



Published in final edited form as:

*Clin Transl Oncol.* 2014 January ; 16(1): 29–35. doi:10.1007/s12094-013-1036-y.

## Diacylglycerol kinase $\eta$ modulates oncogenic properties of lung cancer cells

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**Conflict of interest** The authors declare no conflicts of interest.

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## Abstract

**Purpose**—Lung cancer is a leading cause of cancer deaths and efforts are underway to identify novel therapies to treat these tumors. Diacylglycerol kinase  $\eta$  (DGK $\eta$ ), an enzyme that phosphorylates diacylglycerol to form phosphatidic acid, has been shown to modulate MAPK signaling downstream of EGFR, which is an oncogenic driver in some lung cancers. Since mutations in EGFR and K-Ras are common in lung cancer, we hypothesized that limiting the function of DGK $\eta$  would attenuate oncogenic properties of lung cancer cells.

**Methods**—We determined the expression levels of DGK $\eta$  in a mouse models of mutant EGFR and K-Ras lung cancer and in human lung cancer cell lines with activating mutations in either EGFR or K-Ras. We also tested the effects of shRNA-mediated depletion of DGK $\eta$  in lung cancer cells and tested if DGK $\eta$  depletion augmented the effects of afatinib, a new generation EGFR inhibitor.

**Results**—DGK $\eta$  was expressed in malignant epithelium from mice with mutant EGFR or K-Ras lung cancer. It was also expressed in human lung cancer cell lines with EGFR or K-Ras mutations. Depleting DGK $\eta$  in lung cancer cell lines, harboring mutant EGFR, reduced their growth on plastic and in soft agar and also augmented the effects of afatinib, an EGFR inhibitor. DGK $\eta$  depletion also reduced growth of one of two lung cancer cell lines that harbored mutant K-Ras.

**Conclusions**—Our data indicate that DGK $\eta$  is a potential therapeutic target in lung cancers, especially those harboring EGFR mutations. Our findings warrant further studies to examine the effects of limiting its function in vivo.

## Keywords

Diacylglycerol kinase; Epidermal growth factor receptor; Lipid signaling; Diacylglycerol; Lung cancer

## Introduction

Lung cancer is the leading cause of cancer deaths worldwide. Much of this disease is due to tobacco smoking, but globally up to 15 % of lung cancers in men and almost half of them in women are not attributable to smoking [1]. Often, lung cancers in never smokers harbor specific mutations in genes that are important for tumor maintenance and growth. One gene that is mutated in almost 60 % of lung cancers in never smokers is EGFR [2]. These mutations in EGFR, such as the L858R mutation, increase its kinase activity, leading to a state of oncogene addiction. As such, about 75 % of tumors harboring mutant EGFR respond to small molecule EGFR kinase inhibitors [2], which are superior to chemotherapy as initial treatment for these tumors [3–7]. Unfortunately, most patients eventually develop resistance to them by a variety of mechanisms [8–10]. This has led to additional efforts to find other signaling pathways that are important for tumorigenesis promoted by mutant EGFR.

Diacylglycerol kinases, which phosphorylate the lipid second messenger diacylglycerol to form phosphatidic acid, have been shown to mediate signaling downstream of EGFR. DGK $\delta$ , for example, modulates EGFR abundance and degradation by mediating its de-ubiquitination [11]. DGK $\eta$  also modulates signaling downstream of EGFR by facilitating heterodimerization of B-Raf and C-Raf [12]. Both of these Raf isoforms bind to DGK $\eta$ , which promotes their recruitment to the plasma membrane where they activate the MEK-ERK signaling cascade. RNAi-mediated knockdown of DGK $\eta$  in HeLa cells limited proliferation by about 30 % and reduced EGF-mediated phosphorylation of both MEK and

ERK [12]. Collectively, these data indicated that DGK $\eta$  might be a novel cancer target downstream of EGFR or K-Ras, so we set out to determine the effects of limiting its function in lung cancer cell lines that harbor activating mutations in EGFR or K-Ras.

## Materials and methods

### Cell lines, expression plasmids, cell culture, and transfection

All cell lines (H441, H460, H1650, and H1975) were from American Type Culture Collection and propagated in RPMI 1640 (Invitrogen) with 10 % FBS and antibiotics. The type of lung cancers from which these cells were derived as well as their EGFR and K-Ras mutation status are indicated in Fig. 1a. DGK $\eta$  RNAi was performed using Oligofectamine (Invitrogen) and the following siRNA duplexes: 5'-GGAUCUAGAUUCCGUAGAUTT-3' and 5'-AUCUACGGAAUCUAGAUCGGTT-3'. Scrambled siRNA duplexes were used as controls.

### Generation of stable DGK $\eta$ knockdown cell lines and proliferation, migration, and soft agar assays

To generate stable DGK $\eta$  knockdown H1650 and H1975 cell lines, Dharmacon SMARTvector 2.0 lentiviral particles (#SK-006716-02) were used according to the instructions. Non-targeting particles (#S-005000-01) were used for a control cell line. Polyclonal stable cell lines were isolated under puromycin (2  $\mu$ g/mL) selection. Cell proliferation was assessed by plating 10,000 cells in a 10-cm diameter plate, growing them in 1 % FBS with antibiotics for 5–7 days, and then counting the number of cells. Growth in soft agar was performed as described [13].

### Reverse transcription polymerase chain reaction

Total RNA was prepared from H1650 stable cell lines using Trizol reagent (Invitrogen) and reverse transcribed as described [14]. RT-PCR using 10 ng of total cDNA was performed with the following primers: DGK $\eta$ : 5'-GG ACCTCCAGAAGCATCTG-3' (forward) and 5'-AACGTC ATCCCAAGCTGC-3'; actin: 5'-AGGCACCAGGGCG TGAT-3' (forward) and 5'-TCGTCCAGTTGGTGACG AT-3'.

