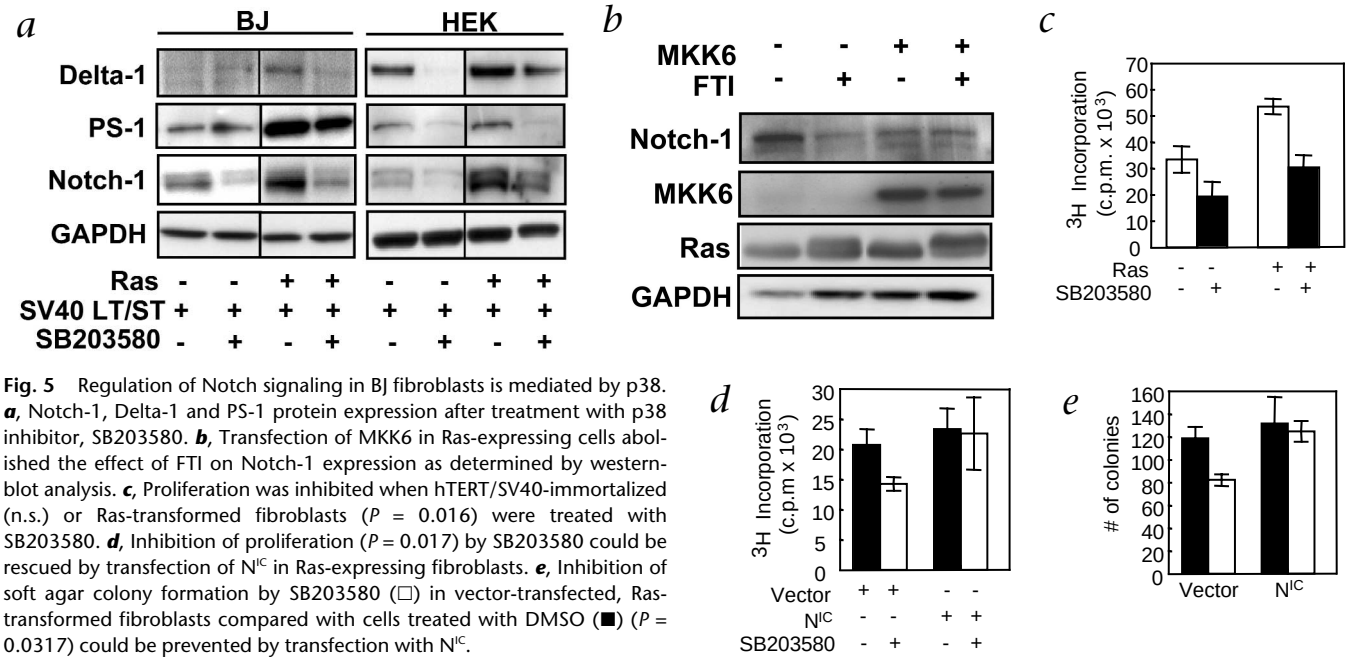

Fig. 5 Regulation of Notch signaling in BJ fibroblasts is mediated by p38. ***a***, Notch-1, Delta-1 and PS-1 protein expression after treatment with p38 inhibitor, SB203580. ***b***, Transfection of MKK6 in Ras-expressing cells abolished the effect of FTI on Notch-1 expression as determined by western-blot analysis. ***c***, Proliferation was inhibited when hTERT/SV40-immortalized (n.s.) or Ras-transformed fibroblasts (P = 0.016) were treated with SB203580. ***d***, Inhibition of proliferation (P = 0.017) by SB203580 could be rescued by transfection of N^{IC} in Ras-expressing fibroblasts. ***e***, Inhibition of soft agar colony formation by SB203580 (□) in vector-transfected, Ras-transformed fibroblasts compared with cells treated with DMSO (■) (P = 0.0317) could be prevented by transfection with N^{IC}.

involved in Notch-1 regulation in our model. Pharmacological inhibitors of MEKK, MEK1/2, PI-3 kinase, p38 and PKC were tested at various concentrations. Moreover, cells were transfected with a dominant-negative JNKK-1 construct[30] and analyzed for Notch-1, PS-1 and Delta-1 protein expression (Supplementary Fig. B online). In this experimental model, only treatment with a p38 inhibitor (SB203580) decreased Notch-1, PS-1 and Delta-1 protein levels (Fig. 5*a*). In HEK cells, PS-1 was strongly downregulated and Delta-1 only slightly reduced by the p38 inhibitor, whereas in BJ fibroblasts this was reversed. These differences may be due to cell-type specific differences in p38 isoforms. We transfected Ras-transformed cells with constitutively active MKK6, a kinase upstream of p38, or vector alone and treated them with FTI to block Ras signaling (Fig. 5*b*). Notch-1 expression was decreased by FTI in vector-transfected cells. Transfection with constitutively active MKK6 abolished the effect of FTI treatment, indicating that p38 signaling mediates Ras effects on Notch-1.

