Oncogene (2014), 1–11

www.nature.com/onc

ORIGINAL ARTICLE

Opposing role of Notch1 and Notch2 in a $Kras^{G12D}$-driven murine non-small cell lung cancer model

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Lung cancer is the leading cause of cancer-related deaths worldwide. Recently, we have shown that Notch1 inhibition resulted in substantial cell death of non-small cell lung cancer (NSCLC) cells *in vitro*. New compounds targeting Notch signal transduction have been developed and are now being tested in clinical trials. However, the tumorigenic role of individual Notch receptors *in vivo* remains largely unclear. Using a $Kras^{G12D}$-driven endogenous NSCLC mouse model, we analyzed the effect of conditional Notch1 and Notch2 receptor deletion on NSCLC tumorigenesis. Notch1 deficiency led to a reduced early tumor formation and lower activity of MAPK compared with the controls. Unexpectedly, Notch2 deletion resulted in a dramatically increased carcinogenesis and increased MAPK activity. These mice died significantly earlier due to rapidly growing tumor burden. We found that Notch1 regulates Ras/MAPK pathway via HES1-induced repression of the DUSP1 promoter encoding a phosphatase specifically suppressing pERK1/2. Interestingly, Notch1 but not Notch2 ablation leads to decreased HES1 and DUSP1 expression. However, Notch2-depleted tumors showed an appreciable increase in β-catenin expression, a known activator of HES1 and important lung cancer oncogene. Characteristically for β-catenin upregulation, we found that the majority of Notch2-deficient tumors revealed an undifferentiated phenotype as determined by their morphology, E-Cadherin and TTF1 expression levels. In addition, these carcinomas showed aggressive growth patterns with bronchus invasion and obstruction. Together, we show that Notch2 mediates differentiation and has tumor suppressor functions during lung carcinogenesis, whereas Notch1 promotes tumor initiation and progression. These data are further supported by immunohistochemical analysis of human NSCLC samples showing loss or downregulation of Notch2 compared with normal lung tissue. In conclusion, this is the first study characterizing the *in vivo* functions of Notch1 and Notch2 in $Kras^{G12D}$-driven NSCLC tumorigenesis. These data highlight the clinical importance of a thorough understanding of Notch signaling especially with regard to Notch-targeted therapies.

Oncogene advance online publication, 10 February 2014; doi:10.1038/onc.2013.592

Keywords: Notch; Ras; mouse model; beta-catenin; DUSP1

INTRODUCTION

Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related deaths worldwide with a median 5-year survival of about 10%.[1] Although specific therapies for distinct subtypes of NSCLC have been developed, treatment options for the majority of patients are unsatisfactory. In order to identify new therapeutic options and targets, a better understanding of the pathogenesis of NSCLC is necessary.

Notch receptors belong to a family of evolutionary conserved transmembrane proteins that are critical for cell fate decisions, tissue homeostasis and tumorigenesis.[2] In mammals, there are four known Notch receptors (Notch1–4). Upon binding to its ligands Notch receptors are proteolytically processed by metalloproteases and the γ-secretase complex. This process releases the intracellular domain of Notch (N^{IC}), which then translocates into the nucleus to modulate expression of its target genes including the basic helix-loop-helix transcriptional repressors of the HES and HEY families.[3]

Notch1 was first identified as an oncogene in T-cell acute lymphoblastic leukemia.[4] Although chromosomal rearrangement of Notch receptors seems to be rare in solid tumors, aberrant expression of Notch receptors and Notch ligands have been linked to a variety of tumors such as carcinomas of the lung, skin, pancreas, breast and cervix.[5–9]

In our previous work, we have shown that Notch1 signaling promotes tumorigenicity and survival in NSCLC cells *in vitro*.[5] Our findings have further been supported by a recent study detecting activation mutations of Notch1 in tumor specimens derived from NSCLC patients.[10] In addition to Notch1, there are several studies suggesting that Notch3 is a potential tumor promoter in NSCLC.[11,12] On the basis of these results, Notch inhibition by γ-secretase inhibitors (GSI) might be a novel therapeutic option, which is being tested in clinical studies.[13] However, a potential limitation of this targeted strategy is the broad inhibition of all Notch receptors independently of their specific function in a given cell type.[14]

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Received 10 April 2013; revised 13 September 2013; accepted 22 November 2013

Interestingly, it has also been shown that Notch receptors can act as tumor suppressors.. Ablation of Notch1 results in epidermal hyperplasia and eventually in the development of malignant skin tumors.[15] A tumor-suppressive function of Notch receptors has also been demonstrated in small cell lung cancer cells, where activation of Notch1 or Notch2 induces cell cycle arrest.[16] Taken together, these data provide strong evidence that the biological functions of the different Notch receptors are highly cell type- and context-specific.

So far, the specific functions of individual Notch receptors in NSCLC remain largely unknown. However, the precise role of each receptor is crucial to decipher therapeutic potential. In order to address this question, we studied Notch1 and Notch2 ablation in a well-established mouse model of lung adenocarcinoma.[17] In this model, Cre-mediated activation of oncogenic $Kras^{G12D}$ is combined with simultaneous deletion of *Notch1* or *Notch2* in epithelial cells of the lung. Surprisingly, we found that loss of Notch1 and Notch2 results in opposite phenotypes of mice with activated $Kras^{G12D}$. Deletion of Notch1 markedly reduced early tumor formation by modulating the MAPK pathway inhibitor DUSP1. In contrast, Notch2 deletion led to a dramatic enhancement of tumor development and loss of differentiation potentially due to enhanced β-catenin signaling. Thus, our results suggest that Notch2 has a tumor-suppressive role in lung cancer development and that unspecific inhibition of Notch receptors might have deleterious effects supporting the requirement for Notch receptor-specific therapies.

RESULTS

Notch1 and Nocth2 have different expression dynamics during mouse lung adenocarcinoma development

We analyzed the abundance of Notch receptor expression in normal lung and tumor tissue. In wild-type (WT) animals Notch1 and Notch2 were predominantly expressed in bronchial epithelium and activity was confirmed by the presence of HES1 (Figure 1a). To determine the expression dynamics of members of the Notch signaling family during lung carcinogenesis, $Kras^{+/LSL\text{-}G12D}$ (refered to as *Kras*) mice were infected by intranasal administration of an adenovirus expressing Cre-recombinase (AdCre).[18] Notch expression was analyzed at 16 and 24 weeks after infection where adenomas and adenocarcinomas are formed, respectively. Notch1 immunohistochemical (IHC) staining indicates robust expression of the receptor in early and advanced tumors. Notch2 expression was strong in early adenomas, whereas adenocarcinomas show a patchy expression within the tumor tissue. Intriguingly, expression of the Notch target HES1 was uniformly strong in early and advanced tumors (Figure 1a). Expression dynamics were further analyzed by immunoblot in tumor biopsies, which confirmed increasing Notch1 and diminished Notch2 levels in advanced tumors. Notch3 expression was also increased, albeit at lower total expression levels (Figure 1b). During tumor progression, we noted a significant increased expression of HES1 and cMYC[7,19] known Notch targets as well as pERK1/2, a marker of advanced lung tumors.[20] In agreement with

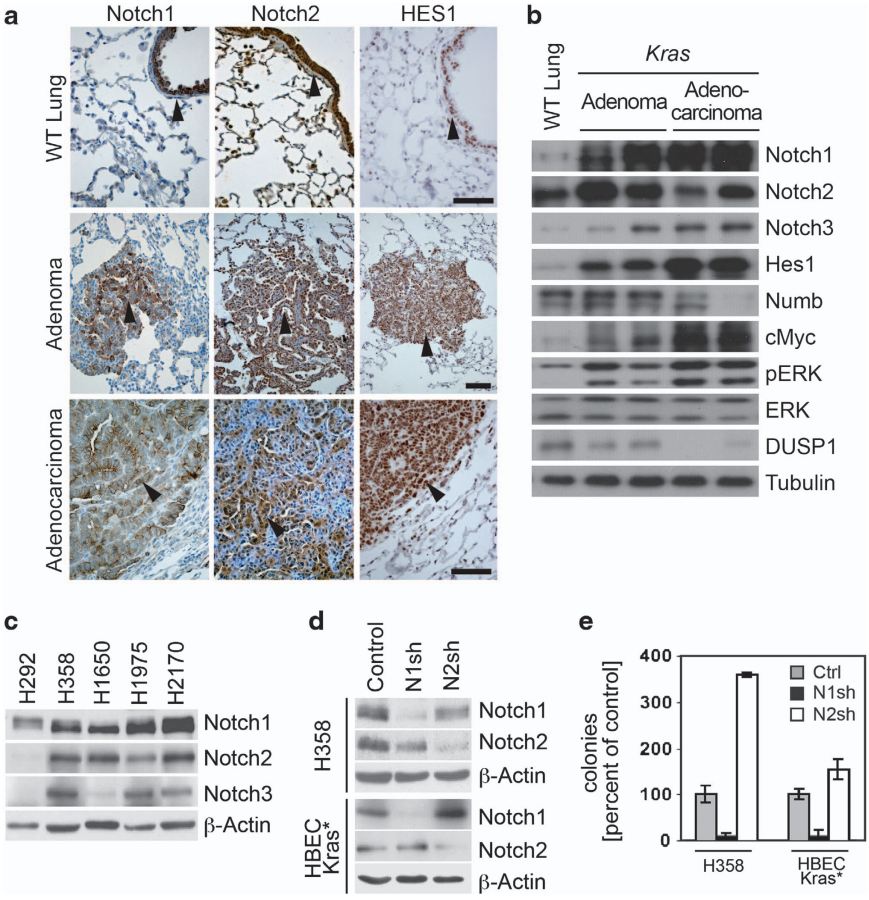


Figure 1. Differential expression dynamics of Notch1 and Nocth2 during lung adenocarcinoma development. (**a**) IHC analysis of Notch1, Notch2 and HES1 expression in WT lung and oncogenic Kras-induced adenoma and adenocarcinoma (arrowheads indicate positive staining). (**b**) Immunoblot analysis of normal lung (WT) and tumor biopsies as indicated. (**c**) Immunoblot analysis of Notch1 and Notch2 protein levels in NSCLC cell lines. (**d**) Analysis of Notch1 and Notch2 knockdown in H358 and HBEC Kras cells using shRNA (N1sh and N2sh, respectively) compared with cells infected with empty vector control. (**e**) Colony formation of Notch1 (N1sh) and Notch2 (N2sh) knockdown cells compared with control-infected cells (Ctrl). Columns, mean ± s.d. Scale bar represents 50 μm.

previous reports, we found a negative correlation between tumor stage and expression of Numb and DUSP1, negative regulators of Notch and MAPK pathways,[10,21] respectively (Figure 1b). Further analysis of human NSCLC cell lines revealed similar to murine tumor expression pattern of Notch receptors, with Notch1 and Notch2 being most prominent (Figure 1c).