### Western blotting

Western blotting was performed according to instructions provided by the suppliers. Anti-EGFR (#2232) antibodies were from Cell Signaling Technologies. Anti-actin (#691001) was from MP Biomedicals. Anti-DGK $\eta$  (#13873-1-AP) was from Proteintech Group. Anti-PCNA (#2714-1) was from Epitomics.

### Mice

All mouse experiments were reviewed and approved by the University of Utah Institutional Review Board. Transgenic mice expressing the reverse tetracycline-controlled transactivator (rtTA) protein under the control of the rat clara cell secretory protein (CCSP) gene promoter were obtained from The Jackson Laboratory (stock#006232). L858REGFR transgenic mice were obtained from the NCI mouse repository (strain#01XEA). Bi-transgenic *CCSP-rtTA x L858R-EGFR* or control *L858R-EGFR* mice were given doxycycline (1gm/L) in their water for 8 weeks. Mouse lungs harboring mutant K-Ras (LSL-*Kras*<sup>G12D/+</sup>) treated with or without adenoviral Cre as described [15] and harvested 12 weeks later were provided by Trudy Oliver (University of Utah).

## Histological analysis

Lungs harvested from control or *L858R-EGFR* mice were fixed in 10 % neutral buffered formalin for 30–48 h, paraffin embedded, sectioned (5  $\mu$ m), and stained with hematoxylin and eosin according to standard protocol. Immunostaining was performed using the ABC reagent (Vector Laboratories) and anti-EGFR (#1902-1) or anti-PCNA (#2714-1) from Epitomics or anti-DGK $\eta$  (#13873-1-AP) from Proteintech Group according to instructions provided by the suppliers.

## Results

### DGK $\eta$ is expressed in human lung cancer cell lines and a mouse model of mutant EGFR lung cancer

To determine if DGK $\eta$  was expressed in human lung cancers, we surveyed a panel of human non-small cell lung cancer cell lines that had activating mutations in either EGFR (H1650 and H1975) or K-Ras (H441 or H460). We found that DGK $\eta$  was expressed in all of them and that the levels of DGK $\eta$  did not correlate with EGFR or K-Ras mutation status (Fig. 1a). This was consistent with prior data demonstrating that the levels of DGK $\eta$  mRNA were not affected by activation of EGFR [16]. To further assess the expression levels of DGK $\eta$  in mutant EGFR lung tumors, we obtained bi-transgenic mice in which doxycycline induces expression of an *L858R-EGFR* transgene in type II lung pneumocytes [17]. In preliminary experiments, we found that doxycycline induced significant tumor load after 8 weeks of treatment (Fig. 1b). As expected, EGFR was highly expressed in the tumors, but not in lungs of mice that were treated with doxycycline but did not carry the *rtTA* transgene necessary to induce expression of the *L858R-EGFR* transgene (Fig. 1c). Proliferating cell nuclear antigen (PCNA), a measure of cell proliferation, was also highly expressed in tumor-bearing lungs but was not detected in normal lungs (Fig. 1c). We found that DGK $\eta$  was expressed in bronchial epithelium of control mice (Fig. 1c) and was highly expressed in malignant epithelium of *L858R-EGFR* mice (Fig. 1c). Collectively, these data indicate that DGK $\eta$  is expressed in human lung cancer cell lines as well as in a mouse model of EGFR mutant lung cancer. Together with its known role in modulating EGFR signaling, these data suggest that DGK $\eta$  might promote tumorigenesis in EGFR or K-Ras mutant lung cancer.

### Depletion of DGK $\eta$ reduces oncogenic properties of lung cancer cells harboring mutant EGFR

To further investigate the potentially oncogenic role of DGK $\eta$ , we knocked down DGK $\eta$  in human H1650 lung cancer cells. These cells express a gain-of-function mutation (delE746-A750) in the gene encoding EGFR. Using lentivirus we generated a polyclonal H1650 cell line stably expressing shRNA targeting DGK $\eta$  that resulted in a ~60 % reduction in DGK $\eta$  mRNA (Fig. 2a) and an ~80 % reduction in DGK $\eta$  protein (Fig. 2b). To evaluate the effects of DGK $\eta$  depletion on cell proliferation, we used cell counting assays and found 5 days after plating the cells in 1 % serum significantly reduced cell numbers of DGK $\eta$ -deficient cells compared to control cells (Fig. 2c). Transient depletion of DGK $\eta$  using siRNA oligonucleotides targeting another region of DGK $\eta$  in H1650 cells also led to a ~30 % reduction in cell number 3–4 days after performing RNAi (Fig. 2d). Together with prior results showing reduced cell proliferation of DGK $\eta$ -deficient HeLa cells [12], these data indicate that DGK $\eta$  depletion leads to reduced cell number in low serum conditions.

To assess the effects of DGK $\eta$  deficiency on additional oncogenic properties, we tested the growth of the stable H1650 cells in conditions of anchorage independence by growing them in soft agar and found significantly reduced colony volumes in DGK $\eta$ -deficient cells compared to control H1650 cells (Fig. 3a, b). Together with the above observations, these data indicated that depleting DGK $\eta$  reduces the oncogenic potential of H1650 cells and led

us to test the effects of its depletion in other EGFR mutant cell lines and to assess the potential therapeutic value of disrupting DGK $\eta$  function in combination with an EGFR inhibitor.

