# Reduced cell proliferation by IKK2 depletion in a mouse lung-cancer model

Yifeng Xia<sup>1</sup>, Narayana Yeddula<sup>1</sup>, Mathias Leblanc<sup>1</sup>, Eugene Ke<sup>1,2</sup>, Yonghui Zhang<sup>3</sup>, Eric Oldfield<sup>3,4</sup>, Reuben J. Shaw<sup>5,6</sup> and Inder M. Verma<sup>1,7</sup>

Lung cancer is one of the leading cancer malignancies, with a five-year survival rate of only ~15%. We have developed a lentiviral-vector-mediated mouse model, which enables generation of non-small-cell lung cancer from less than 100 alveolar epithelial cells, and investigated the role of IKK2 and NF- $\kappa$ B in lung-cancer development. IKK2 depletion in tumour cells significantly attenuated tumour proliferation and significantly prolonged mouse survival. We identified *Timp1*, one of the NF- $\kappa$ B target genes, as a key mediator for tumour growth. Activation of the Erk signalling pathway and cell proliferation requires Timp-1 and its receptor CD63. Knockdown of either *lkbkb* or *Timp1* by short hairpin RNAs reduced tumour growth in both xenograft and lentiviral models. Our results thus suggest the possible application of IKK2 and Timp-1 inhibitors in treating lung cancer.

Inflammation and nuclear factor-κB (NF-κB) activation have long been linked to cancer development<sup>1</sup>. Recent studies have indicated the involvement of NF-κB in v-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue (*Kras*)-mutation-induced lung adenocarcinoma<sup>2–4</sup>. Expressing IκBαM super-repressor or conditional-knockout p65 in the tumour cells significantly reduced tumour size and tumour incidence. In addition, ablation of IKK2 in myeloid cells inhibited tobacco-smokeinduced lung-tumour promotion and malignant-cell proliferation in the Kras<sup>LA2</sup> model<sup>5</sup>. A very recent study has also indicated the involvement of NF-κB in epidermal growth factor receptor tyrosine kinase inhibitor resistance<sup>6</sup>. IKK2, as the essential kinase for NF-κB activation, is thus thought to be a good candidate for drug design<sup>7–9</sup>.

Lung cancers along with other human cancers are initiated from a few cells bearing certain mutations that give growth advantage<sup>10–13</sup>. Previous studies have indicated that different cell origins are responsible for different subtypes of lung cancer<sup>14</sup>. These tumour-initiating cells are surrounded by normal cells and this microenvironment is critical for studying tumour development. Mouse tumour models based on crossing a conditional allele of the tumorigenic element with tissue-specific Cre recombinase may not aptly reflect the real tumour initiation, as the genetic lesions usually take place in the whole organ<sup>15,16</sup>.

We used lentiviral vectors for establishing tumours in mice for the following reasons: (1) lentiviruses infect almost any type of cell, and transgene expression can be controlled by a tissue-specific promoter, which enables a more precise tracing of the origin of the cancer cell; (2) lentiviruses integrate into genomic DNA so that it is possible to stably deliver oncogenes and short hairpin RNAs (shRNAs) against tumour suppressors, and bypass the requirement of numerous conventional genetic crossings; (3) viral titres can be controlled to infect only a few cells, to more faithfully recapitulate human cancer initiation<sup>17</sup>. Moreover, in principle, a lentiviral vector carrying certain oncogenes and shRNAs against tumour suppressors is sufficient to initiate tumorigenesis in a mouse of any genomic background, although in this study we used a series of tissue-specific Cre-expressing lentiviral vectors to initiate tumours in LSL-Kras<sup>G12D</sup> mice<sup>18</sup>, for biosafety considerations.

In the present study, we have investigated the underlying mechanisms involved in NF- $\kappa$ B promoting lung-cancer cell proliferation by knocking out inhibitor of kappaB kinase beta (*Ikbkb*, also known as *IKK2*) specifically in tumour cells. We found that downregulation of *Timp1* (tissue inhibitor of metalloproteinase 1), one of the NF- $\kappa$ B target genes, contributed to slower tumour-cell proliferation, consistent with the fact that NF- $\kappa$ B and Timp-1 are usually highly expressed in advanced lung-cancer patients with poor prognosis<sup>19,20</sup>. Furthermore, knocking down *Ikbkb* or *Timp1* with shRNAs in tumour cells significantly reduced Erk activation and cell proliferation, thereby attenuated tumour progression in our mouse model.

<sup>7</sup>Correspondence should be addressed to I.M.V. (e-mail: verma@salk.edu)

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<sup>&</sup>lt;sup>1</sup>Laboratory of Genetics, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, California 92037, USA. <sup>2</sup>Graduate Program in Bioinformatics, University of California San Diego, La Jolla, California 92093, USA. <sup>3</sup>Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, USA. <sup>4</sup>Center for Biophysics and Computational Biology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, USA. <sup>5</sup>Molecular and Cellular Biology Laboratory, The Salk Institute for Biological Studies, La Jolla, California 92037, USA. <sup>6</sup>Howard Hughes Medical Institute, The Salk Institute for Biological Studies, La Jolla, California 92037, USA.



**Figure 1** Lentiviral-vector-mediated mouse lung-cancer model. (a) Diagram of lentiviral vector design. Oncogene or Cre is controlled by a tissue-specific promoter and shRNAs are driven by U6 or H1 promoter. CA2, carbonic anhydrase 2 promoter. (b) Kras<sup>G12D</sup> mice were infected intratracheally with CA2Cre or CA2Cre-shp53 lentivirus. Mice were harvested at 2, 3 or 4 months after the infection. Lung sections showed different tumour

#### RESULTS

#### Mouse lung-cancer model mediated by lentiviral vectors

We developed a set of lentiviral vectors that can induce oncogenic mutations in a small number of lung epithelial cells and initiate lung cancer in mice. As shown in Fig. 1a, a typical lentivector for this purpose has a combination of a tissue-specific oncogene (or Cre) and several shRNAs to knock down tumour-suppressor genes. We successfully induced lung adenoma and adenocarcinoma in the LSL-Kras<sup>G12D</sup> mouse with carbonic anhydrase 2 (CA2)-driven Cre lentiviral vector (Supplementary Fig. S1b). Notably, the result from CA2-Cre virus infection in LSL-Rosa26<sup>lacZ</sup> reporter mice indicated that the CA2 promoter is expressed mainly in alveolar epithelial cells<sup>21</sup> and the tumours were initiated in very few cells tranduced by lentiviral vectors (Supplementary Fig. S1a).