Generation of a mouse model with $Kras^{G12D}$-driven adenocarcinomas of the lung and conditional Notch1 and Notch2 knockout

For numerous solid tumors, it has been shown that Notch1 and Notch2 can have distinct effects on tumor growth in the same cell type.[6,22,23] However, the detailed mechanisms of Notch1 action in NSCLC are not fully understood and functions of Notch2 have not been studied yet. To assess the function of Notch1 and Notch2 in NSCLC cell lines, we specifically inhibited Notch1 and Notch2 receptor expression by shRNA (Figure 1d) and analyzed the effects on colony formation in a soft agar assay (Figure 1e). We analyzed immortalized human bronchial epithelial cells (HBECs) with $Kras^{G12D}$ mutation (HBEC Kras) and H358 cells, which express $Kras^{G12D}$.[24,25] Notch1 knockdown led to a significant reduction of colonies in H358 cells (Figure 1e, left panel). Surprisingly, Notch2 deficiency led to a marked increase of colonies. In HBEC Kras cells, we again found decreased colony growth in Notch1 knockdown cells and increased colony formation in Notch2 knockdown cells (Figure 1b, right panel). Intriguingly, *Kras* WT cells (NCI-H520 and p53-deficient HBECs) in contrast to mutants, did not show an increase in colony formation upon Notch2 downregulation (data not shown). These results indicate that Notch1 and Notch2 have distinct and even opposite roles in maintaining the oncogenic-driven malignant phenotype of NSCLC cells.

To determine the biological function of Notch1 and Notch2 in the pathogenesis of NSCLC *in vivo,* we used the well-established *lox-stop-lox* $Kras^{G12D}$ (*Kras*) mouse model.[17] Mice were crossed with conditional knockout alleles of *Notch1*[26] (*Kras;N1*$^{-/-}$) or *Notch2*[27] (*Kras;N2*$^{-/-}$) and infected by intranasal administration of an Cre-expressing adenovirus.[18] To verify recombination, mice were additionally crossed with ROSA-*LacZ* knockin reporter mice[28] (Supplementary Figure 1a). In addition, we could not detect any AdCre-mediated recombination of *Kras, Notch1* or *Notch2* in any other organ but the lungs (Supplementary Figure 1b). To assess possible effects of the deletion of Notch receptors alone, control cohort of *N1*$^{-/-}$ and *N2*$^{-/-}$ animals were infected and aged (Supplementary Figure 2).

Deletion of Notch1 or Notch2 modulates $Kras^{G12D}$-induced lung tumorigenesis

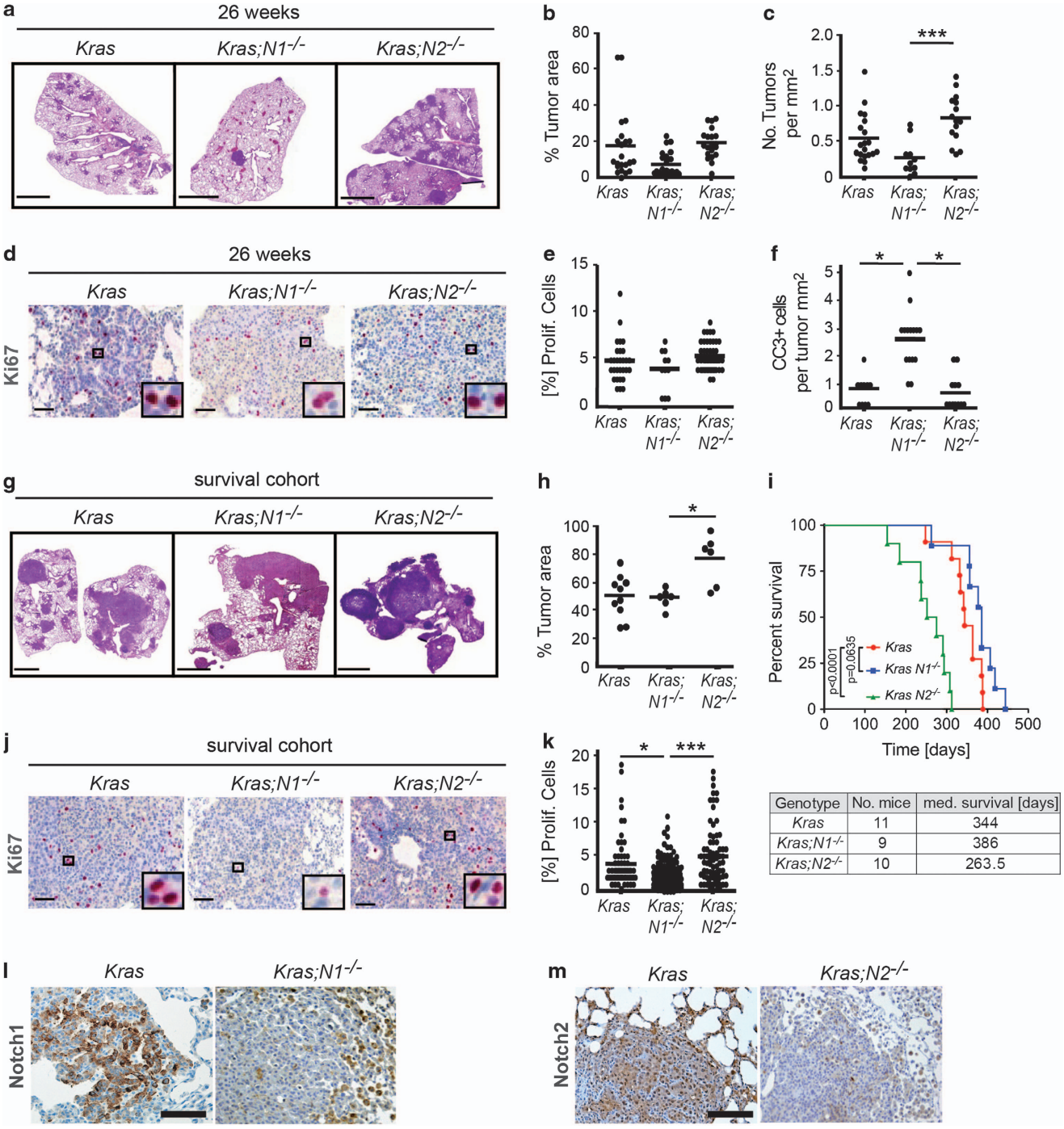
To assess the effect of Notch1 or Notch2 deletion on tumorigenesis, mice from each genotype were aged and lungs examined histologically (Figure 2a). At 26 weeks post induction, the tumor area of *Kras;N1*$^{-/-}$ mice was smaller than the tumor area of *Kras* control mice, however not to the level of statistical significance ($P = 0.062$) (Figure 2b). *Kras;N2*$^{-/-}$ mice showed a similar tumor area compared with the *Kras* mice. In addition, in *Kras;N1*$^{-/-}$ mice, we found less tumors per mm^2 compared with *Kras* control mice. In contrast, Notch2 deletion resulted in a higher number of tumors per mm^2 (Figure 2c). To further elucidate the biological differences of Notch receptors, we analyzed cell proliferation and apoptosis by IHC staining of Ki67 and cleaved caspase 3, respectively. However, there were no significant differences in proliferation between the various genotypes (Figures 2d and e), whereas we found significantly increased levels of apoptosis in *Kras;N1*$^{-/-}$ tumors (Figure 2f). Thus, these data provide strong evidence that Notch1 signaling modulates tumorigenesis through the regulation of tumor initiation.

A cohort of mice of each genotype was followed for disease progression and survival. Although the difference in survival between *Kras* and *Kras;N1*$^{-/-}$ mice did not reach statistical significance ($P = 0.0635$), there was noticeable difference in median age of death, 344 and 386 days, respectively. In contrast, *Kras;N2*$^{-/-}$ mice had a median survival of only 263.5 days and thus died significantly earlier than *Kras* and *Kras;N1*$^{-/-}$ mice (Figure 2i). In addition, we applied AdCre to N1$^{-/-}$ and *N2*$^{-/-}$ mice without oncogenic Kras. Interestingly, these mice showed no difference in survival time compared with WT mice suggesting that Notch signaling modulates oncogene-induced tumor growth rather than facilitating spontaneous tumor development (Supplementary Figure 2).