Inhibition of p38 with SB203580 decreased proliferation in both hTERT/SV40 immortalized and Ras-transformed cells (P = 0.016) (Fig. 5*c*). This effect was abolished by transfection of N^{IC} in Ras-transformed cells (Fig. 5*d*). Similarly, inhibition of anchorage-independent growth by SB203580 (Fig. 5*e*) was abrogated by transfection of N^{IC} in Ras-transformed cells.

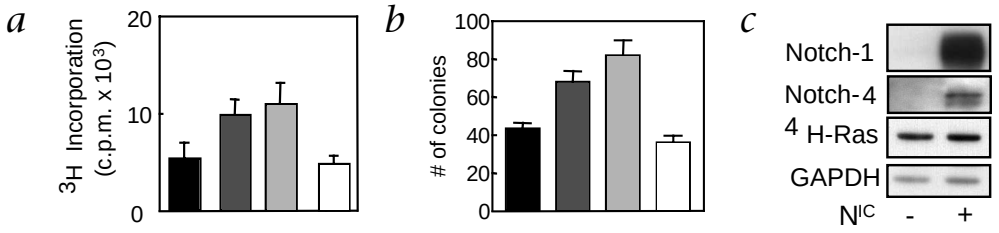

Fig. 6 N^{IC} expression can partially substitute for oncogenic Ras and induces Notch-4. ***a***, Transfection of N^{IC} or H-RasV12 in human hTERT/SV40-immortalized BJ fibroblasts significantly increased proliferation compared with vector-transfected cells (P = 0.0372 and P = 0.05, respectively). c-Myc did not have an effect on proliferation in these cells. Values represent the mean of three wells ± s.d. ***b***, Soft agar assay performed using human hTERT/SV40-immortalized BJ fibroblasts transfected with N^{IC}, H-RasV12 or c-Myc. Experiments were performed in triplicate and values represent the mean colony number ± s.d. A statistically significant difference was detected between vector- and N^{IC}- or Ras-transfected cells using an unpaired t-test (P = 0.0171 and P = 0.013 respectively). For *a* and *b*: ■, vector; ■, N^{IC}; ■, H-RasV12; □, c-Myc ***c***, Western-blot analysis of H-RasV12-negative fibroblasts that were transiently transfected with a N^{IC} and analyzed at the peak of N^{IC} protein production 48 h after transfection. Ras protein expression and electrophoretic migration were analyzed and no significant differences occurred between N^{IC}- and vector-transfected cells, but Notch-4 protein was prominently induced in N^{IC}-transfected cells.

Active Notch-1 can partially substitute for oncogenic Ras

Whether active wild-type Notch-1 requires the concomitant presence of oncogenic Ras to maintain the transformed phenotype is unclear. Ras enables the transforming activity of constitutively active Notch-4[31]. Conversely, constitutively active Notch-1 and -2 with adenovirus E1A transform rodent kidney cells in the absence of H-RasV12 (refs. 5,6). When we introduced N^{IC} into hTERT/SV40-immortalized human fibroblasts in the absence of H-RasV12, proliferation and anchorage independent growth in soft agar were significantly increased ($P < 0.001$ and $P < 0.01$, respectively) compared with vector-transfected fibroblasts (Fig. 6*a* and *b*). In these cells, a different oncogene, c-myc, did not increase proliferation or anchorage-independent growth. These observations are consistent with previous studies[5,6] and indicate that active Notch-1 can independently mediate at least some of the effects of oncogenic Ras. However, Notch-1 overexpression in the absence of oncogenic Ras was consistently unstable in BJ fibroblasts and in other cell lines (MCF-7 and DO11) that do not carry Ras mutations (data not shown). This suggests that enforced overexpression of intracellular Notch-1 in the absence of Ras activation may be toxic and Ras activation enables transformed cells to tolerate sustained Notch-1 activation. Overexpression of N^{IC} did not affect the level or apparent molecular mass of wild-type Ras (Fig. 6*c*), but it prominently induced Notch-4, as predicted by CBF-1-responsive elements in the Notch-4 promoter[32]. The possible role of Notch-4 in Notch-1 signaling warrants further investigation.

Discussion

There is mounting evidence that deregulated expression and/or activity of wild-type Notch receptors occurs frequently in human malignancies and that constitutively active Notch receptors have transforming activity. However, thus far the possible role of wild-type Notch signaling in transformation has been unknown. We showed that in Ras-transformed human cell lines, Notch-1 expression and activity are upregulated and that Notch-1 is a key downstream mediator of oncogenic Ras. Strong upregulation of PS-1 and Delta-1 was also observed in Ras-transformed cells.