### Depletion of DGK $\eta$ augments the inhibitory effects of EGFR inhibitors

EGFR mutations in lung cancer can be targeted by several small molecule inhibitors that are either in clinical use or in late stage clinical trials. One of these inhibitors, afatinib (BIBW-2992), showed promising results in EGFR mutant lung cancer [18]. Since DGK $\eta$  depletion reduced growth of cells harboring EGFR mutations, we tested the effects of combining afatinib with DGK $\eta$  depletion. For these experiments, we generated a second cell line to test using H1975 cells, which harbor another activating EGFR mutation (L858R), to make stable DGK $\eta$  knockdown cells (Fig. 4a). In this cell line, we were only able to reduce DGK $\eta$  protein by about 35 %, which was less than the \*80 % reduction that we achieved in the H1650 cells.

We first tested growth of the control cell lines in the presence or absence of afatinib and in preliminary experiments we found that afatinib, alone, reduced growth of control H1650 cells more substantially than it reduced growth of control H1975 cells. This finding is consistent with the known T790 M mutation that is also harbored by H1975 cells, which reduces the affinity of EGFR for small molecule inhibitors. We then compared growth of control and DGK $\eta$ -depleted cells and found in the absence of afatinib that DGK $\eta$ -deficient H1975 cells proliferated more slowly than control H1975 cells (Fig. 4b), but the reduction in proliferation caused by DGK $\eta$  depletion was not as substantial as in the H1650 cells. The reduced growth inhibition in the H1975 cells might reflect the limited knockdown of DGK $\eta$  that we were able to achieve compared to the H1650 cells. In both cell lines, we found that DGK $\eta$  depletion combined with afatinib led to more pronounced reductions in cell counts compared to afatinib alone (Fig. 4b, c). All differences between control and DGK $\eta$  knockdown cells in the presence or absence of afatinib were statistically significant. Together, these data demonstrated that depleting DGK $\eta$  reduced growth of another EGFR mutant cell line and also suggested that inhibitors of DGK $\eta$  might be useful as adjuvant therapy combined with EGFR inhibitors in lung cancers harboring EGFR mutations.

### DGK $\eta$ depletion reduces growth of some lung cancer cell lines harboring activating mutations in K-Ras

The reduced growth of DGK $\eta$  deficient cell lines was consistent with the known effects of reducing expression of DGK $\eta$  in HeLa cells [12], but the reduction of cell number was significantly greater in H1650 cells compared to the approximately 30 % reduction that was observed in HeLa cells [12]. Since HeLa cells express wild-type EGFR, the more pronounced reduction in the H1650 cells compared to HeLa cells was possibly due to the activating mutation in EGFR that the H1650 cells harbor. To further investigate the effects of DGK $\eta$  deficiency on cell growth and determine if they are more pronounced in EGFR mutant cell lines, we set out to test the effects of depleting DGK $\eta$  in lung cancer cell lines harboring activating mutations in K-Ras, which is mutated in about 16 % of lung cancers according to the catalog of somatic mutations in cancer (COSMIC) database. We first examined the expression levels of DGK $\eta$  in a mouse model of mutant K-Ras lung cancer [15]. Similar to our observations in mutant EGFR lung tumors in mice, we found high levels of DGK $\eta$  in mutant K-Ras lung tumors (Fig. 5a), indicating that DGK $\eta$  might also be important in K-Ras-driven lung tumors.

To determine if DGK $\eta$  contributed to cell growth in lung tumor cell lines harboring activating mutations in K-Ras, we used transient siRNA-mediated depletion of DGK $\eta$  and compared H460 and H441 cell lines, both of which harbor activated K-Ras, to H1650 cells.

We found 3–4 days after performing RNAi and growing the cells in 1 % serum that depleting DGK $\eta$  in H441 cells reduced cell number to the same extent as H1650 cells (Fig. 5b). But DGK $\eta$  depletion caused only a small, non-significant reduction in H460 cell counts (Fig. 5b). These data indicated that the effects of DGK $\eta$  depletion are not limited to cells harboring EGFR mutations.

Under the conditions of these growth assays, we could not measure consistent differences in the apoptosis markers cleaved PARP or caspase-3 in control or DGK $\eta$ -depleted cells (data not shown), indicating that the reduced DGK $\eta$ -deficient cell numbers that we observed were likely due to changes in the levels of proliferation. To assess the level of proliferation, we assayed the abundance of PCNA and found reduced levels in DGK $\eta$ -deficient H441 and H1650 cells, but not in H460 cells (Fig. 5c). This lack of effect of DGK $\eta$  depletion on PCNA levels in H460 cells was consistent with the lack of change in cell number caused by DGK $\eta$  depletion. Together, these data indicate that DGK $\eta$  has a prominent role in proliferation of EGFR mutant cell lines and can reduce proliferation of some cells harboring mutant K-Ras.

## Discussion

Our data suggest that DGK $\eta$  modulates oncogenic properties in lung cancer cells harboring EGFR mutations and in some lung cancer cells harboring K-Ras mutations. These findings are consistent with prior studies showing that DGK $\eta$  depletion also reduced HeLa cell growth [12], indicating that DGK $\eta$  modulates oncogenic properties in other types of cancer cell lines that do not harbor EGFR or K-Ras mutations. The broad effects of DGK $\eta$  depletion on oncogenic behavior are not surprising given its role in facilitating Raf heterodimerization and membrane recruitment downstream of EGFR [12]. Since functional activation of EGFR is found in a majority of human epithelial cancers [19] and activating K-Ras mutations are common as well, our data indicate that DGK $\eta$  is a novel cancer target and that inhibiting its function might affect a broad array of cancers.