Analysis of a survival cohort revealed numerous tumors in mice of all genotypes harboring oncogenic Kras (Figure 2g). Although quantification of tumor areas showed a similar value for *Kras* and *Kras;N1*$^{-/-}$ mice (Figure 2h), the proliferation of Notch1-deficient tumors was significantly reduced (Figure 2k). These results indicate that loss of Notch1 might be important for tumor progression. Strikingly, *Kras;N2*$^{-/-}$ mice displayed large tumor masses depleting large parts of normal lung tissue (Figures 2g and h and Supplementary Figure 3). The tumor burden as determined by the tumor area was significantly higher compared with *Kras* and *Kras;N1*$^{-/-}$ mice, although mice of this genotype had to be analyzed $\sim$80 days earlier due to reduced survival. Notch2-deficient tumors derived from *Kras;N2*$^{-/-}$ mice also revealed a higher median proliferation rate than the *Kras;N1*$^{-/-}$ and *Kras* controls (Figures 2j and k). However, Ki67 staining of *Kras;N2*$^{-/-}$ tumors was more heterogeneous with more highly proliferative adenocarcinomas compared with *Kras* tumors. Heterogeneity might suggest possible incomplete Cre recombination of Notch alleles, however detailed analysis confirmed knockout of respective receptors (Figures 2l and m). These results suggest a possible tumor suppressor function of Notch2 in Kras-induced tumorigenesis. Notably, lungs of *N1*$^{-/-}$ and *N2*$^{-/-}$ mice without oncogenic Kras were morphologically virtually identical to those derived from respective control mice with no evidence for hyperplasia, adenomas or adenocarcinomas. This data indicates that Notch2 receptor knockout alone is not sufficient to induce abnormal epithelial cell growth (Supplementary Figure 2). These findings further support the idea that Notch2 exerts tumor suppressor function in oncogene-driven carcinogenesis.

Notch1 regulates MAPK signaling via HES1-induced DUSP1 expression

Oncogenic activation of the Ras pathway is a frequent event in lung adenocarcinoma and activation of MAPK is essential for tumor progression.[20,29] Thus, we quantified the percent of tumors that show pERK1/2-positive IHC staining. Intriguingly, we found lower levels of ERK1/2 activation in tumors with Notch1 deletion compared with *Kras* control mice. *Kras;N2*$^{-/-}$ showed a slight albeit not significant increase in pERK1/2 status (Figures 3a and b). Interestingly, several reports suggested MAPK and Notch pathway crosstalk.[11,21,30–32] A recent report by Maraver *et al.*[21] showed that γ-secretase inhibitor suppresses ERK1/2 phosphorylation through HES1 de-repression of DUSP1 phosphatase promoter.[21] Thus, we assessed the Notch target HES1 status by IHC staining. As expected *Kras* tumors almost universally expressed HES1, surprisingly so did the *Kras;N2*$^{-/-}$ whereas *Kras;N1*$^{-/-}$ lesions were largely negative (Figures 3a and c). Furthermore, we found that tumors isolated from various *Kras* animals showed an association between high expressions of Notch1 and its targets HES1, cMYC and high levels of pERK1/2 as well as low levels of DUSP1 expression (Figure 3d). We next confirmed that in *HES1* depletion by shRNA in H358 cells increases levels of DUSP1 promoter activity as previously shown[21] (Figures 3e and f). In addition, H358 cells upon HES1 knockdown showed a higher expression of DUSP1 and phosphorylation of ERK1/2 was less



Genotype	No. mice	med. survival [days]
Kras	11	344
Kras;N1$^{-/-}$	9	386
Kras;N2$^{-/-}$	10	263.5

Figure 2. Distinct role of Notch1 and Notch2 in Kras-driven lung adenocarcinoma. (**a**) Notch receptor deletion modifies Kras^{G12D}-induced tumorigenesis. Representative pictures of lung histology (HE staining) derived from all genotypes 26 weeks post induction. (**b**) Quantification of the tumor area in the lungs from mice 26 weeks post induction of at least $n=5$ per genotype ($P=0.062$). (**c**) Quantification of tumors per mm^2 lung area from mice 26 weeks post induction of at least $n=4$ per genotype (***$P=0.003$). (**d** and **e**) Representative IHC images and quantification of proliferating, Ki67-positive cells per tumor 26 weeks post induction of at least $n=3$ per animal ($P=0.1$). (**f**) Quantification of Cleaved Caspase 3 (CC3) positive cells per lung. (**g**) Representative pictures of lung histology (HE staining) derived from all genotypes from survival cohort. (**h**) Quantification of the tumor area in the lungs from mice of the survival cohort of at least $n=5$ per genotype (*$P<0.05$). Scale bar, 2000 μm. (**i**) Kaplan–Meier survival curve with detailed description as indicated. (**j** and **k**) Representative IHC images and quantification of proliferating, Ki67-positive cells per tumor of the survival cohort of at least $n=4$ per animal (*$P=0.03$; ***$P<0.0001$). (**l** and **m**) Confirmation of Notch1 and Notch2 ablation in *Kras;N1*$^{-/-}$ and *Kras;N2*$^{-/-}$, respectively.

responsive to EGF stimulation (Figure 3g). Together, those data suggest a crosstalk between Notch1 and MAPK signaling via HES1-mediated activation of ERK1/2 phosphatase DUSP1.

Notch2 ablation correlates with Wnt/β-catenin signal amplification

Tumor suppressor role of Notch in the skin, pancreatic and colorectal cancer leads to misregulation of β-catenin

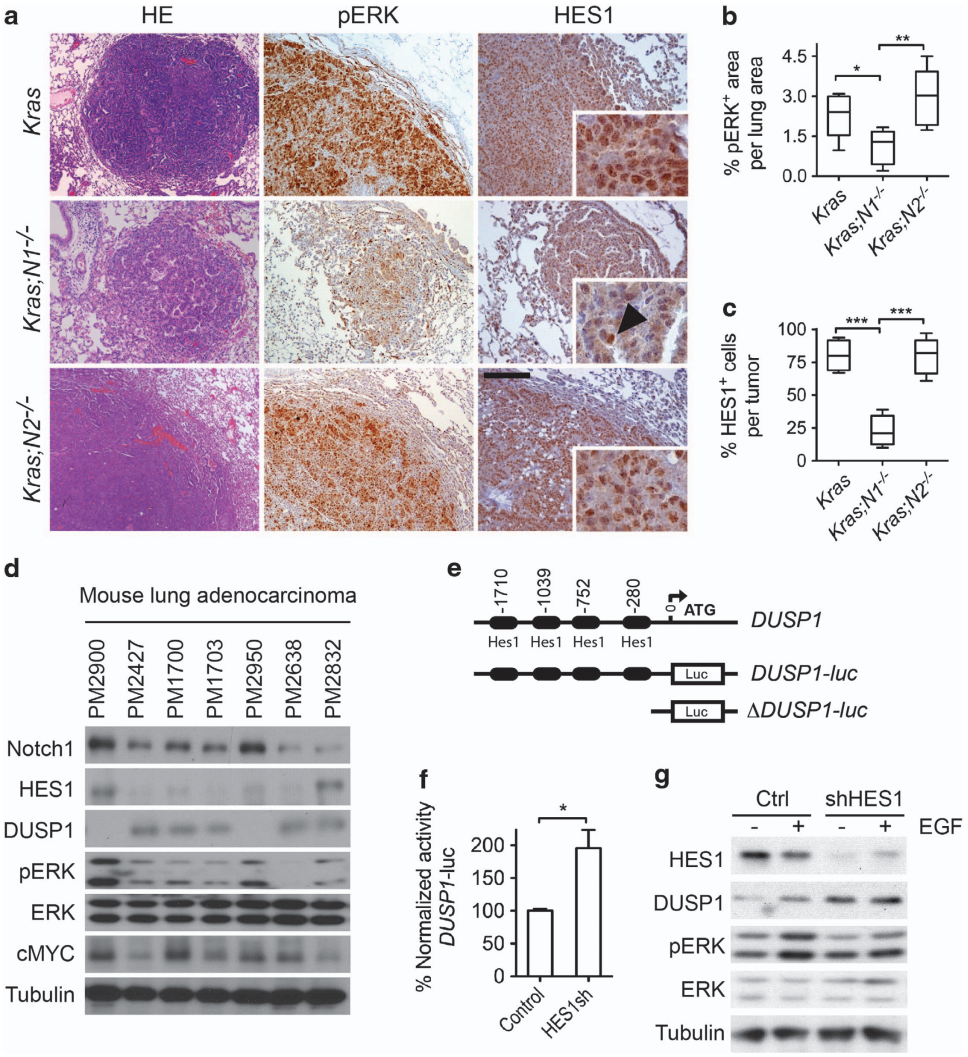


Figure 3. Notch1 regulates MAPK signaling via HES1-induced DUSP1 expression. (**a**) Histology (HE) and IHC analysis of pERK and HES1 levels in *Kras*, *KrasN1*$^{-/-}$, *KrasN2*$^{-/-}$ tumors. (**b**) Quantification of pERK1/2 positive are per lung. (**c**) Percent of HES1-positive cells per tumor. (**d**) Immunoblot analysis of eight independent lung adenocarcinomas isolated from *Kras* mice. (**e**) Location of Tcf and RBP-Jκ-binding sites on human *DUSP1* promoter. (**f**) *DUSP1* promoter activity in H358 cells with shRNA-depleted *HES1* or control shRNA. (**g**) Immunoblot analysis of effects of HES1 depletion in H358 cells on DUSP1 and ERK pathway levels upon EGF stimulation. Data are represented as mean ± s.e.m. **P*-value <0.05; ***P*-value <0.01; ****P*-value <0.001 (two-tailed unpaired Student's *t*-test).

signaling.[15,22,33] Thus, we next investigated, if the tumor suppressor function of Notch2, which emerges from our study, is facilitated by β-catenin signaling modulation. We performed IHC staining, which showed cell membrane β-catenin localization in the normal lung that serves as an indication of pathway inactivity. In *Kras* and *Kras;N1*$^{-/-}$ tumors, both membranous and cytoplasmic localization of β-catenin were noted. *Kras;N2*$^{-/-}$ adenocarcinomas showed a strong β-catenin staining which was additionally prominent in the cytoplasm and nucleus (Figure 4a) that is commonly regarded as indicators of active canonical Wnt/β-catenin signaling.[33] Activation of Wnt/β-catenin signaling accelerates Kras-driven lung cancer,[34] which potentially accounts for Notch2 ablation phenotype.