Our observations suggest that deregulated Notch-1 activation is necessary to maintain the neoplastic phenotype in Ras-transformed cells. Complex cross-talk between the Ras and Notch signaling pathways has been documented in *Drosophila*[33–36]. Recent data indicate that during *Drosophila* myogenesis and cardiogenesis, Ras and Notch pathways interact with both cooperative and antagonistic effects[36]. Constitutively active Ras increases Notch and Delta, similar to our observations, as well as EGFR antagonist Argos. In turn, Notch increases itself, decreases Delta and inhibits Ras signaling by an unknown mechanism. The fact that Notch inhibits Ras signaling in invertebrates may provide a clue as to why unopposed overexpression of constitutively active Notch-1 is toxic in the absence of active Ras. In fact, the aberrant Notch-1 activation we observed in cells expressing oncogenic Ras may represent the pathological counterpart of a physiological negative feedback circuit in which wild-type Ras extinguishes its own signaling through Notch. Deregulated Ras activation would result in persistent activation of Notch signaling with consequences that may be completely different from the physiological effects of Notch activation in untransformed cells. In fact, there is evidence that in the absence of other oncogenes, physiological activation of Notch signaling is necessary for differentiation programs in various mammalian cells[37–39].

Our results suggest that deregulated Notch-1 signaling has a role in the maintenance of the transformed phenotype in Ras-transformed cell lines and cell lines derived from primary tumors that spontaneously overexpress Notch-1, such as cervical carcinomas. Additionally, our data suggest that Notch signaling may be activated in breast carcinomas. These observations indicate the need for detailed investigations of Notch signaling in

human malignancies in which Ras signaling is upregulated due to Ras mutations, increased expression of wild-type Ras isoforms or other mechanisms. Further studies will be necessary to establish whether the molecular mechanisms underlying Notch activation in our experimental model operate in specific human neoplasms and whether other Notch receptors and ligands have biological roles in some malignancies similar to the role of Notch-1 in our models. Evidence of Notch signaling deregulation has been described anecdotally in a variety of human malignancies[11–16]. However, a systematic exploration of Notch expression and signaling in spontaneous human cancers has not been conducted as yet.

Plausible mechanisms for Notch effects in transformed cells include growth promotion[7,16,40], inhibition of terminal differentiation[41] and inhibition of apoptosis[42–44]. Different mechanisms may predominate in different cell types. In this study, Notch-1 downregulation by ASN expression inhibited growth in Ras-transformed fibroblasts but caused death in cervical cancer cells.

Our results place Notch signaling among the key cell fate regulatory pathways that are deregulated in human Ras-transformed cells and indicate that wild-type Notch-1 has a crucial role in Ras-mediated transformation in human cells *in vitro* and *in vivo*. These data suggest that the Notch signaling network may offer a promising target for the development of novel antineoplastic agents.

Methods

Cell lines and drugs. Foreskin fibroblast (BJ) and human epithelial kidney (HEK) cell lines were generated as described[18]. CaSki cells were obtained from ATCC (CRL-1550, Manassas, Virginia). ASN- and vector-transfected cells were treated with 1 μg/ml (BJ) or 0.1 μg/ml (CaSki) doxycycline (Sigma, St. Louis, Missouri) for 48 h. Cell lines were treated with the following drugs for 24 h to inhibit MEKK, MEK1/2, PI-3 kinase, PKC, p38, activated Ras or γ-secretase: 10 μM PD98059 (Calbiochem, San Diego, California), 10 μM U1026 (Calbiochem), 50 nM Wortmannin (Calbiochem), 5 μM GF109203X (Alexis, San Diego, California), 10 μM SB203580 (Alexis), 3 μM FTI-277 (Calbiochem), and 25 μM of cbz-IL-aldehyde, a γ-secretase inhibitor[28], respectively.

Western blot, immunofluorescence and immunohistochemistry. Immunoblotting of cellular extracts was performed using antibodies to Notch-1, Notch-4, Delta-1, Presenilin-1 (Santa Cruz Biotechnology, Santa Cruz, California), c-H-Ras (Calbiochem), and GAPDH (Chemicon, Temecula, California). Secondary antibodies were anti-goat IgG-HRP (Vector Laboratories, Burlingame, California) and anti-mouse/rabbit $F(ab)_2$ IgG-HRP (Roche, Mannheim, Germany). For confocal immunofluorescence and immunohistochemistry, antibodies against Notch-1 or non-immune goat IgG (Sigma) were used. Biotinylated anti-goat antibody (Vector Laboratories) and fluorescein labeled goat anti-rabbit IgG (KPL, Gaithersburg, Maryland) were the secondary antibodies. Immunochemistry was performed on a formalin-fixed, paraffin-embedded surgical specimen of breast cancer from 7 patients. Serial sections were stained for 3 h. Detection was performed using Vectastain (Vector). H-ras detection was performed using a mouse c-H-Ras antibody (Oncogene Research Products, San Diego, California) or non-specific mouse IgG as described[27]. Detection of the antigen–antibody complex was performed using the Mouse ABC staining kit (Santa Cruz Biotechnology).