Given the role of EGFR in lung cancer [2, 19], we focused our efforts on understanding the effects of depleting DGK $\eta$  in lung cancer cell lines. We found evidence that DGK $\eta$  promotes oncogenic properties of lung cancer cells. Consistent with this conclusion, DGK $\eta$  was recently identified as part of a seventeen gene microarray signature that predicted likelihood of death from non-small cell lung cancer (NSCLC) [20]. Based on the cohort used in that study, low levels of DGK $\eta$  mRNA correlated with longer survival in patients with NSCLC (Ref. [20] and S. Philipsen personal communication). The favorability of reduced DGK $\eta$  expression in that study is in concert with our findings that DGK $\eta$  depletion reduced oncogenic properties of lung cancer cells. Given its possible role in human lung cancer, we additionally tested growth of our stable control or DGK $\eta$ -deficient H1650 or H1975 cell lines as xenografts in nude mice, but we could not maintain knockdown of DGK $\eta$  over the 3 weeks of the experiments. Thus, it will take a more robust knockdown system or genetic models to further study the anti-oncogenic properties of depleting DGK $\eta$  in vivo.

Targeted therapies directed against EGFR have been developed and used clinically during the past few decades. They work best when used to treat tumors “addicted” to EGFR signaling. Unfortunately, most tumors that initially respond to the inhibitors eventually develop resistance by a variety of mechanisms [8–10]. This acquired resistance to EGFR inhibitors and the inherent EGFR inhibitor resistance of some tumors has spawned efforts to find other ways of blocking the EGFR pathway. Our data indicate that inhibiting the function of DGK $\eta$  in conjunction with an EGFR inhibitor might be a viable approach to treat tumors. Indeed, we discovered that when combined with afatinib, DGK $\eta$  depletion further reduced growth of both H1650 and H1975 cell lines. Collectively, our data suggest

that inhibitors of DGK $\eta$  might have clinical efficacy either when used alone or when combined with EGFR inhibitors.

DGK $\eta$  is a type II DGK that is structurally similar to DGK $\delta$ . We discovered that DGK $\delta$  also modulates EGFR signaling [11, 21] and that its depletion significantly reduced proliferation of H1650 cells in both two and three dimensions (M.K.T. manuscript in preparation). As such, targeting both DGKs  $\delta$  and  $\eta$  might have additive or synergistic effects and could be a very effective therapeutic approach to limit EGFR signaling. Type II DGKs are unlike other DGK isoforms because they have a split catalytic domain [22]. This structural difference suggests that it might be possible to design inhibitors that specifically target both DGKs  $\delta$  and  $\eta$ . This possibility and the antineoplastic effects of depleting DGK $\eta$  warrant further studies to examine its role in human cancer, to study the effects of depleting DGK $\eta$  in animal models of lung cancer and other cancers, and to develop strategies to inhibit its tumorigenic functions.

## Acknowledgments

This work was supported by the Huntsman Cancer Foundation, the R. Harold Burton Foundation, the National Institutes of Health Grants R01-CA95463 (to M.K.T.) and by P30-CA042014.

## Abbreviations

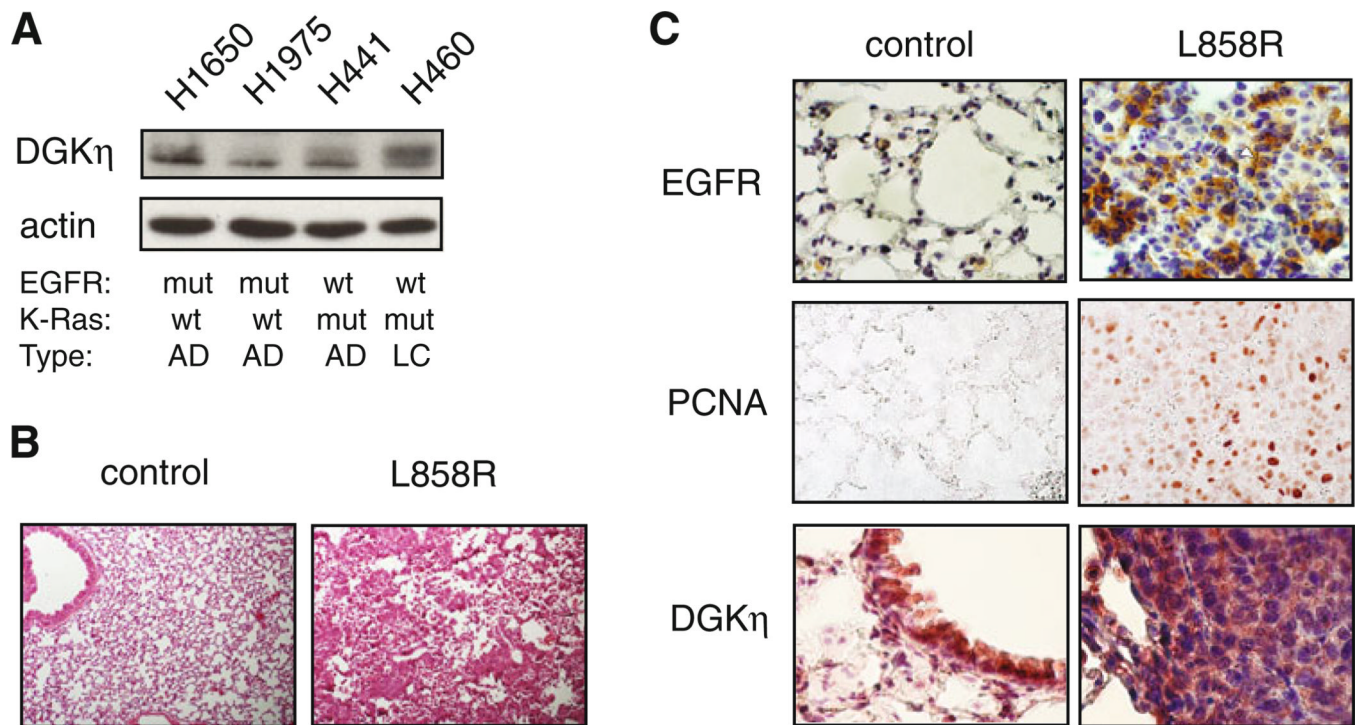
<b>EGFR</b>	Epidermal growth factor receptor
<b>DGK</b>	Diacylglycerol kinase
<b>DMEM</b>	Dulbecco's modified Eagle's medium
<b>rtTA</b>	Reverse tetracycline-controlled transactivator
<b>CCSP</b>	Clara cell secretory protein
<b>PCNA</b>	Proliferating cell nuclear antigen
<b>NSCLC</b>	Non-small cell lung cancer