Transduction by lentiviral vectors generating shRNA against p53 (U6-shp53) significantly accelerated tumour progression and resulted in rapid development of adenocarcinomas with more advanced tumour grading (Fig. 1b). Figure 1c-e shows the evolution of tumour lesion from an atypical adenomatous hyperplasia to small adenoma and to advanced adenocarcinoma<sup>22</sup>. Twelve weeks after infection, the majority of the tumour lesions reached grade 3 and grade 4, showing pleomorphic nuclei and aberrant mitosis (Fig. 1f). More importantly, massive stromal cell infiltration was found in  $\sim 20\%$  of the advanced tumours, which recapitulates well the pathology observed in human malignancies (Fig. 1g,h). All tumours we have analysed in the CA2Cre-shp53 lentiviral model were typical adenocarcinomas with SPC (pro-surfactant protein C)-positive and CC10 (Clara-cell-specific antigen)-negative staining patterns (Fig. 1i,j). The Ki-67-positive rate is 5-20% in these tumours, which is similar to what has been reported in human adenocarcinomas23 (Fig. 1k).



burdens. (**c**–**e**) Histology of atypical adenomatous hyperplasia, adenoma and adenocarcinoma respectively. (**f**) Pleomorphic nuclei (arrowhead) and aberrant mitosis (arrow) were found in advanced adenocarcinoma. (**g**,**h**) Stromal cell infiltration (arrows) identified by haematoxylin and eosin staining and Masson's trichrome staining. (**i**–**k**) Tumour sections stained with SPC, CC10 and Ki-67 antibodies respectively. Scale bars,  $100 \,\mu$ m.

#### Ikbkb knockout in tumour cells impairs tumour proliferation

Given that the NF- $\kappa$ B pathway plays an important role in tumour development and that IKK2 is the seminal kinase responsible for NF- $\kappa$ B pathway activation, we were particularly interested in exploring the therapeutic potential of IKK2 inhibition in non-small-cell lung cancers. We therefore crossed IKK2<sup>fl/fl</sup> and Kras<sup>G12D</sup> mice so that we could simultaneously activate *Kras*<sup>G12D</sup> to initiate tumour formation and inactivate *Ikbkb*, on transduction by Cre lentiviral vectors. Compared with Kras<sup>G12D</sup>IKK2<sup>WT</sup> mice (wild type), Kras<sup>G12D</sup>IKK2<sup>fl/fl</sup> mice (IKK2<sup>-/-</sup>) developed lung tumours with much longer latency (162 days median survival time versus 114 days when infected with CA2Cre-shp53 vector, Fig. 2a). At 100 days post lentiviral infection, the average tumour burden in IKK2<sup>-/-</sup> mice was only about one-third to one-half of that in wild-type mice (Fig. 2b).

Although IKK2<sup>-/-</sup> mice had less tumour burden than wild-type mice when collected at an early time, they developed comparable tumours at the endpoint (Fig. 2b,c). The tumours found in both genotypes of mice were adenocarcinomas, exhibiting SPC-positive and CC10-negative staining (Fig. 2d,e). Similar percentages of the tumour areas were scored positive for Erk phosphorylation (Erk-p), indicating their advanced tumour stage<sup>24,25</sup> (Fig. 2f).

As, in various cancers, inflammatory-cell infiltration stimulates tumour growth, we hypothesized that IKK2-deficient tumours might produce less chemokines and attract fewer inflammatory cells. We therefore analysed inflammatory-cell infiltration in the bronchioalveolar lavage at different times after tumour initiation. Macrophage counts from wild-type mice increased significantly during the three months of tumour development. IKK2<sup>-/-</sup> mice showed less macrophage infiltration for the first three months; however, the cell numbers were similar to those found in wild-type mice at the endpoint, suggesting that inflammatory-cell infiltration was



**Figure 2** IKK2 depletion in Kras<sup>G12D</sup> tumour cells prolonged mouse survival. (a) Kaplan–Meier curve showing Kras<sup>G12D</sup> (wild type, WT) and Kras<sup>G12D</sup> IKK2<sup>II/fI</sup> (IKK2<sup>-/-</sup>) mouse survival after CA2Cre-shp53 lentivirus infection. Median survival time of the two groups was 114 days (wild type, n = 15) and 162 days (IKK2<sup>-/-</sup>, n = 19) respectively (P < 0.0001). (b) Representative whole lungs collected from wild-type and IKK2<sup>-/-</sup> mice at indicated times after infection. Scale bars, 5 mm. (c-j) Tumours from wild-type and IKK2<sup>-/-</sup> mice collected at endpoint were stained with haematoxylin and eosin, anti-SPC, CC10, Erk-p, von Willebrand factor (green) and CD68 (red), cleaved caspase 3, Ki-67 and PCNA antibodies respectively. Scale bars,  $100 \,\mu$ m. (k) Quantification of Ki-67-positive cells at multiple fields. (I) Bronchioalveolar lavage (BAL) macrophage counts were analysed from wild-type and IKK2<sup>-/-</sup> mice at indicated times after infection. Error bars, s.d. (Normal mice, n = 2; 6 weeks, n = 1; 12 weeks, n = 4; endpoint, n = 3.)

correlated with the size of the tumour burden instead of the IKK2 status of the tumour (Fig. 2g,l). This might be due to the fact that tumour-surrounding tissues were IKK2 wild type, which could compensate for chemokine production when inflamed by nearby tumour formation. These results rule out the possibility that the  $IKK2^{-/-}$  mice developed fewer and smaller tumours owing to reduced macrophage chemotaxis and inflammation.