Intriguingly, β-catenin signaling regulates the HES1 expression via conserved Tcf-binding sites on the *HES1* promoter, close to canonical RBP-Jκ-binding site[35] (Figure 4b). To test this regulatory mechanism, we performed luciferase reporter assay in H358 cells to evaluate the activity of human *HES1* promoter. Indeed activation of Wnt/β-catenin signaling by Wnt3a or LiCl, a known inhibitor of GSK3β, resulted in increased *HES1* promoter activity (Figure 4b). Moreover, we found that activation of Wnt/β-catenin signaling can rescue γ-secretase inhibitor (DAPT)-induced suppression of *HES1* expression and ERK1/2 phosphorylation (Figure 4d). Together our results suggest that Notch2 is a lung tumor suppressor contributing to Wnt/β-catenin signaling modulation (Figure 6f).

Deletion of Notch2 regulates differentiation and morphology of Kras^{G12D}-induced adenocarcinomas

Notch signaling is involved in cell-fate decisions and differentiation processes.[3] To elucidate the effect of Notch1 and Notch2 deletion on tumor differentiation, we classified the tumors of each genotype into five grades according to the criteria published by Jackson. Grade 1 (G1) describes epithelial hyperplasia and grade 5 (G5) resembles poorly differentiated, advanced tumors with desmoplastic tissue.[36] At 26 weeks post induction, the majority of tumors derived from *Kras* and *Kras;N1*$^{-/-}$ mice were well differentiated and thus graded G1 or G2 (Figure 5a). In *Kras;N2*$^{-/-}$ mice, we found less-differentiated tumors, with the majority being grade G2 or G3. In mice of the survival cohorts, we observed a shift toward higher grades in each genotype (Figure 5a). In *Kras* and

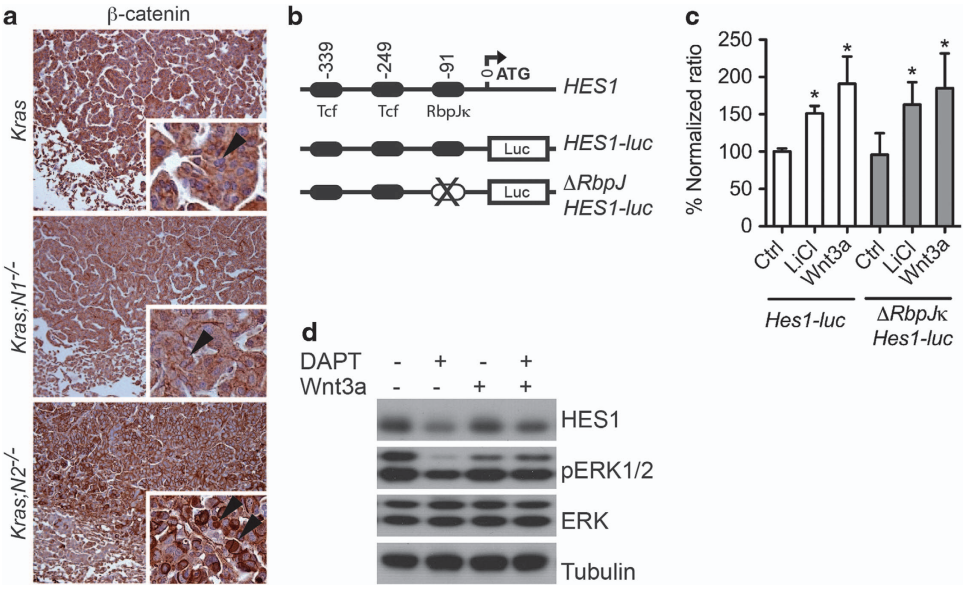


Figure 4. Wnt/β-catenin signaling pathway status in *Kras*, *KrasN1*$^{-/-}$, *KrasN2*$^{-/-}$. (**a**) IHC staining for β-catenin. (**b**) Schematic of the *Hes1* promoter structure. (**c**) Luciferase assay shows the *Hes1* promoter activity upon Wnt/β-catenin signaling stimulation by LiCl or Wnt3a. (**d**) Immunoblot shows HES1 and ERK pathway regulation upon DAPT GSI inhibitor treatment and Wnt3a mediated β-catenin activation. Data are represented as mean ± s.e.m. **P*-value <0.05 (two-tailed unpaired Student's *t*-test).

Kras;N1$^{-/-}$ mice, tumors were less differentiated but still showed tubulo-papillary structures. In contrast, *Kras;N2*$^{-/-}$ mice showed large undifferentiated tumor masses (Supplementary Figure 3). Most of these tumors were graded G4 and G5 (Figure 5a). In addition to the undifferentiated state of *Kras;N2*$^{-/-}$ tumors, we frequently observed invasion of tumor cells into bronchioles and inflammatory infiltrates as a consequence of mucus retention (Supplementary Figure 3). This could not be seen in any other genotype.

In order to confirm the de-differentiated status of tumors derived from *Kras;N2*$^{-/-}$ mice, we analyzed Thyroid transcription factor 1 (TTF1) and E-cadherin expression in *Kras;N2*$^{-/-}$, *Kras;N1*$^{-/-}$ and *Kras* control mice. TTF1 was expressed in the majority of tumor cells of all genotypes 26 weeks post induction (Figure 5c, left panel). However, it was markedly reduced in tumor cells of tumors derived from *Kras;N2*$^{-/-}$ mice of the survival cohort (Figure 5c, right panel). In contrast, TTF1 levels in tumors derived from *Kras;N1*$^{-/-}$ mice and *Kras* mice were similar (Figure 5c). At 26 weeks post induction, we detected E-cadherin (low and high) in all *Kras, Kras;N1*$^{-/-}$ and *Kras;N2*$^{-/-}$ tumors (Figure 5b, left panel). In mice of the survival cohort almost all tumors derived from *Kras* and *Kras;N1*$^{-/-}$ mice were still expressing E-cadherin. In contrast, 75% of the tumors of *Kras;N2*$^{-/-}$ lacked E-cadherin expression (Figure 3b, right panel). Low E-cadherin expression and enhanced Wnt/β-catenin signaling activation in *Kras;N2*$^{-/-}$ is consistent with previous studies indicating that alternation in β-catenin levels can lead to changes in E-cadherin/catenin interaction and loss of E-cadherin stability.[34,37–40] The major consequence of E-cadherin loss is associated with an invasive phenotype.[37,38,41–43] As we also observed Notch2 downregulation in advanced adenocarcinomas (Figure 1b), we speculated that Notch2 is a key regulator in acquiring a metastatic phenotype of NSCLC. We tested the hypothesis using shRNA-induced Notch2 knockdown (N2sh) in H358 cells and performed cell migration and invasion assays. N2sh induced a modest decrease of E-cadherin levels, which was further lowered by TGFβ stimulation (Figure 6a). Motility of cells was assessed in wound healing assay. Quantification of the wound closure revealed that N2sh has significantly enhanced cell migration (Figures 6b and c). Similarly, cell invasiveness in *Boyden* chamber assays was increased upon Notch2 depletion (Figures 6d and e). Although Notch2 deletion conferred a more aggressive tumor phenotype with substantial bronchus invasion and obstruction (Supplementary Figure 3), we did not observe metastases in other organs (data not shown), which might be due to mouse model limitation.

Taken together, these data show that deletion of Notch2 leads to a substantially less-differentiated phenotype of Kras^{G12D}-driven tumors. In contrast, Notch1 deletion does not seem to alter differentiation in these tumors (Figure 6f).

Notch2 expression is enhanced in human lung tumor samples

We have previously shown that Notch1 expression can be detected in normal tissue and NSCLC specimens to the same extent.[11] However, the Notch downstream target Hes1 showed a significantly higher abundance within the tumor group suggesting constitutive Notch activation in NSCLC. So far the expression of Notch2 in normal and cancerous lung tissue has not been analyzed systematically. Therefore, we have analyzed 85 NSCLC samples and 10 normal lung tissues for Notch2 expression using tissue arrays (Figures 7a and b). Strikingly, 67% of NSCLC samples showed either no or only weak Notch2 expression, whereas all normal tissues revealed medium to strong Notch2 expression ($P<0.001$). Among these specimens, we found five G1 tumors, 37 G2 tumors and 43 G3 tumors. There was a (non-significant) correlation between Notch2 expression level and grading showing lower Notch2 expression in tumors with higher grades (Supplementary Table 1 and data not shown). In addition, we determined the mutational status of Kras ($n=78$ total, $n=27$ mutated) and protein expression of p53 indicating p53 mutations ($n=83$ total, $n=42$ expressing). We found a higher percentage of Kras mutations (40 vs 27%) and p53 'wild-type' expression (70 vs 53%) within the Notch2 low-expressing NSCLC group (IHC 0 and 1), however not to the level of statistical significance (Figures 7c and d and Supplementary Table 1). These results strongly suggest a role of Notch2 in the development of (Kras-driven) lung cancer development and support the notion of a possible tumor suppressor function of Notch2 in NSCLC.