## References

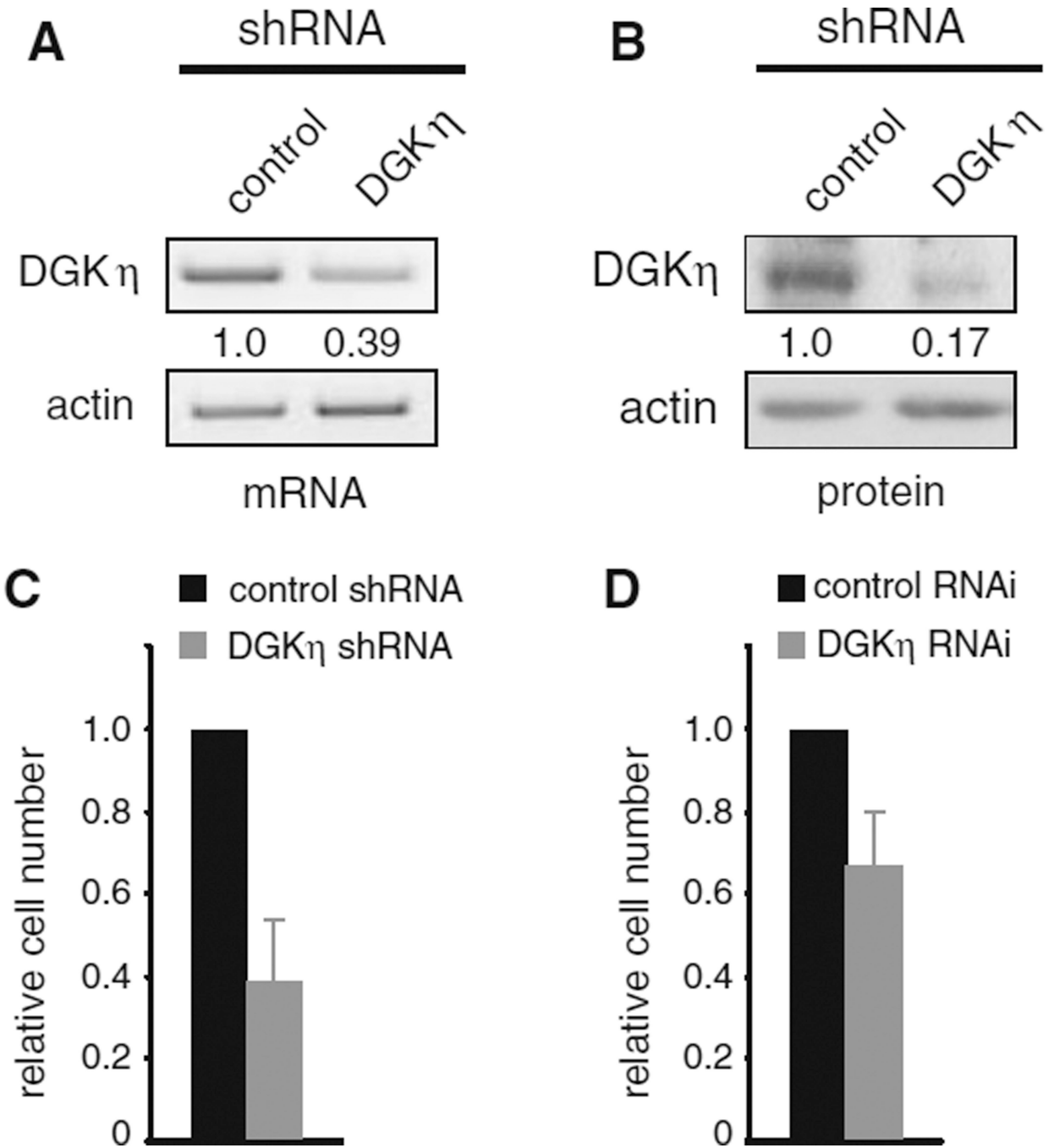
1. Sun S, Schiller JH, Gazdar AF. Lung cancer in never smokers—a different disease. *Nat Rev Cancer*. 2007; 7:778–790. [PubMed: 17882278]
2. Pao W, Chmielecki J. Rational, biologically based treatment of EGFR-mutant non-small-cell lung cancer. *Nat Rev Cancer*. 2010; 10:760–774. [PubMed: 20966921]
3. Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, et al. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *New Engl J Med*. 2010; 362:2380–2388. [PubMed: 20573926]
4. Mitsudomi T, Morita S, Yatabe Y, Negoro S, Okamoto I, Tsurutani J, et al. Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. *Lancet Oncol*. 2010; 11:121–128. [PubMed: 20022809]
5. Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *New Engl J Med*. 2009; 361:947–957. [PubMed: 19692680]
6. Rosell R, Carcereny E, Gervais R, Vergnenegre A, Massuti B, Felip E, et al. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EORTC): a multicentre, open-label, randomised phase 3 trial. *Lancet Oncol*. 2012; 13:239–246. [PubMed: 22285168]