To investigate the real contribution of IKK2 and the NF- $\kappa$ B pathway in this lung-cancer model, we examined other possible roles of NF- $\kappa$ B, such as pro-proliferation, anti-apoptosis and pro-angiogenesis. We were unable to find any significant differences between IKK2<sup>-/-</sup> and wild-type tumours regarding the apoptotic marker (cleaved caspase 3) or vascular endothelial marker (von Willebrand factor; Fig. 2h,g). In contrast, we found surprisingly high staining of proliferation markers Ki-67 and PCNA in wild-type tumours (12% Ki-67 positive in wild-type tumours versus 6% in IKK2<sup>-/-</sup> tumours at 11 weeks, and 8% in wild type versus 2% in IKK2<sup>-/-</sup> at endpoint, Fig. 2i–k). Low Ki-67 staining was found throughout the whole period of tumour progression in IKK2<sup>-/-</sup> mice (Supplementary Fig. S2a). Similarly, cell lines derived from IKK2<sup>-/-</sup> tumours had less 5-bromodeoxyuridine (BrdU) incorporation in cell culture (Supplementary Fig. S2b). The microarray data further support this notion because the majority of the differences between wild-type and IKK2<sup>-/-</sup> tumours are in the genes in cell-cycle progression (Supplementary Fig. S2c–e and Table S1). Taken together, these results indicate that the most likely major contribution of NF-κB pathway in our lung-cancer model is pro-proliferation.

#### NF-KB activation in lung cancer

Constitutive NF-KB activation has been reported in various tumours, including different forms of lung cancer<sup>26</sup>. Therefore, we next examined NF-KB activity in our primary lung-tumour samples. Nuclear extracts from wild-type tumours showed high NF-kB binding activity, whereas IKK2<sup>-/-</sup> tumours remained at a basal level. Supershift using specific antibodies confirmed the bands as canonical NF-KB dimers containing p65 and p50, but not c-Rel (Fig. 3a). We next asked whether oncogenic stress induced by  $Kras^{G12D}$  is responsible for NF- $\kappa B$  activation in these cells. Using a tetracycline-response element (TRE)-regulated Kras-expressing system, we detected high NF-kBactivity within 3-4 days of Kras<sup>G12D</sup> expression (Supplementary Fig. S3a). Kras expression upregulated the PI3K-Akt and MEK-Erk pathways, as well as the DNA-damage response, which all activated the NF-KB pathway<sup>27,28</sup> (Fig. 3b and Supplementary Fig. S3b-d). In addition, inhibitors of MEK, PI3K and ATM all suppressed the NF-kB reporter activity induced by Kras (Fig. 3c).

As anticipated, IKK2 was required for the NF- $\kappa$ B activation by Kras, as *Ikbkb* shRNA knockdown as well as the small-molecule inhibitor TPCA-1 significantly reduced NF- $\kappa$ B activity. Furthermore, *p53* shRNA knockdown resulted in higher NF- $\kappa$ B activity (Fig. 3d), consistent with a previous report<sup>4</sup>. All these results supported the idea that oncogenic Kras, together with p53 deficiency, contributed to NF- $\kappa$ B activation in these tumour cells.

# Timp-1 is one of the mediators for NF- $\kappa$ B-induced tumour proliferation

We next sought to elucidate how NF- $\kappa$ B contributed to tumour proliferation. Multiple NF- $\kappa$ B-regulated cytokines stimulate tumourcell proliferation through autocrine and paracrine pathways<sup>29</sup>. We thus investigated the expression of a set of 40 cytokines in wild-type and IKK2<sup>-/-</sup> tumour lysates using an antibody array, and further verified by using quantitative PCR with reverse transcription (RT–PCR) (Supplementary Fig. S4a–c).

Timp-1 was identified among the cytokines that were significantly downregulated in the IKK2<sup>-/-</sup> tumour lysate. We also saw reductions in the levels of some other cytokines (IL-23 and TREM-1; Supplementary Fig. S4b); however, their protein levels were very low in the tumour lysates and could not be detected in cell cultures derived from the tumours, indicating that they might be from tumour-infiltrating inflammatory cells. Interestingly, *Timp1* has been reported to be expressed in different human tumours, including lung adenocarcinomas<sup>30</sup>. Although the role of Timp-1 in cancer growth and metastasis is controversial<sup>31</sup>, high *Timp1* expression levels are always linked to poor prognoses in lung-cancer patients<sup>19,20</sup>. The *Timp1* 



**Figure 3** *Timp1* expression induced by NF-κB activation in Kras<sup>G12D</sup> tumours. (a) Nuclear extracts from primary tumour lesions were analysed by electrophoretic mobility shift assay for NF-κB binding. Antibodies were used in supershift to specify the composition of NF-κB dimers. (b) Kras<sup>G12D</sup> was expressed under the regulation of TREs in U2OS cells. Cell lysates were collected at different times after adding doxycycline and subjected to immunoblotting analysis. (c) Exogenous Kras<sup>G12D</sup> expression in U2OS cells activated NF-κB-luciferase reporter, which was suppressed by ATM (KU55933), MEK1 (PD0325901), PI3K (wortmannin) and IKK2 (TPCA-1) inhibitors. (d) NF-κB activation by Kras<sup>G12D</sup> was down- or upregulated by different shRNAs or the IKK2 inhibitor TPCA-1. Error bars, s.d. (c,d,

gene promoter has putative NF- $\kappa$ B binding sites<sup>32</sup>, so its expression could be suppressed by the I $\kappa$ B $\alpha$ M super-repressor, or *Ikbkb* shRNA, in wild-type tumour cells (Fig. 3e). The difference of *Timp1* expression in primary wild-type and IKK2<sup>-/-</sup> tumours was then further verified at messenger RNA and protein levels (Fig. 3f–h; Supplementary Fig. S4e).