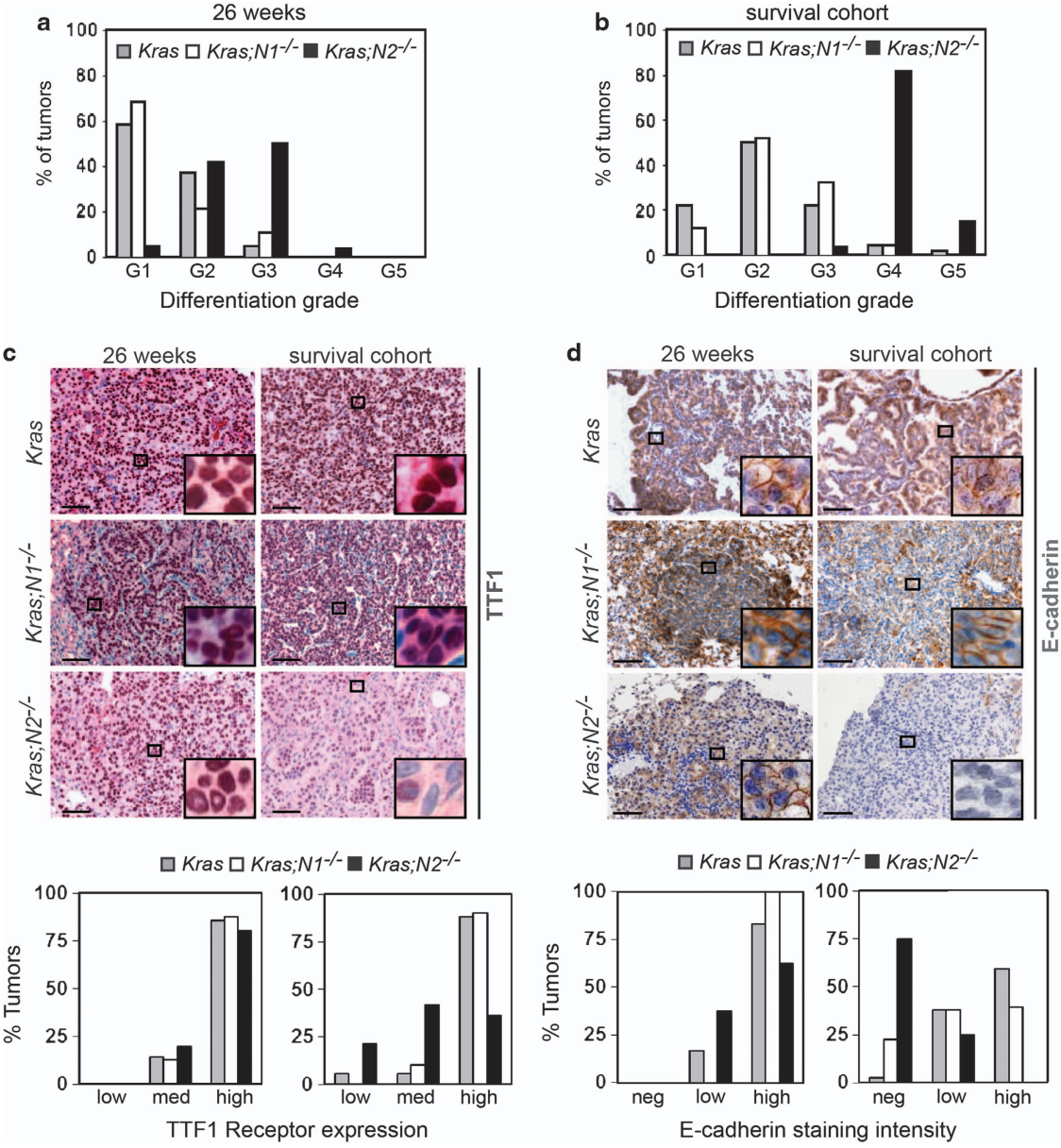


Figure 5. Deletion of Notch2 increases dedifferentiation of lung adenocarcinomas. (**a** and **b**) Quantification of tumor grades ranging from most differentiated (G1) to least differentiated (G5). (**c**) E-Cadherin expression in Kras and *Kras;N2*$^{-/-}$ tumors 26 weeks post induction and at the time of killing. Representative pictures of lungs (top) and quantification (bottom). Staining intensities: Negative (neg), weak intensity (low), intermediate and strong intensity (high). (**d**) TTF1 expression in Kras and *Kras;N2*$^{-/-}$ tumors 26 weeks post induction and at the time of killing (survival cohort). Representative pictures (top) and quantification (bottom). Number of positive cells: 0–33.3% positive cells (low), 33.4–66.6% positive cells (medium) and 66.7–100% positive cells (high). Scale bar, 50 μm.

DISCUSSION

The Notch signaling pathway has been implicated in tumorigenesis of various tumors including carcinomas of the lung, skin, pancreas, breast, colon and cervix.[5–10,15,22,33]

To therapeutically target Notch signaling, GSIs as long-known effective Notch signaling inhibitors are now being tested in clinical trials. However, this compound class is limited by Notch-independent effects, toxicity issues and non-specificity for individual Notch receptors. The rational for an employment of GSI is based on numerous, mainly *in vitro* studies with cell lines derived from various entities.[11,12,21,44,45] These studies show an anti-tumor effect of GSIs on tumor growth and survival. In Kras^{G12D}-expressing cells, our results using shRNA knockdown approaches suggest that Notch1 and Notch2 have different or even opposed biological functions on tumor growth. It also implies that unspecific treatment might be ineffective or even adverse due to distinct tumor biological functions of individual Notch receptors. Several other groups have shown that general inhibition of Notch signaling affects the survival of NSCLC cell lines. This was ascribed to the inhibition of Notch1, Notch3 or HES1 signaling.[10–12,21,45] However, in these *in vitro* studies only few cell lines were Notch2-positive and the genetic background of the cell lines was not taken into account or only late stage of tumors were treated. In addition to unspecific molecular targeting, broad inhibition of all Notch receptors can cause substantial toxicity, for example, in intestinal epithelial cells. In this line, Wu *et al.*[46] demonstrated that the use of a specific antibody for Notch1 induces substantial anti-tumor effects with significant

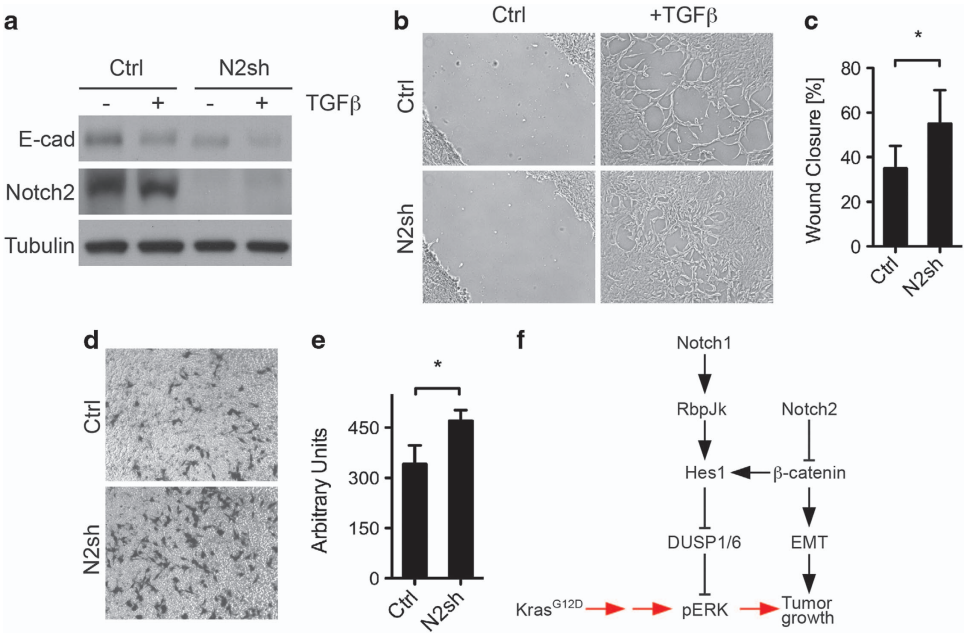
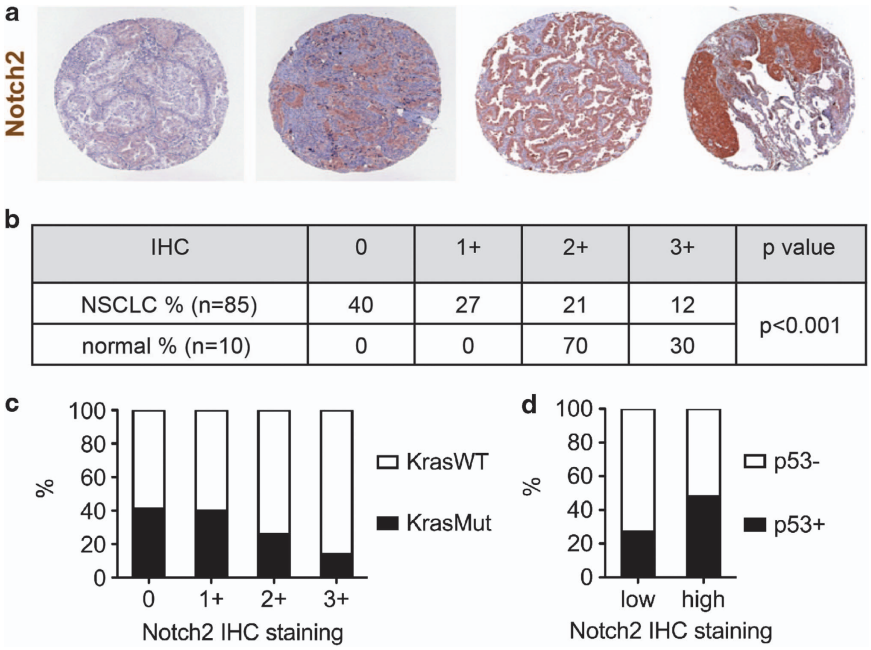


Figure 6. Notch2 regulates E-cadherin levels, cell migration and invasiveness. (**a**) Immunoblot shows control (ctrl) and Notch2 shRNA knockdown (N2sh) regulation of E-cadherin protein levels in H538 cells upon TGFβ stimulation. (**b** and **c**) Wound healing assays representative picture and quantification, respectively. Assays were performed using N2sh and control H358 cells stimulated for 24 h with TGFβ. (**d** and **e**) Transwell migration assay representative picture and quantification, respectively. N2sh and control H358 cells were stimulated with TGFβ. (**f**) Summary model for Notch1 and Notch2 regulation of tumor signaling. Data are represented as mean ± s.e.m. *P-value <0.05 (two-tailed unpaired Student's t-test).