7. Zhou C, Wu YL, Chen G, Feng J, Liu XQ, Wang C, et al. Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): a multicentre, open-label, randomised, phase 3 study. *Lancet Oncol.* 2011; 12:735–742. [PubMed: 21783417]
8. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science.* 2007; 316:1039–1043. [PubMed: 17463250]
9. Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, Meyerson M, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *New Engl J Med.* 2005; 352:786–792. [PubMed: 15728811]
10. Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med.* 2005; 2:e73. [PubMed: 15737014]
11. Cai J, Crotty TM, Reichert E, Carraway KL, Stafforini DM, Topham MK. Diacylglycerol kinase delta and protein kinase C(alpha) modulate epidermal growth factor receptor abundance and degradation through ubiquitin-specific protease 8. *J Biol Chem.* 2010; 285:6952–6959. [PubMed: 20064931]
12. Yasuda S, Kai M, Imai S, Takeishi K, Taketomi A, Toyota M, et al. Diacylglycerol kinase eta augments C-Raf activity and B-Raf/C-Raf heterodimerization. *J Biol Chem.* 2009; 284:29559–29570. [PubMed: 19710016]
13. Al-Salihi MA, Ulmer SC, Doan T, Nelson CD, Crotty T, Prescott SM, et al. Cyclooxygenase-2 transactivates the epidermal growth factor receptor through specific E-prostanoid receptors and tumor necrosis factor-alpha converting enzyme. *Cell Signal.* 2007; 19:1956–1963. [PubMed: 17572069]
14. Ding L, Traer E, McIntyre TM, Zimmerman GA, Prescott SM. The cloning and characterization of a novel human diacylglycerol kinase DGKi. *J Biol Chem.* 1998; 273:32746–32752. [PubMed: 9830018]
15. Oliver TG, Mercer KL, Sayles LC, Burke JR, Mendus D, Lovejoy KS, et al. Chronic cisplatin treatment promotes enhanced damage repair and tumor progression in a mouse model of lung cancer. *Genes Dev.* 2010; 24:837–852. [PubMed: 20395368]
16. Murakami T, Sakani F, Imai S, Houkin K, Kanoh H. Identification and characterization of two splice variants of diacylglycerol kinase  $\eta$ . *J Biol Chem.* 2003; 278:34364–34372. [PubMed: 12810723]
17. Politi K, Zakowski MF, Fan P, Schonfeld EA, Pao W, Varmus HE. Lung adenocarcinomas induced in mice by mutant EGF receptors found in human lung cancers respond to a tyrosine kinase inhibitor or to down-regulation of the receptors. *Genes Dev.* 2006; 20:1496–1510. [PubMed: 16705038]
18. Yang JC, Shih JY, Su WC, Hsia TC, Tsai CM, Ou SH, et al. Afatinib for patients with lung adenocarcinoma and epidermal growth factor receptor mutations (LUX-Lung 2): a phase 2 trial. *Lancet Oncol.* 2012; 13:539–548. [PubMed: 22452895]
19. Ciardiello F, Tortora G. EGFR antagonists in cancer treatment. *New Engl J Med.* 2008; 358:1160–1174. [PubMed: 18337605]
20. Hou J, Aerts J, den Hamer B, van Ijcken W, den Bakker M, Riegman P, et al. Gene expression-based classification of non-small cell lung carcinomas and survival prediction. *PLoS ONE.* 2010; 5:e10312. [PubMed: 20421987]
21. Crotty T, Cai J, Sakane F, Taketomi A, Prescott SM, Topham MK. Diacylglycerol kinase delta regulates protein kinase C and epidermal growth factor receptor signaling. *Proc Natl Acad Sci USA.* 2006; 103:15485–15490. [PubMed: 17021016]
22. Shulga YV, Topham MK, Epand RM. Regulation and functions of diacylglycerol kinases. *Chem Rev.* 2011; 111:6186–6208. [PubMed: 21800853]