These observations led to the question of whether high *Timp1* expression in wild-type tumours contributes to their rapid proliferation *in vivo*. To address this question, we examined tumour-cell growth in a subcutaneous xenograft model in nude mice using two cell lines derived from wild-type and IKK2<sup>-/-</sup> primary tumours, respectively. Wild-type line 4A3 formed a much larger tumour mass than did IKK2<sup>-/-</sup> line 1D3, within three weeks after transplantation. Knocking down either *Ikbkb* or *Timp1* expression in 4A3 reduced tumour size considerably (Fig. 3i). Although ectopic expression of *Ikbkb* in line 1D3 completely rescued its proliferation as judged by the size of the tumours (Fig. 3j), surprisingly, ectopic expression of *Timp1* did not significantly influence tumour size. It thus seems that IKK2 and NF- $\kappa$ B play an essential role in tumour-cell proliferation, whereas Timp-1 may be a necessary but not sufficient mediator of this effect.

n = 4 biological replicates). (e) *Timp1* expression was reduced in wild-type tumour cells stably infected with 1kBαM super-repressor (green fluorescent protein, GFP fusion) or *Ikbkb* shRNA virus. (f) Immunostaining of Timp-1 in wild-type and IKK2<sup>-/-</sup> tumours. Scale bars, 100 μm. (g,h) *Timp1* mRNA and protein levels were examined by quantitative RT–PCR and enzyme-linked immunosorbent assay (ELISA) in individual primary tumours collected from wild-type and IKK2<sup>-/-</sup> mice. (i) shRNA knockdown of *Ikbkb* or *Timp1* reduced 4A3 (wild-type) cell proliferation in a nude-mice xenograft model. (j) 1D3 (IKK2<sup>-/-</sup>) cell proliferation in the xenograft model was rescued by exogenous *Ikbkb* expression, but not *Timp1*. Uncropped images of blots are shown in Supplementary Fig. S7.

#### Timp-1 stimulates cell proliferation through the Erk pathway

There is increasing evidence indicating that Timp-1 has paradoxical effects on tumour growth<sup>31,33</sup>. When Kras<sup>G12D</sup> was expressed in U2OS cells, significant activation of Erk, Akt and NF-KB pathways was observed, together with upregulation of Timp1 expression (Fig. 4a; Supplementary Fig. S5a,b). Given that the Erk pathway is critical for cell proliferation, we asked whether Timp1 expression stimulates cell proliferation in a positive-feedback loop through Erk activation. Interestingly, Erk but not Akt phosphorylation was severely impaired when Ikbkb was knocked down in U2OS cells (Fig. 4a). Moreover, doxycycline (Dox)-induced expression of Timp1 completely rescued the defect of Erk phosphorylation in Ikbkb-knockdown cells, which had low endogenous Timp1 induction owing to lack of NF-KB activation (Fig. 4a). Similarly, an Erk phosphorylation defect was seen in Timp1knockdown cells (Supplementary Fig. S5c). These results point to a positive-feedback loop: KrasG12D-Erk-p-NF-KB-Timp-1-Erk-p, which might play an important role in tumour-cell proliferation.

We next examined this effect in cell lines derived from  $IKK2^{-/-}$  tumour, which show low *Timp1* expression and slow proliferation.



**Figure 4** IKK2 and Timp-1 are required to maintain high Erk activation. (a) TRE-Kras<sup>G12D</sup> was expressed in U2OS cells stably infected with control shRNA, *Ikbkb* shRNA or *Ikbkb* shRNA plus TRE-Timp-1. Erk and Akt phosphorylation was examined by immunoblotting analysis and quantified with ImageJ. \*Dox-induced *Timp1* expression was shown at a shorter exposure time. (**b,c**) Dox-induced *Timp1* expression in 1D3 (IKK2<sup>-/-</sup>) cells upregulated Erk phosphorylation and BrdU incorporation. The increase of

When ectopic *Timp1* expression in these cells was turned on by addition of Dox, both Erk phosphorylation and cell proliferation, determined by BrdU incorporation, were substantially increased (Fig. 4b,c). Importantly, the increase of cell proliferation by Timp-1 could be abolished by adding MEK inhibitor PD0325901, which further supports our contention that the Erk pathway mediates the pro-proliferation effect of Timp-1 (Fig. 4c).

Indeed, there was higher *Timp1* expression in wild-type tumours than in IKK2<sup>-/-</sup> tumours at all stages of tumour development, owing to NF- $\kappa$ B activation (Supplementary Fig. S4e), correlating with the rapid proliferation and high percentage of Erk-p staining observed in wild-type tumours, compared with IKK2<sup>-/-</sup> tumours (Fig. 4d). Although the majority of the IKK2<sup>-/-</sup> tumours at the endpoint showed elevated Erk-p staining, as observed with wild-type tumours (Supplementary Fig. S5d), clear differences could be observed in tumours collected 11 weeks after lentiviral infection and stable cell lines derived from the tumours (Fig. 4d,e and Supplementary Fig. S5d,e). p19ARF was also found at low levels in IKK2<sup>-/-</sup> tumours, owing to the low Erk activation (Fig. 4e, tumours 3, 4 and 5, and Supplementary Fig. S5e). Overall, Erk phosphorylation was found in ~30% of wild-type tumour lesions, as compared with less than 10% in IKK2<sup>-/-</sup> tumours. These results clearly indicate a critical role for

BrdU incorporation was abolished by adding MEK inhibitor PD0325901. Error bars, s.d. (n = 3 biological replicates). (d) Tumour samples from wild-type and IKK2<sup>-/-</sup> mice collected at 11 weeks were analysed for Timp-1, Erk-p and Ki-67 staining. Scale bars, 100 µm. (e) Individual tumour lesions collected at 11 weeks were lysed and protein expression was analysed by immunoblotting. Uncropped images of blots are shown in Supplementary Fig. S7.

IKK2 and Timp-1 in maintaining high proliferation and advanced pathology in lung cancers.

#### Activation of the Erk pathway by Timp-1 requires CD63

As Timp-1 was originally identified as a tissue inhibitor of metalloproteinases (MMPs), we first asked whether this inhibitory property is responsible for the its pro-proliferation activity. Addition of recombinant Timp-1 protein (rTimp-1) to IKK2<sup>-/-</sup> tumour cells precipitously increased both focal adhesion kinase (FAK) and Erk phosphorylation, whereas the potent MMP inhibitor GM6001 had no effect (Fig. 5a). Furthermore, unlike rTimp-1, GM6001 failed to stimulate cell proliferation in the BrdU incorporation assay (Fig. 5b). These results indicate that Timp-1's function in cell proliferation is independent of its MMP inhibitor activity.