IHC	0	1+	2+	3+	p value
NSCLC % (n=85)	40	27	21	12	p<0.001
normal % (n=10)	0	0	70	30	

Figure 7. Notch2 expression is reduced in human NSCLC. (**a**) Examples of NSCLC specimens with different IHC staining intensities for Notch2. (**b**) Quantification of Notch2 staining intensities ($P<0.001$). (**c**) Correlation between Kras mutation status (details in Supplementary Table 1) and Notch2 staining intensity. (**d**) Correlation between p53 IHC status and Notch staining intensity.

reduction of intestinal toxicity compared with GSI application or Notch1/Notch2 dual antibody treatment.[47] Thus, in the light of individualized tumor therapy, the genetic background such as Kras mutation as well as thorough target evaluation might be crucial for the efficacy and toxicity of future treatment strategies.

In addition, in *Kras;N1*$^{-/-}$ mice we observed less tumors and a reduced tumor area 26 weeks after tumor induction and a modestly beneficial effect on overall survival. On the basis of these results, we conclude that Notch1 is critically involved in tumor initiation and progression. This is consistent with a recent report, showing activated Notch1 to induce lung adenomas and a need to cooperate with activated Myc to progress further.[48] Our data are in line with *in vitro* data generated by Weijzen *et al.*[8] They demonstrated that Notch1 expression was required for oncogenic

Ras-induced transformation *in vitro*, which constitutes an early step in cancer development. This concept is further supported by Osanyingbemi-Obidi *et al.*[49] proposing Notch signaling in the initial steps of tumorigenesis in the same mouse model. On the other hand, we and others have previously demonstrated that Notch1 is critical for the survival of NSCLC cell lines suggesting that Notch1 is also required for the maintenance of the transformed phenotype.[5,10,50] However, these NSCLC cell lines might depend on Notch1 signaling because they are cultured *ex vivo* and might therefore only incompletely reflect the *in vivo* situation. Our results of Notch1 deletion are in agreement with Maraver *et al*. They demonstrated that the inhibition of Notch signaling *in vivo* leads to a reduced growth of $Kras^{G12D}$-driven lung tumors via HES1 de-repression of DUSP1 phosphatase. GSI treatment reduces levels of MAPK activity and induces growth arrest.[21] However, animals were only treated for 3 weeks due to intestinal toxicity of the compound and tumors were evaluated by imaging without evaluating long-term efficacy and survival.[14]

Our results provide strong evidence that–at least in Kras-mutated NSCLC–the biological function of Notch signaling requires thorough analysis. In contrast to *Kras;N1*$^{-/-}$ mice, *Kras;N2*$^{-/-}$ mice had more tumors and died significantly earlier, developing larger tumors in a shorter period of time. These results strongly suggest that Notch2 functions as a tumor suppressor in bronchial epithelial cells modulating both, tumor initiation and progression, as has been shown for malignant mesothelioma.[51] Our findings describing distinct tumor biological functions of Notch1 and Notch2, is in accordance with previous reports.[7,22,23] Fan *et al.*[23] demonstrated that medulloblastoma growth was inhibited by Notch1 and promoted by Notch2 *in vitro* and this antagonistic effect was consistent with Notch receptor expression *in vivo*. They further showed differential regulation of Notch target genes, which might mediate the opposite functions of Notch1 and Notch2. Also in pancreatic cancer distinct roles of Notch1 and Notch2 were suggested. In our previous study, we showed that Notch2 deficiency stopped the progression of pancreatic intraepithelial neoplasia (PanINs) and prolonged the survival of tumor-bearing mice, whereas Notch1 deficiency had non-significant effects on tumorigenesis.[7] Another report suggested that Notch1 deficiency can accelerate PanIN development due to β-catenin activation.[22]

These studies highlight the complexity and context-dependent role of Notch signaling and emphasize that the detailed mechanisms of how Notch receptors orchestrate in different and even opposed functions in cancer are still largely unknown.

Histological examination revealed that tumors of *Kras;N1*$^{-/-}$ mice showed no difference in differentiation compared with control Kras mice. In contrast, tumors derived from *Kras;N2*$^{-/-}$ mice were bigger, more invasive and less differentiated. In addition, we frequently observed bronchial obstruction with subsequent retention pneumonia in *Kras;N2*$^{-/-}$ mice. To date, there are only few studies describing the specific role of Notch2 on differentiation. We have shown previously that Notch signaling has a central role in the development of the pancreas and in pancreatic cancer where Notch2 leads to differentiation and fate selection of PanINs to pancreatic ductal adenocarcinoma.[7] Another study shows the requirement of Notch2 for proximal fate decision in the mammalian nephron.[52] In addition to those reports, our data strongly suggests that Notch2 seems to function as a critical regulator or modulator of differentiation in $Kras^{G12D}$-driven adenocarcinomas. The poorly differentiated state of *Kras;N2*$^{-/-}$ tumors is additionally reflected by reduced E-cadherin expression levels and infiltrations of tumor cells into the bronchioles of *Kras;N2*$^{-/-}$ tumors. Reduction of E-cadherin is associated with epithelial–mesenchymal transition and our results suggest that β-catenin de-repression by Notch2 deletion may lead to this malignant phenotype (Figure 6f). It has been shown that aberrant Wnt/β-catenin signaling dramatically accelerates mouse lung tumorigenesis and imposes an embryonic phenotype manifested by a switch from bronchiolar epithelium to highly proliferative distal progenitors found in the embryonic lung. Interestingly, our preliminary analysis suggests a similar phenotypic switch and overexpression of SOX9 embryonic marker in *Kras;N2*$^{-/-}$ mice (data not shown). Moreover, our preliminary study indicates that Notch2 ablation leads to the expansion of a Notch3-positive compartment (data not shown). It has been recently shown that Notch3-positive cells confine a progenitor cell compartment and drive tumor propagation and self-renewal.[12]

Importantly, the results observed in our murine NSCLC model were substantiated by the analysis of human specimens. In contrast to normal lung, NSCLC samples displayed a significant reduction of Notch2 expression possibly critical for normal lung tissue integrity. A similar correlation of reduced Notch2 expression with poorly differentiated tumors has also been reported in colon carcinoma.[53] In addition, we found an association between low expression levels of Notch2 and oncogenic *Kras* mutations as well as p53 WT status, supporting a possible tumor suppressor function of Notch2. However, owing to a limited number of available specimens, the correlations could not reach the level of statistical significance.

In summary, we demonstrate that Notch signaling modulates tumorigenesis of $Kras^{G12D}$-driven adenocarcinomas of the lung and provide evidence for distinct roles of Notch1 and Notch2 in NSCLC *in vivo*. These findings are of high clinical relevance because they question the benefit of unspecific Notch inhibition as a therapeutic option and suggest that inhibition of Notch2 might have deleterious effects on tumor progression (Figure 6f). Thus, careful evaluation and a deeper understanding of Notch signaling in NSCLC and other tumors is a stringent prerequisite before compounds targeting this pathway are used in clinical trials.

MATERIALS AND METHODS

Mice

LSL-Kras^{G12D}, *Notch1*$^{lox/lox}$, *Notch2*$^{lox/lox}$ and *LSL-Rosa26*^{lacZ} have previously been described.[17,27,28,54] All experiments were performed according to the guidelines of the local Animal Use and Care Committees. All the mice were in a mixed C57BL/6;129SV background and we systematically used sex-matched littermates as controls.

AdCre infection

AdCre infection has previously been described.[18] Briefly, mice were infected at 6–8 weeks of age by intranasal application of 1×10^6 plaque-forming units AdCre:CaPi coprecipitates in two 62.5 μl instillations.

Histologic analysis and IHC

Hematoxylin and eosin (H&E) and IHC were performed as previously described.[7] Antibodies included E-cadherin (24E10, 1:75) and Notch1 (D1E11, 1:50), Cleaved Caspase 3 (5A1E, 1:100) from Cell Signaling (Danvers, MA, USA), TTF1 (8G7G3/1, 1:200) from Santa Cruz Biotechnology (Dallas, TX, USA), Ki67 (Tec3, 1:50) from DAKO (Hamburg, Germany), β-catenin (610153, 1:300) from BD Biosciences (San Jose, CA, USA), Notch2 (c651.6DbHN, 1:300) from the Developmental Studies Hybridoma Bank (DSHB, Iowa City, IA, USA) and Hes1 was a gift from T. Sudo (1:100, Torey Industries Inc, Tokyo, Japan). For semi-quantitative analysis of E-Cadherin, a three-tiered grading system was used as follows: neg (negative), low (weak intensity), high (intermediate and strong intensity) and for TTF1 staining, the percentage of positive cells per tumor was depicted as follows: low (0–33.3% positive cells), medium (33.4–66.6% positive cells), and high (66.7–100% positive cells). For semi-quantitative analysis of Notch2, a four-tiered grading system was used as follows: 0 (negative), 1+ (weak intensity), 2+ (intermediate intensity) and 3+ (strong intensity). Mutation analyses of the hot spot regions of KRAS were performed by a combination of different techniques, as previously shown.[55] Mutation status of p53 was assessed by IHC (DO-7 clone, DAKO). Staining was performed on an automated immunostainer (Ventana Medical Systems, Inc, Tucson, AZ, USA) according to previously published procedures.[56]

Recombination analysis

PCR analysis was performed as described.[7] For visualization of recombination of the *lacZ* gene, mice were killed 72 h after application of 2×10^8 plaque-forming units AdCre. Staining of cryosections (10 μm) with X-Gal was performed according to standard protocols.