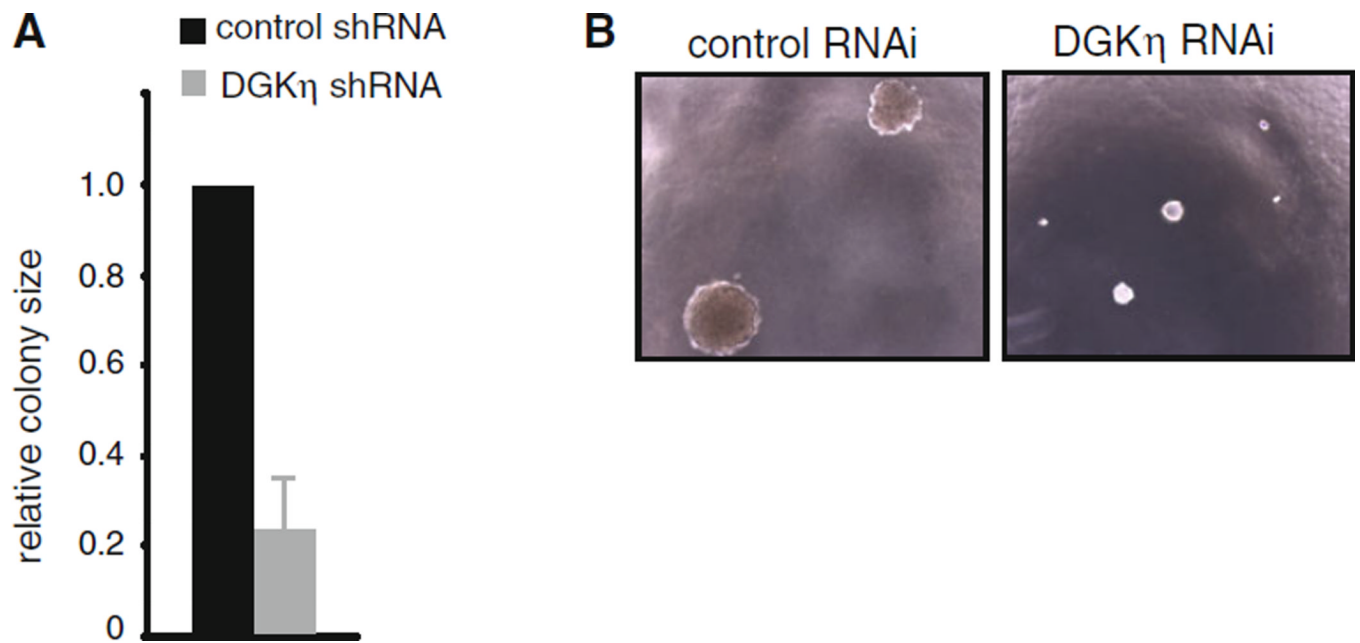


**Fig. 1.** Expression of DGK $\eta$  in lung cancer cell lines and in a mouse model of mutant EGFR lung cancer. **a** Lung cancer cell lines were grown to confluence, harvested, and then the levels of DGK $\eta$  and actin were detected in the cell lysates by Western blotting. The mutation status of EGFR or K-Ras in each cell line is shown below the blot (*mut* mutation, *wt* wild-type). The type of lung carcinoma from which each line was obtained is also indicated (*AD* adenocarcinoma, *LC* large cell carcinoma). **b** Lungs from *rtTA(-) × EGFR-L858R* (control) or *rtTA(+)* × *EGFR-L858R* (L858R) mice treated with doxycycline for 8 weeks were harvested, fixed, sectioned, and stained with eosin. **c** Lung sections from control or L858R mice were immunostained to detect EGFR, PCNA, or DGK $\eta$ .