It has previously been shown that Timp-1 can bind to CD63, a member of the tetraspanin family, and activate its downstream pathways including FAK and Erk phosphorylation<sup>34</sup>. We used two shRNAs to knock down *Cd63* expression in the IKK2<sup>-/-</sup> tumour cells (Fig. 5c) and checked if *Cd63* knockdown would eliminate the effect of Timp-1 treatment. As shown in Fig. 5d,e, Timp-1-induced Erk phosphorylation and cell proliferation were impaired in *Cd63* shRNA 1 and *Cd63* shRNA 2 cells, but not in control shRNA cells. Finally,



**Figure 5** Activation of FAK–Erk pathway and cell proliferation requires Timp-1 and CD63. (a) IKK2<sup>-/-</sup> tumour cells were treated with 0.5  $\mu$ g ml<sup>-1</sup> rTimp-1 or 10  $\mu$ M GM6001 for different times. Erk, Akt and FAK phosphorylation was examined by immunoblotting and quantified with ImageJ. (b) Cell proliferation was analysed by BrdU incorporation assay. Error bars, s.d. (n = 3 biological replicates). (c) *Cd63* mRNA levels were examined in cells infected with control shRNA or *Cd63* shRNAs by quantitative RT–PCR (upper panel). Error bars, s.d. (n = 4 biological replicates). CD63 protein levels were examined by flow cytometry (lower

FAK inhibitor (FAK I-14) successfully repressed Erk phosphorylation induced by ectopic *Timp1* expression, supporting a link in the signal transduction from CD63 to FAK and Erk (Fig. 5f). Our cumulative results confirm that binding of Timp-1 to CD63 and activation of downstream signal transduction are crucial for its pro-proliferation effect.

# *Timp1* knockdown recapitulates IKK2 deficiency in lung-cancer progression

To further confirm the importance of IKK2 and Timp-1 in lung adenocarcinoma progression *in vivo*, we designed a set of lentiviral vectors that included shRNAs against *Ikbkb* or *Timp1* (Fig. 6a). We then applied these vectors to initiate lung tumours, and followed their growth. When tumours were collected after the same inoculation time, we observed reduced tumour size from the groups of mice transduced with *Ikbkb* and *Timp1* shRNA vectors (Fig. 6b). Immunostaining confirmed that *Ikbkb* shRNA and *Timp1* shRNA tumours had less *Timp1* expression, less Erk phosphorylation and lower Ki-67 percentage as compared with controls (Fig. 6b,c). Mice receiving either *Ikbkb* or *Timp1* shRNA vectors (202 days and 192 days versus 129 days median survival time, Fig. 6d). All these results strongly support the possibility

panel). APC, allophycocyanin. (d) Cells infected with different shRNAs (indicated by GFP expression) were treated with rTimp-1 for 24 h and Erk phosphorylation was examined by immunoblotting. (e) BrdU incorporation assay was carried out 2 days after rTimp-1 treatment. Error bars, s.d. (n=3 biological replicates). (f) *Timp1* expression was induced by Dox in the cells for 1, 2 or 3 days, and the cells were treated with PD0325901 or FAK I-14 for 4 h before the collection. Phosphorylation of Erk was examined by immunoblotting. Uncropped images of blots are shown in Supplementary Fig. S7.

of designing inhibitors that target IKK2 or Timp-1 for treatment of lung adenocarcinomas.

As proof of principle, we tested the effect of TPCA-1, a preclinical IKK2 inhibitor<sup>35,36</sup>, in treating lung cancer in our mouse model. We induced tumours in Kras<sup>G12D</sup>Rosa26<sup>luc</sup> mice with CA2Cre-shp53 lentivirus and initiated therapy 10 weeks after viral infection for 6 weeks (Fig. 7a). The luciferase imaging results indicated that TPCA-1 treatment led to slower tumour growth when compared with vehicle control (Fig. 7b,c). We were not able to continue treatment during the whole tumour development, owing to the toxicity of chronic NF-KB inhibition; however, administration of TPCA-1 for 6 weeks already increased the median survival time from 141 days to 153 days, with statistical significance (P = 0.0007 by log-rank test), indicating high therapeutic potential of IKK2 inhibition (Fig. 7d). Although we have not been able to test Timp-1-neutralizing antibodies in treating primary mouse lung cancer owing to the lack of reagents, follow-up experiments using Timp-1 antibodies or inhibitors will hopefully offer opportunities of treating lung cancer.

#### DISCUSSION

In this study, we have described a mouse lung-cancer model mediated by lentiviral vectors. Compared with the widely used adeno-Cre



**Figure 6** Knockdown of *lkbkb* or *Timp1* attenuated tumour growth. (a) Diagram of the lentiviral vectors used in the experiments. (b) Tumours from Kras<sup>G12D</sup> mice infected with  $2 \times 10^4$  control shRNA, *lkbkb* shRNA or *Timp1* shRNA lentiviral particles were collected at 4 months. Scale bars, 5 mm. Tumour histology, Timp-1, Ki-67 and Erk-p immunostaining were

vector<sup>18</sup>, the lentiviral vectors used here give more versatility in terms of tissue specificity, viral titre and sustained expression of genes and shRNAs to be delivered. In the Rosa26<sup>lacZ</sup> reporter mouse experiment, we found that fewer than 100 cells were transduced and gene expression was observed when  $5 \times 10^4$  lentiviral particles carrying CA2Cre were given intratracheally (Supplementary Fig. S1a). The tissue-specific promoter enables tumour initiation from a defined type of cell, which makes it possible to trace the cellular origins of different types of lung cancer.