Tumor grading

Tumor grading was analyzed by AB and TD blinded to the phenotype on H&E-stained sections of each lung. On the basis of published criteria,[36] each lesion was given a score from grade 1 (G1, well differentiated) to grade 5 (G5, poorly differentiated).

Quantification of the tumor area and proliferating tumor cells

H&E-stained slides were scanned at × 20 objective magnification by a Mirax Scanner System (Zeiss, Jena, Germany). Lung and tumor areas were quantified using Definiens Architect XD Software (Definiens, Munich, Germany) in manual measurement mode. For tumor growth after 26 weeks, three levels of the lung were measured with a distance of at least 100 μm between each level. For proliferation, a parameter set was configured with Definiens TissueMap to quantify Ki67 positive and negative nuclei per tumor.

Two-chamber invasion assays

The Millipore Cell Invasion Assay is performed in an Invasion Chamber, based on the Boyden chamber principle. In a serum-free medium, 2.5×10^4 H358 cells were seeded into the upper chamber and invaded toward 10% FCS as a chemoattractant in the lower chamber for 24 h. Each experiment was repeated in triplicate. Upper chamber contains an 8 μm pore size polycarbonate membrane coated with a thin layer of Matrigel extracellular matrix proteins. The Matrigel layer occludes the membrane pores, blocking non-invasive cells from migrating through. Invasive cells, on the other hand, migrate through the polymerized collagen layer and cling to the bottom of the polycarbonate membrane. Invaded cells on the bottom of the insert membrane are incubated with Cell Stain Solution, then subsequently extracted and detected on a standard microplate reader (560 nm) according to the manufacturer's instructions.

Wound healing assays

H385 cells (1×10^6 per well) were plated in a six-well plate and cultured until confluent. A scratch was made to the monolayer with a 200 μl pipette tip and photographs (magnification, × 400) were taken immediately at 0 and 48 h after wounding. The size of the wound was measured. Statistical analysis of the data was carried out using a paired Student's *t*-test. Each experiment was done in triplicate.

Dual luciferase reporter assay

Aliquots of 4×10^4 H358 cells were transiently transfected with the 400 ng shRNA expressing plasmid and 100 ng of *DUSP1-luc* plasmid,[21] or *HES1-luc*[35] and 2 ng of pRL-TK (Promega, Madison, WI, USA) as an internal control using FuGENE6 (Roche, Mannheim, Germany). In each experiment, total amounts of plasmids were adjusted to 500 ng by the addition of pcDNA plasmid (Invitrogen, Darmstadt, Germany). After overnight incubation, LiCl (5 mM) or recombinant Wnt3a (100 ng/ml) was added to the medium for stimulation.

Cell lines, soft agar assays and reagents

The NCI-H358 cell line was purchased from the American Type Culture Collection. HBECs were generously provided by John D. Minna (UT Southwestern Medical Center, Dallas, TX, USA). For anchorage-independent growth, cells were plated in soft agar in duplicates as previously described.[5] EGF 20 μg/ml, TGFβ 10 μg/ml, LiCl (5 mM) or recombinant Wnt3a (100 ng/ml) was added to the culture medium as indicated in the text.

Western blots

Western blots were performed as previously described[5] with antibodies to Notch1 (Pharmingen, San Jose, CA, USA), Notch2 (DSHB) and β-Actin and β-Tubulin (Sigma, St Louis, MO, USA), pERK1/2, ERK, Notch3, Hes1, E-cadherin, Numb, DUSP1 and cMYC (Cell Signaling).

Lentiviral transfection

Lentiviral shRNA in pLKO.1 vector (Sigma) was used to generate stable cell lines with Hes1, Notch1 and Notch2 knockout as previously described.[5] Turbofect (Fermentas, Schwerte, Germany) was used for infection of packaging cells according to manufacturer's protocol.

Statistics

Kaplan–Meier curves were calculated using the individual survival time for the indicated number of mice from each genotype. The log-rank test was used to test for significant differences between the groups. Other values were calculated using ANOVA followed by a multiple comparisons test or are presented as mean ± s.d. of a representative experiment.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We would like to thank Dr Tibor Schuster for the help with statistical calculations, Nadya Mitova and Birgit Geist for her excellent technical help with the experiments and Joachim Alfer for providing histological pictures. We also thank Freddy Radtke for the *Notch1*$^{lox/lox}$ mice, Ursula Zimber-Strobl for the *Notch2*$^{lox/lox}$ mice and David Tuveson and Tyler Jacks for the *Kras*$^{+/LSL\text{-}G12D}$ mice. TD received a grant from the Wilhelm Sander-Stiftung (#2005.158.1). TD and JTS have been supported by grants from the Deutsche Krebshilfe (#109606 to TD and JTS) and the Deutsche Forschungsgemeinschaft (SI 1549/1-1 to JTS). PKM was supported by the Tobacco-Related Disease Research Program, a Dean's Fellowship from Stanford University and the Child Health Research Institute and Lucile Packard Foundation for Children's Health at Stanford.

AUTHOR CONTRIBUTIONS

AB and PKM contributed equally to this work and were responsible for the experimental design, execution, data analysis and manuscript preparation. MA, MR, KS, AF, KB, AW, RB, CP and JD acquired data, provided materials. JTS and TD were equally responsible for supervision of research, data interpretation and manuscript preparation.

REFERENCES

1 Jemal A, Siegel R, Xu J, Ward E. Cancer statistics. *CA: a cancer journal for clinicians* 2010; **60**: 277–300.
2 Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science* 1999; **284**: 770–776.
3 Bray SJ. Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol* 2006; **7**: 678–689.
4 Ellisen LW, Bird J, West DC, Soreng AL, Reynolds TC, Smith SD *et al.* TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* 1991; **66**: 649–661.
5 Baumgart A, Seidl S, Vlachou P, Michel L, Mitova N, Schatz N *et al.* ADAM17 regulates epidermal growth factor receptor expression through the activation of Notch1 in non-small cell lung cancer. *Cancer Res* 2010; **70**: 5368–5378.
6 Mazur PK, Gruner BM, Nakhai H, Sipos B, Zimber-Strobl U, Strobl LJ *et al.* Identification of epidermal Pdx1 expression discloses different roles of Notch1 and Notch2 in murine Kras(G12D)-induced skin carcinogenesis *in vivo*. *PLoS One* 2010; **5**: e13578.
7 Mazur PK, Einwachter H, Lee M, Sipos B, Nakhai H, Rad R *et al.* Notch2 is required for progression of pancreatic intraepithelial neoplasia and development of pancreatic ductal adenocarcinoma. *Proc Natl Acad Sci USA* 2010; **107**: 13438–13443.
8 Weijzen S, Rizzo P, Braid M, Vaishnav R, Jonkheer SM, Zlobin A *et al.* Activation of Notch-1 signaling maintains the neoplastic phenotype in human Ras-transformed cells. *Nature Med* 2002; **8**: 979–986.
9 Talora C, Sgroi DC, Crum CP, Dotto GP. Specific down-modulation of Notch1 signaling in cervical cancer cells is required for sustained HPV-E6/E7 expression and late steps of malignant transformation. *Genes Develop* 2002; **16**: 2252–2263.
10 Westhoff B, Colaluca IN, D'Ario G, Donzelli M, Tosoni D, Volorio S *et al.* Alterations of the Notch pathway in lung cancer. *Proc Natl Acad Sci USA* 2009; **106**: 22293–22298.
11 Konishi J, Kawaguchi KS, Vo H, Haruki N, Gonzalez A, Carbone DP *et al.* Gamma-secretase inhibitor prevents Notch3 activation and reduces proliferation in human lung cancers. *Cancer Res* 2007; **67**: 8051–8057.