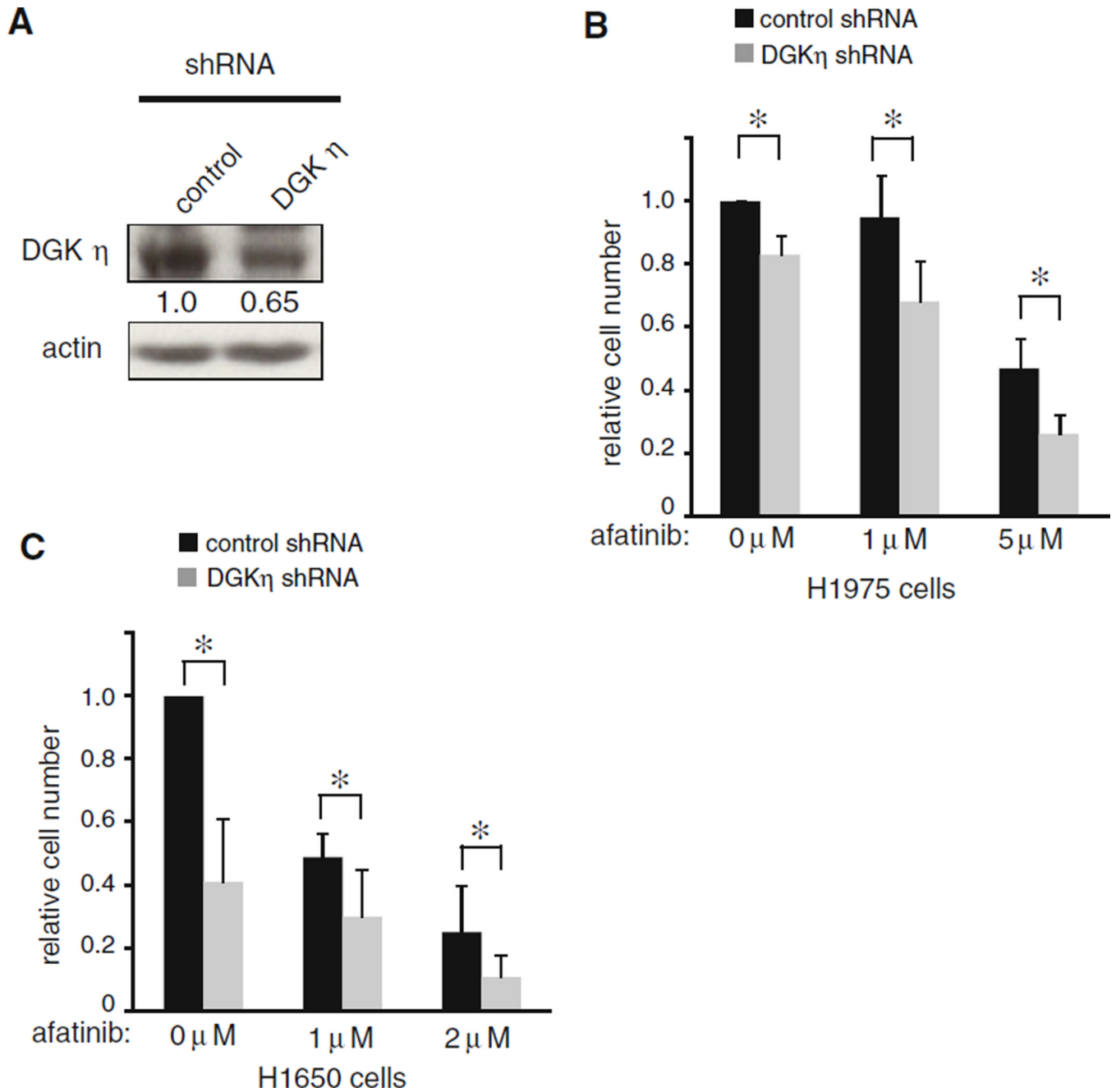


**Fig. 2.** DGK $\eta$  depletion reduces cell proliferation. H1650 cells were used to generate control or DGK $\eta$  shRNA polyclonal cell lines. The cells were grown to confluence, harvested, and then DGK $\eta$  and actin were detected using semi-quantitative RT-PCR (**a**) or Western blotting (**b**). The relative levels of DGK $\eta$  normalized to actin are shown below the DGK $\eta$  images. **c** Equal numbers of control or DGK $\eta$  shRNA H1650 cells were grown for 5 days in medium with 1 % serum and then counted. The difference in cell numbers between control and DGK $\eta$  shRNA cells was statistically significant ( $n = 3$ ;  $p < 0.02$ ). **d** Control or siRNA oligonucleotides were used for transient depletion of DGK $\eta$  in H1650 cells grown in 1 %

serum. Cell numbers were determined 3–4 days later. The difference in cell number between control and DGK $\eta$  RNAi cells was statistically significant ( $n = 3$ ;  $p < 0.05$ )

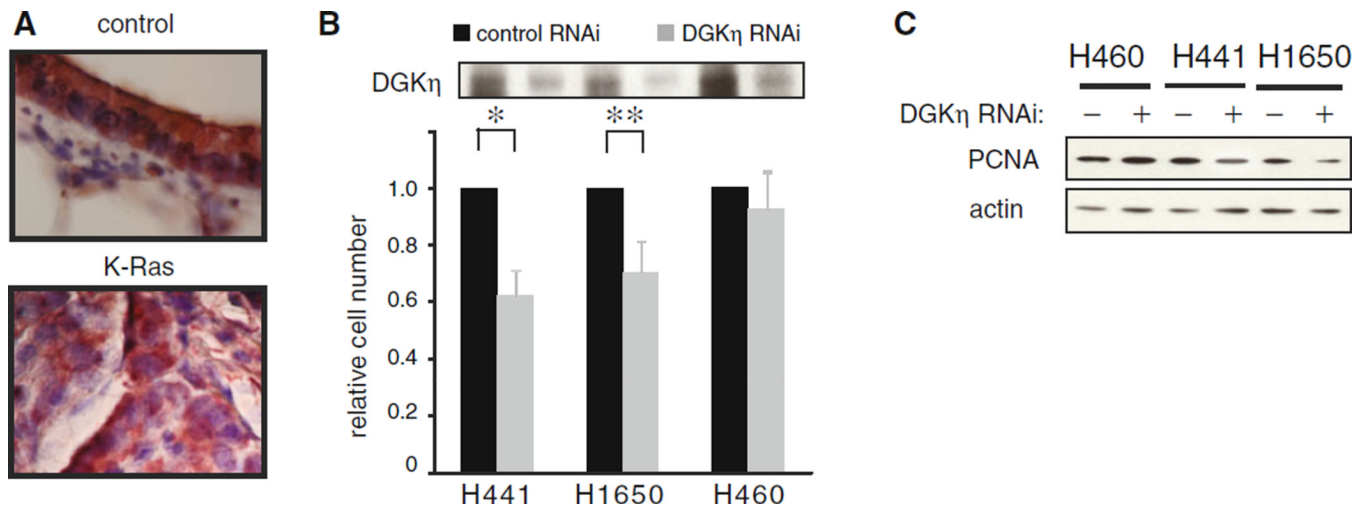


**Fig. 3.** DGK $\eta$  depletion reduces growth in soft agar. **a** Control or DGK $\eta$  shRNA H1650 cells were grown in soft agar for 14 days and then colony size was evaluated. The difference in colony size between control and DGK $\eta$  shRNA cells was statistically significant ( $n > 50$  colonies;  $p < 0.02$ ). **b** Representative photos of colonies evaluated in panel **a**



**Fig. 4.** Depletion of DGK $\eta$  augments the afatinib-induced growth inhibition. **a** H1975 cells were used to generate control or DGK $\eta$  shRNA polyclonal cell lines. The cells were grown to confluence, harvested, and then DGK $\eta$  and actin were detected in the cell lysates by Western blotting. The relative expression levels of DGK $\eta$  normalized to actin are shown below the DGK $\eta$  blot. **b** Control or DGK $\eta$  shRNA H1975 cells were grown for 5 days in medium with 1 % serum in the absence or presence of escalating concentrations of afatinib and then counted. The *asterisk* indicates that the changes were statistically significant ( $n = 3$ ;  $p < 0.05$ ). **c** Control or DGK $\eta$  shRNA H1650 cells were grown 5 days in medium with 1 %

serum in the absence or presence of escalating concentrations of afatinib and then counted. The *asterisk* indicates that the changes were statistically significant ( $n = 3$ ;  $p < 0.05$ )



**Fig. 5.** DGK $\eta$  is expressed in K-Ras mutant lung tumors and its depletion reduces proliferation of some K-Ras mutant cell lines. **a** DGK $\eta$  was detected by immunostaining in lungs from control (sham-treated) or LSL-*Kras*<sup>G12D/+</sup> mice. **b** Control or DGK $\eta$  depleted lung cancer cell lines grown in 1 % serum were counted 3–4 days after RNAi. The *asterisk* indicates  $p < 0.04$  ( $n = 3$ ) and the *double asterisk* indicates  $p < 0.02$  ( $n = 3$ ). DGK $\eta$  was detected by immunoblotting in each cell line and shown above the graph. **c** Levels of PCNA and actin in control or DGK $\eta$  depleted cells grown in 1 % serum and harvested 3 days after RNAi were detected by immunoblotting