NF-κB is a major anti-apoptotic transcription factor, and its activation has been investigated in different types of human cancer<sup>1,7</sup>. It is not surprising that inhibition of NF-κB activity impairs tumour progression. However, the role of NF-κB in cancer is apparently more than anti-apoptotic. Several groups have reported tumour inhibition when NF-κB activity was abolished in lung-cancer cells by removing p65 (ref. 3) or IKK2 (this study), or by overexpressing IκBαM (ref. 4). Contrary to expectation, none of these groups identified changes in classic anti-apoptotic gene expression when NF-κB was inhibited (Supplementary Fig. S4d). In contrast, we observed significant decrease

examined. Scale bars,  $100 \,\mu$ m. (c) Quantification of Ki-67-positive cells at multiple fields. (d) Kaplan–Meier curve showing survival of mice infected with shRNA vectors. The median survival time of control shRNA, *lkbkb* shRNA and *Timp1* shRNA mice was 129 days (n = 7), 202 days (n = 8) and 192 days (n = 5) respectively.

of cell-proliferation markers (both Ki-67 and PCNA) in IKK2<sup>-/-</sup> tumours. Consistent with this result, we identified predominantly changes of cell-cycle profiling in microarray analysis of wild-type and IKK2<sup>-/-</sup> tumour samples (Supplementary Fig. S2d and Table S1), and, impressively, the changes of cell-cycle-related genes could be rescued in the IKK2<sup>-/-</sup> tumour cells by IKK2 reconstitution (Supplementary Fig. S2e).

In the search for NF- $\kappa$ B target genes that are related to stimulating cell proliferation, we have identified *Timp1*. In both *in vitro* and *in vivo* assays, Timp-1 showed pro-proliferation activity by maintaining high Erk activation. It is worth mentioning that Erk activation has been found in mouse lung adenocarcinomas at advanced stage<sup>24,25</sup> (Supplementary Fig. S5d). Furthermore, complete Erk1 and Erk2 ablation eliminated Kras<sup>G12V</sup>-induced lung cancers<sup>37</sup>. In experiments reported here, *Timp1* or *Ikbkb* knockdown in tumour cells markedly reduced Erk phosphorylation, and hence impaired tumour proliferation (Fig. 6). These results identify an important role of NF- $\kappa$ B as well as Timp-1 in maintaining Erk activation and accelerating lung-cancer progression.





**Figure 7** Lung-cancer treatment using IKK2 inhibitor TPCA-1. (a) Schematic representation of TPCA-1 treatment. Kras<sup>G12D</sup>Rosa26<sup>luc</sup> mice were induced for lung cancer with  $2 \times 10^4$  CA2Cre-shp53 lentiviral particles. Mice were given TPCA-1 treatment (15 mg kg<sup>-1</sup> d<sup>-1</sup>) 10 weeks after the infection for 6 weeks. (**b**,**c**) The

Clearly in our model, depletion of IKK2 impaired the progression of all tumours carrying p53, p16INK4a, Pten or Lkb1 shRNA (Supplementary Fig. S6). Actually, NF-KB has been found to be aberrantly upregulated in a large number of human cancers<sup>1,8</sup>, and targeting NF-kB with small molecules for the treatment of cancer as well as other diseases has attracted much attention in the past decades. However, safety and efficacy are still of concern owing to the involvement of NF-KB in so many biological activities<sup>8,38</sup>. Our work of identifying target genes of NF-KB that mediate its role in tumour development may offer an alternative approach . For example, Timp-1 inhibition reduced tumour size and prolonged animal survival, similarly to what is seen with IKK2 inhibition in our mouse model (Fig. 6b,d), so developing Timp-1-neutralizing antibodies or inhibitors that can abolish its pro-proliferation role may be an attractive alternative to IKK2 inhibition and probably has fewer side-effects. Our study indicates that the pro-proliferation function of Timp-1 relies on the presence of CD63 (Fig. 5). Thus, future work of developing Timp-1 antibodies and inhibitors should focus on its carboxy-terminus region that is required for binding to CD63 (refs 34,39). The preclinical studies on Timp-1 inhibition will definitely benefit those lung-cancer patients carrying Kras mutations, against which there is no effective therapeutic strategy available at present. 

tumour burden was monitored by IVIS imaging on days 0, 7 and 14 of the treatment. (d) Kaplan–Meier curve showing improved survival of mice given TPCA-1 treatment. The median survival time of the untreated group and TPCA-1 group was 141 days (n = 13) and 153 days (n = 14) respectively (P = 0.0007).

#### METHODS

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

Note: Supplementary Information is available on the Nature Cell Biology website

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#### AUTHOR CONTRIBUTIONS

Y.X. designed and carried out the experiments and wrote the paper. N.Y. carried out Timp-1-related experiments and microarray analysis. M.L. examined tumour samples and interpreted tumour histology. E.K. analysed the microarray data. Y.Z.

and E.O. synthesized IKK2 inhibitors. R.J.S. provided mouse lines and gave advice on the project. I.M.V. supervised the project and wrote the paper.

#### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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#### METHODS

**Mice.** The LSL-Kras<sup>G12D</sup> mouse was originally generated in T. Jacks lab<sup>18</sup>. The IKK2<sup>fl/fl</sup> mouse was obtained from M. Pasparakis<sup>40</sup> (University of Cologne). LSL-Rosa26<sup>luc</sup>, LSL-Rosa26<sup>luc</sup>, LSL-Rosa26<sup>luc</sup> and nude/J mice were purchased from Jackson Laboratory. IKK2<sup>fl/fl</sup> and LSL-Rosa26<sup>luc</sup> were crossed with the LSL-Kras<sup>G12D</sup> mouse to obtain Kras<sup>G12D</sup> IKK2<sup>fl/fl</sup> for depletion of IKK2 in tumour cells and Kras<sup>G12D</sup> Rosa26<sup>luc</sup> for live-animal tumour imaging<sup>41</sup>. All mouse studies were carried out according to the protocols that were approved by the Institutional Animal Care and Use Committee of Salk Institute.

**Lentiviral production and mice infection.** Lentiviruses were produced as described previously<sup>42</sup>. Human CA2 promoter (-1603 to +66) was provided by T. Takeya<sup>43</sup> (Nara Institute of Science and Technology). The 2.4-kilobase rat CC10 promoter sequence<sup>44</sup> was provided by H. Nakajima (Chiba University). The shRNA sequences used were: 5'-GTACATGTGTAATAGCTCC-3' (*p53*); 5'-GAAGATACTTGAACCAGTT-3' (*lkbkb*); 5'-CCACCTTATACCAGC-GTTATA-3' (*Timp1*); 5'-GCAGTGGGATTGATCGCCATT-3' and 5'-CAGAT-GCAGAATTACCTTAAA-3' (*Cd63*); 5'-CTAACACTGGGTTATAACAA-3' (control, HPV-18 E6). Biological viral tire was determined by infecting 293-switch cells (express GFP after Cre recombination) with serial diluted viruses. Eight-week-old mice were infected intratracheally with 5 × 10<sup>4</sup> lentiviral particles (or 2 × 10<sup>4</sup> where specified) using a similar protocol as described before<sup>45</sup>.