Pulse-chase assay. Fibroblasts were incubated for 1 h in methionine/cysteine-free medium (ICN, Costa Mesa, California) and pulsed with 250 μCi of Tran^{35}S-label (ICN) for 20 min at 37 °C. 100 μg/ml cold methionine/cysteine containing medium was added and at different times, cells were collected and lysed in RIPA buffer. Immunoprecipitation was performed on equal amounts of total protein using anti-Notch-1 (Santa Cruz Biotechnology). Gels were fixed in 25% isopropanol and 10% acetic acid for 30 minutes, soaked in Amplify (Amersham, Arlington Heights, Illinois) for 30 min and fluorographed. hTERT-immortalized fibroblasts were transfected with Notch-1 N^{IC} or N^{TM} constructs and total lysates were run simultaneously with the immunoprecipitated samples as band-identity controls. Protein bands from N^{IC} or N^{TM} transfected cells were blotted and detected with a Notch-1 antibody.

Transfections and luciferase assay. Fibroblasts transformed with hTERT, SV40 and HrasV12 were transfected with human antisense Notch-1 (ASN) (bp 4790–7270) cloned in antisense orientation under a doxycycline-inducible promoter in pRetroOn (Clontech, Palo Alto, California) or empty vector. Fibroblasts without HrasV12 were transfected with human N^{IC} (bp 5309-7655) or vector alone (pcDNA3). CaSki cells were stably transfected with pRevTet-On (Clontech) and maintained under neomycin selection. These cells were transfected with pRevTre-ASN or empty pRevTre and kept under double selection (0.1 mg/ml neomycin and 0.1 μg/ml hygromycin). Cells were transfected with a dominant-negative mutant of H-Ras (N17) in pLZRS, Rlf-CAAX[24], wild-type H-Ras (Upstate Biotechnology, Lake Placid, New York), dominant-negative JNKK/SEK1 plasmid pEBG-SEK1(K129R)[30], constitutively active MKK6[45], or empty vector. Cells were plated in 6-well plates and transfected using Lipofectamine Plus (Life Technologies, Gaithersburg, Maryland). For luciferase assays, cells were transfected with a CBF1 luciferase reporter construct[20] or pUC18 as a negative control and pEGFP (Clontech). Luciferase activity was measured after 48 h using a kit from Promega (Madison, Wisconsin) and values were normalized for protein content and transfection efficiency as established from the percentage of GFP-positive cells. With a *Renilla* luciferase control plasmid devoid of CBF-1 responsive elements, Ras-transformed cells showed only a minor increase in transcription (< 2-fold).

Soft Agar assay and tumorigenicity assays. Cells were pre-treated with doxycycline, SB203580 or GSI for 48 or 24 h, respectively. Cell viability was assessed and 1×10^4 cells were suspended in IMDM containing 10% FBS and 0.1% agarose (Promega). Cell suspensions were added to a solid base of IMDM containing 10% FBS, 0.6% agarose and the appropriate selection antibiotics. Both bottom and top agarose layers contained 1 μg/ml doxycycline, 10 μM SB203580, 25 μM GSI or DMSO, and plates were overlaid with 6 ml of complete medium containing doxycycline, SB203580, GSI or DMSO and the appropriate selection antibiotics. Cells were maintained in culture at 37 °C in a humidified, 5% CO_2 atmosphere until colonies could be counted. For *in vivo* tumorigenicity assays, SCID/NOD mice (Jackson, Bar Harbor, Maine) were housed under specific pathogen–free conditions. Mice were injected s.c. in the left flank with 3×10^6 human Ras-transformed fibroblasts transfected with ASN or empty vector (n = 10). Mice were injected daily s.c. in the right flank with 100 μl of 150 μg/ml doxycycline from the day of tumor injection or 14 days after tumor injection. Tumor growth was measured every 3–4 days in a 3-dimensional fashion using a caliper. All animal studies were conducted under IACUC-approved protocols at Loyola University Chicago.

Note: Supplementary information is available on the Nature Medicine *website.*

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Competing interests statement

The authors declare that they have no competing financial interests.

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