12 Zheng Y, de la Cruz CC, Sayles LC, Alleyne-Chin C, Vaka D, Knaak TD *et al.* A rare population of CD24(+)ITGB4(+)Notch(hi) cells drives tumor propagation in NSCLC and requires Notch3 for self-renewal. *Cancer Cell* 2013; **24**: 59–74.
13 ClinicalTrals.gov http://clinicaltrials.gov/.
14 Maraver A, Serrano M. Notching up a new therapeutic strategy for non-small cell lung carcinoma (NSCLC). *Oncotarget* 2012; **3**: 917–918.
15 Nicolas M, Wolfer A, Raj K, Kummer JA, Mill P, van Noort M *et al.* Notch1 functions as a tumor suppressor in mouse skin. *Nat Genet* 2003; **33**: 416–421.
16 Sriuranpong V, Borges MW, Ravi RK, Arnold DR, Nelkin BD, Baylin SB *et al.* Notch signaling induces cell cycle arrest in small cell lung cancer cells. *Cancer Res* 2001; **61**: 3200–3205.
17 Jackson EL, Willis N, Mercer K, Bronson RT, Crowley D, Montoya R *et al.* Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras. *Genes Develop* 2001; **15**: 3243–3248.
18 DuPage M, Dooley AL, Jacks T. Conditional mouse lung cancer models using adenoviral or lentiviral delivery of Cre recombinase. *Nat Protocols* 2009; **4**: 1064–1072.
19 Kopan R, Ilagan MX. The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell* 2009; **137**: 216–233.
20 Junttila MR, Karnezis AN, Garcia D, Madriles F, Kortlever RM, Rostker F *et al.* Selective activation of p53-mediated tumour suppression in high-grade tumours. *Nature* 2010; **468**: 567–571.
21 Maraver A, Fernandez-Marcos PJ, Herranz D, Canamero M, Munoz-Martin M, Gomez-Lopez G *et al.* Therapeutic effect of gamma-secretase inhibition in KrasG12V-driven non-small cell lung carcinoma by derepression of DUSP1 and inhibition of ERK. *Cancer Cell* 2012; **22**: 222–234.
22 Hanlon L, Avila JL, Demarest RM, Troutman S, Allen M, Ratti F *et al.* Notch1 functions as a tumor suppressor in a model of K-ras-induced pancreatic ductal adenocarcinoma. *Cancer Res* 2010; **70**: 4280–4286.
23 Fan X, Mikolaenko I, Elhassan I, Ni X, Wang Y, Ball D *et al.* Notch1 and notch2 have opposite effects on embryonal brain tumor growth. *Cancer Res* 2004; **64**: 7787–7793.
24 Rodenhuis S, Slebos RJ, Boot AJ, Evers SG, Mooi WJ, Wagenaar SS *et al.* Incidence and possible clinical significance of K-ras oncogene activation in adenocarcinoma of the human lung. *Cancer Res* 1988; **48**: 5738–5741.
25 Sato M, Vaughan MB, Girard L, Peyton M, Lee W, Shames DS *et al.* Multiple oncogenic changes (K-RAS(V12), p53 knockdown, mutant EGFRs, p16 bypass, telomerase) are not sufficient to confer a full malignant phenotype on human bronchial epithelial cells. *Cancer Res* 2006; **66**: 2116–2128.
26 Radtke F, Raj K. The role of Notch in tumorigenesis: oncogene or tumour suppressor? *Nature Rev Cancer* 2003; **3**: 756–767.
27 Besseyrias V, Fiorini E, Strobl LJ, Zimber-Strobl U, Dumortier A, Koch U *et al.* Hierarchy of Notch-Delta interactions promoting T cell lineage commitment and maturation. *J Exp Med* 2007; **204**: 331–343.
28 Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 1999; **21**: 70–71.
29 Feldser DM, Kostova KK, Winslow MM, Taylor SE, Cashman C, Whittaker CA *et al.* Stage-specific sensitivity to p53 restoration during lung cancer progression. *Nature* 2010; **468**: 572–575.
30 Haruki N, Kawaguchi KS, Eichenberger S, Massion PP, Olson S, Gonzalez A *et al.* Dominant-negative Notch3 receptor inhibits mitogen-activated protein kinase pathway and the growth of human lung cancers. *Cancer Res* 2005; **65**: 3555–3561.
31 Kim MY, Park JH, Choi EJ, Park HS. Presenilin acts as a positive regulator of basal level activity of ERK through the Raf-MEK1 signaling pathway. *Biochem Biophys Res Commun* 2005; **332**: 609–613.
32 Michie AM, Chan AC, Ciofani M, Carleton M, Lefebvre JM, He Y *et al.* Constitutive Notch signalling promotes CD4 CD8 thymocyte differentiation in the absence of the pre-TCR complex, by mimicking pre-TCR signals. *Int Immunol* 2007; **19**: 1421–1430.
33 Kim HA, Koo BK, Cho JH, Kim YY, Seong J, Chang HJ *et al.* Notch1 counteracts WNT/beta-catenin signaling through chromatin modification in colorectal cancer. *J Clin Invest* 2012; **122**: 3248–3259.
34 Pacheco-Pinedo EC, Durham AC, Stewart KM, Goss AM, Lu MM, Demayo FJ *et al.* Wnt/beta-catenin signaling accelerates mouse lung tumorigenesis by imposing an embryonic distal progenitor phenotype on lung epithelium. *J Clin Invest* 2011; **121**: 1935–1945.
35 Peignon G, Durand A, Cacheux W, Ayrault O, Terris B, Laurent-Puig P *et al.* Complex interplay between beta-catenin signalling and Notch effectors in intestinal tumorigenesis. *Gut* 2011; **60**: 166–176.
36 Jackson EL, Olive KP, Tuveson DA, Bronson R, Crowley D, Brown M *et al.* The differential effects of mutant p53 alleles on advanced murine lung cancer. *Cancer Res* 2005; **65**: 10280–10288.
37 Asnaghi L, Vass WC, Quadri R, Day PM, Qian X, Braverman R *et al.* E-cadherin negatively regulates neoplastic growth in non-small cell lung cancer: role of Rho GTPases. *Oncogene* 2010; **29**: 2760–2771.
38 Bremnes RM, Veve R, Hirsch FR, Franklin WA. The E-cadherin cell-cell adhesion complex and lung cancer invasion, metastasis, and prognosis. *Lung Cancer* 2002; **36**: 115–124.
39 Wang XQ, Li H, Van Putten V, Winn RA, Heasley LE, Nemenoff RA. Oncogenic K-Ras regulates proliferation and cell junctions in lung epithelial cells through induction of cyclooxygenase-2 and activation of metalloproteinase-9. *Mol Biol Cell* 2009; **20**: 791–800.
40 Ma L, Young J, Prabhala H, Pan E, Mestdagh P, Muth D *et al.* miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis. *Nat Cell Biol* 2010; **12**: 247–256.
41 Saad AG, Yeap BY, Thunnissen FB, Pinkus GS, Pinkus JL, Loda M *et al.* Immunohistochemical markers associated with brain metastases in patients with nonsmall cell lung carcinoma. *Cancer* 2008; **113**: 2129–2138.
42 von Burstin J, Eser S, Paul MC, Seidler B, Brandl M, Messer M *et al.* E-cadherin regulates metastasis of pancreatic cancer *in vivo* and is suppressed by a SNAIL/HDAC1/HDAC2 repressor complex. *Gastroenterology* 2009; **137**: 361–371371 e361-365.
43 Myong NH. Reduced expression of E-cadherin in human non-small cell lung carcinoma. *Cancer Res Treatment* 2004; **36**: 56–61.
44 Sjolund J, Johansson M, Manna S, Norin C, Pietras A, Beckman S *et al.* Suppression of renal cell carcinoma growth by inhibition of Notch signaling in vitro and *in vivo*. *J Clin Invest* 2008; **118**: 217–228.
45 Huynh C, Poliseno L, Segura MF, Medicherla R, Haimovic A, Menendez S *et al.* The novel gamma secretase inhibitor RO4929097 reduces the tumor initiating potential of melanoma. *PLoS One* 2011; **6**: e25264.
46 van Es JH, van Gijn ME, Riccio O, van den Born M, Vooijs M, Begthel H *et al.* Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature* 2005; **435**: 959–963.
47 Wu Y, Cain-Hom C, Choy L, Hagenbeek TJ, de Leon GP, Chen Y *et al.* Therapeutic antibody targeting of individual Notch receptors. *Nature* 2010; **464**: 1052–1057.
48 Allen TD, Rodriguez EM, Jones KD, Bishop JM. Activated Notch1 induces lung adenomas in mice and cooperates with Myc in the generation of lung adenocarcinoma. *Cancer Res* 2011; **71**: 6010–6018.
49 Osanyingbemi-Obidi J, Dobromilskaya I, Illei PB, Hann CL, Rudin CM. Notch signaling contributes to lung cancer clonogenic capacity in vitro but may be circumvented in tumorigenesis *in vivo*. *Mol Cancer Res* 2011; **9**: 1746–1754.
50 Eliasz S, Liang S, Chen Y, De Marco MA, Machek O, Skucha S *et al.* Notch-1 stimulates survival of lung adenocarcinoma cells during hypoxia by activating the IGF-1R pathway. *Oncogene* 2010; **29**: 2488–2498.
51 Graziani I, Eliasz S, De Marco MA, Chen Y, Pass HI, De May RM *et al.* Opposite effects of Notch-1 and Notch-2 on mesothelioma cell survival under hypoxia are exerted through the Akt pathway. *Cancer Res* 2008; **68**: 9678–9685.
52 Cheng HT, Kim M, Valerius MT, Surendran K, Schuster-Gossler K, Gossler A *et al.* Notch2, but not Notch1, is required for proximal fate acquisition in the mammalian nephron. *Development* 2007; **134**: 801–811.
53 Chu D, Zheng J, Wang W, Zhao Q, Li Y, Li J *et al.* Notch2 expression is decreased in colorectal cancer and related to tumor differentiation status. *Ann Surg Oncol* 2009; **16**: 3259–3266.
54 Radtke F, Wilson A, Stark G, Bauer M, van Meerwijk J, MacDonald HR *et al.* Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* 1999; **10**: 547–558.
55 Bettstetter M, Berezowska S, Keller G, Walch A, Feuchtinger A, Slotta-Huspenina J *et al.* Epidermal growth factor receptor, phosphatidylinositol-3-kinase catalytic subunit/PTEN, and KRAS/NRAS/BRAF in primary resected esophageal adenocarcinomas: loss of PTEN is associated with worse clinical outcome. *Human Pathol* 2013; **44**: 829–836.
56 Slotta-Huspenina J, Koch I, de Leval L, Keller G, Klier M, Bink K *et al.* The impact of cyclin D1 mRNA isoforms, morphology and p53 in mantle cell lymphoma: p53 alterations and blastoid morphology are strong predictors of a high proliferation index. *Haematologica* 2012; **97**: 1422–1430.

Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)