Histology, immunofluorescence staining, ELISA and immunoblotting analysis. Mouse lung-tumour samples collected at different times were fixed with 10% formalin, paraffin-embedded and sectioned for haematoxylin and eosin staining and immunofluorescence staining. Elite ABC system (Vector labs) was applied where staining signal was weak. ELISA and immunoblotting analysis was carried out according to standard protocol. Antibodies were purchased from Millipore (SPC, 1:2,000; CC10, 1:2,000; PCNA, 1:1,000;  $\gamma$ -H2AX, 1:200), Cell Signaling (phos-Erk, total Erk, phos-Akt, total Akt, cleaved Caspaes 3, phos-ATM, all 1:1,000), Abcam (von Willebrand factor, 1:200; p19, 1:1,000), AbD Serotec (CD68, 1:200), Vector Labs (Ki-67, 1:500; p53, 1:4,000), R&D Systems (Timp-1, CD63, both 1 µg ml<sup>-1</sup>), Santa Cruz Biotechnology (Kras, 53BP1, IĸB $\alpha$ , tubulin, actin, all 1:1,000) and Biosource (IKK2, 1:1,000).

**Quantitative RT–PCR.** Total RNA isolated from homogenized tumour samples or cell lines was reverse transcribed using the Superscript III system (Invitrogen) with random primers. Quantitative PCR was carried out in triplicate using an 7900HT fast real-time PCR system with the SYBR Green method (Applied Biosystems). Results were analysed for the relative expression of mRNAs normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and cyclophilin. A complete list of primers used in the study is shown in Supplementary Table S2.

**Chemical inhibitors and IVIS imaging.** Chemical inhibitors for MEK1 (PD0325901), ATM (KU55933), PI3K (wortmannin), MMP (GM6001) were purchased from Calbiochem. FAK inhibitor 14 was purchased from Tocris Bioscience. IKK2 inhibitor TPCA-1 was synthesized according to reported procedures (PCT Int. Appl. WO/2003/029241) and fully characterized. TPCA-1 was administered in a vehicle consisting of 0.9% dimethylsulphoxide, 7% DMA and 10% Cremophor El (ref. 35). The dosage used was 15 mg kg<sup>-1</sup> intraperitoneally every day for 42 days starting on day 70 after the infection. Live-animal tumour imaging was carried out on days 0, 7 and 14 of the TPCA-1 treatment using an IVIS 100 imaging system from Caliper Lifesciences.

Stable cell lines, cell proliferation assay and xenograft model. 4A3 and 1D3 cell lines were subcloned from wild-type and IKK2<sup>-/-</sup> primary tumours respectively by serial dilution. 4A3 shRNA lines (control shRNA, *Ikbkb* shRNA and *Timp1* shRNA), 1D3 overexpression lines (GFP, IKK2, Timp-1 and Tet-on Timp-1) and *Cd63* shRNA lines were generated by lentiviral infection and fluorescence-activated cell sorting.

Xenograft experiments were done by subcutaneously transplanting 10<sup>6</sup> tumour cells in the flank region of a nude/J mouse. Tumour sizes were measured 7, 14 and 21 days after the transplantation. Recombinant Timp-1 proteins were purchased from R&D Systems. Cell proliferation was measured using Cell Proliferation ELISA (BrdU) from Roche.

**Statistics.** The statistical significance of the differences of gene expression or tumour size was evaluated using Student's unpaired two-tailed t-test. The Kaplan–Meier curves were analysed by the log–rank test.

Microarray analysis. RNA isolated from finely dissected tumour nodules and tumour cell lines (4A3 and 1D3) was applied to microarray analysis using Affymetrix Mouse Gene 1.0 ST arrays according to the manufacturer's instructions. We used Affymetrix power tools (http://www.affymetrix.com/partners\_programs/programs/ developer/tools/powertools.affx) with the parameters '-a mas5-bg, med-norm, pm-only, med-polish' to normalize and summarize probe sets. This analysis was carried out for the Sweet-Cordero data set<sup>46</sup>, Affymetrix sample data (http://www. affymetrix.com/support/technical/sample\_data/gene\_1\_0\_array\_data.affx) and our microarrays. Significantly expressed genes were called with Cybert-T (ref. 47) using thresholds of  $\ln(p) < 0.05$  and PPDE(< p) > 0.5. Genes were mapped across array platforms (U74av2 and Mogene ST 1.0) using slightly modified annotation files from the Molecular Signatures Database<sup>48,49</sup> (MSigDB) to correct for outdated or ambiguous names. These genes were then analysed with Gene Set Enrichment Analysis (GSEA) permutating across gene sets using c2.cp.v3 and c5.bp.v3 gene sets from MSigDB. In addition, gene sets specific for Kras were derived using original probe-set annotations from Sweet-Cordero. NF-KB target genes (total 413) were gathered from a list maintained by Thomas Gilmore (http://www. bu.edu/nf-kb/gene-resources/target-genes/). Heat maps were generated using the Matrix2png program<sup>50</sup>.

**Accession numbers.** Raw data of microarray analysis have been deposited to the NCBI Gene Expression Omnibus with accession number GSE30049.

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**Figure S1** CA2Cre and CC10Cre lentiviruses initiated lung adenoma by targeting alveolar epithelial cells. (a) Rosa26<sup>lacZ</sup> reporter mice were infected intra-tracheally with CA2Cre or CC10Cre lentiviruses.  $\beta$ -galactosidase expression was examined by X-gal staining 14 days after the infection. Cells infected with lentiviruses and had Cre expression were stained blue (arrows). (b) Kras<sup>G12D</sup> mice were infected with CA2Cre or CC10Cre lentiviruses. Lung tumours were examined 2 (top) or 4 (bottom) months after the infection.

Lesions were found only in the alveoli. In contrast, in a small cell lung cancer model (SCLC, Xia et al. unpublished), lesions were found exclusively in the bronchioles. (c) Kras<sup>G12D</sup> mice were infected with CA2Cre-shp53-shp16 or CC10Cre-shp53-shp16 lentiviruses. Tumours were typical adenocarcinomas with SPC positive and CC10 negative staining. Scale bars, 100  $\mu$ m. These two groups of mice didn't show big difference in median survival time, n=12 and 6 respectively (d).



**Figure S2** Reduced proliferation was found in IKK2-/- tumour. (a) Ki-67 staining was done on tumour sections of different stages (from top to bottom: early to late stages). Compared to WT counterparts, IKK2-/- tumours had lower Ki-67 positive percentage. Scale bars,  $100 \ \mu m$ . (b) BrdU uptake was measured in a collection of cell lines derived from WT and IKK2-/- tumours. The results indicated lower proliferation rate in IKK2-/- cells. (c) Kras signature of WT and IKK2-/- tumours. Comparisons of normal (N) versus tumour (T) samples of up- or down- regulated KrasLA genes as defined by Sweet-Cordero are shown by heat maps. Red indicates higher expression levels than blue. Each row is one gene and each column is one array. Rows are normalized to zero with a variance of one for display purposes. Genes are first segregated by their presence in the data sets, and then are ordered by decreasing significance as determined by GSEA. Missing values are either due to lack of mapping or in most instances insignificant differential gene

expression, are represented as black and values close to zero are in white. Normalized Enrichment Scores (NES) from GSEA are shown on the top. Positive numbers indicate enrichment of gene set in normal lung tissue while negative numbers indicate enrichment in tumour. Overall, scores of WT and IKK2-/- tumours are very close, indicating that both tumours are lung adenocarcinomas with Kras signature. (d) Differential gene expression between WT and IKK2-/- tumours. Comparison of WT (4A3) and IKK2-/-(1D3) tumour cell lines of NF-κB genes as defined by Gilmore. Total 103 genes are up-regulated in WT cells. NES for NF-κB target genes is 2.41. (e) Nineteen cell-cycle related genes that showed changes in the microarray analysis (both tumours and cells) were picked for further verification. RNAs from WT (4A3), IKK2-/- (1D3) and IKK2 reconstitution (1D3-IKK2) cells were analyzed. IKK2 reconstitution in 1D3 cells restored the gene expression pattern. Results are shown as average of two biological replicates.



**Figure S3** Kras<sup>G12D</sup> induced DNA damage response and NF- $\kappa$ B activation. (a) Kras<sup>G12D</sup> was expressed under the regulation of tetracycline responsible elements (TRE) in U2OS cells for different time. NF- $\kappa$ B activity was measured by luciferase reporter assay. Error bars, s.d. (*n*=4 biological replicates.) (b) DNA damage response was

shown by immunostaining of ATM-p, phos- $\gamma$ H2A and 53BP1 foci. (c) Quantification of 53BP1 foci positive cells at different time point. Error bars, s.d. (*n*=10 random fields.) (d) Immunostaining of 53BP1 in early lung tumour lesion induced by CA2Cre-shp53 lentivirus. Scale bars, 10 µm.



**Figure S4** Cytokine array analysis of WT and IKK2-/- tumours identified Timp-1. (a) Primary tumours of similar size were precisely dissected and whole lysates were used for cytokine array. Visible dots corresponding to different cytokines were quantified with Image J. (**b,c**) Those genes showed difference in cytokine array were further verified by quantitative RT-PCR with more tumour samples. Three genes were confirmed lower in the IKK2-/- tumours (b), while other genes showed no statistical difference (c). (d) Anti-apoptotic genes regulated by NF- $\kappa$ B didn't show difference between WT and IKK2-/- tumours. (e) Timp-1 expression was examined by immunohistochemistry in WT and IKK2-/- tumours at different stages (from left to right: early to late stages). Scale bars, 100 µm.



**Figure S5** Timp-1 expression is required for maintaining high Erk activation. (**a,b**) TRE-Kras<sup>G12D</sup> expression in U2OS cells induced Timp-1 expression via NF- $\kappa$ B, which was abolished by IKK2 inhibitor TPCA-1 or I $\kappa$ B $\alpha$ M super repressor. (**c**) Erk but not Akt phosphorylation was attenuated by shTimp-1, indicating the role of IKK2/Timp-1 in maintaining high Erk activation downstream of Kras. (**d**) Erk phosphorylation was examined in WT and IKK2-/- tumours at 11 weeks and endpoint. In 11-week WT samples, Erk-p was found in mosaic pattern. Notice that Erk-p was visible even in some early lesions. In contrast, 11-week IKK2-/- samples had very little positive staining. In endpoint samples, IKK2-/- tumours had slightly lower staining than WT, however very strong staining was also found in some tumours. These tumours might have obtained certain mutations that could bypass the requirement of IKK2/Timp-1 for maintaining high Erk activation. Scale bars, 100  $\mu$ m. (e) Stable cell lines derived from IKK2-/- tumours showed lower Timp-1 expression and Erk phosphorylation, compared to WT counterparts.



**Figure S6** IKK2 depletion in tumour cells prolonged median survival time. Tumours were induced in Kras<sup>G12D</sup> mice by lentiviral vectors carrying different mutations. In all cases, IKK2 depletion in tumour

cells increased median survival time, indicating the role of NF- $\kappa B$  and IKK2 in tumour progression is not limited to tumours with certain mutations.



Figure S7 Full scans of the immunoblotting analysis in Figure 4 and Figure 5. Cropped regions are indicated by boxes.

#### Supplementary Table legends

Table S1 Normalized Enrichment Scores (NES) comparing WT and IKK2-/- tumours. Positive numbers indicate enrichment of gene set in WT tumours, while negative numbers indicate enrichment in IKK2-/- tumours. Cell cycle related terms are the major changes, which are consistent with the high proliferation rate found in WT tumours.

Table S2 Full list of primers used in quantitative RT-PCR.