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

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Abstract

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 allows generation of non-small cell lung cancer from less than one hundred alveolar epithelial cells, and investigated the role of IKK2 and NF-κB in lung cancer development. IKK2 depletion in tumour cells significantly attenuated tumour proliferation and significantly prolonged mouse survival. We identified Timp-1, one of the NF-κB target genes, as a key mediator for tumour growth. Activation of Erk signalling pathway and cell proliferation requires Timp-1 and its receptor CD63. Knockdown of either IKK2 or Timp-1 by shRNAs 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.

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Author Contributions Y.X. designed and performed the experiments and wrote the paper. N.Y. performed Timp-1 related experiments and microarray analysis. M.L. examined tumour samples and interpreted tumour histology. E.K. analyzed the microarray data. Y.Z. and E.O. synthesized IKK2 inhibitors. R.J.S. provided mouse lines and gave advices on the project. I.M.V. supervised the project and wrote the paper.

Introduction

Inflammation and NF-κB activation have long been linked to cancer development[1]. Recent studies have indicated the involvement of NF-κB in Kras mutation induced lung adenocarcinoma[2-4]. 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 smoke induced lung tumour promotion and malignant cell proliferation in $Kras^{LA2}$ model[5]. A very recent study has also indicated the involvement of NF-κB in EGFR tyrosine kinase inhibitor resistance[6]. IKK2, as the essential kinase for NF-κB activation, is thus thought to be a good candidate for drug design[7-9].

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

We used lentiviral vectors for establishing tumours in mice for the following reasons: 1) lentiviruses infect almost any type of cells, and transgene expression can be controlled by a tissue-specific promoter, which allows 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 shRNAs against tumour suppressors, and bypass the requirement of numerous conventional genetic crossings; 3) viral titres can be controlled so as to infect only a few cells, in order to more faithfully recapitulate human cancer initiation[17]. Moreover, in principle, a lentiviral vector carrying certain oncogenes and shRNAs against tumour suppressors is sufficient to initiate tumourigenesis 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-$Kras^{G12D}$ mice[18], for biosafety considerations.

In the present study, we have investigated the underlying mechanisms involved in NF-κB promoting lung cancer cell proliferation by knocking out IKK2 specifically in tumour cells. We found that down-regulation of Timp-1, one of the NF-κB target genes, contributed to slower tumour cell proliferation, consistent with the fact that NF-κB and Timp-1 are usually highly expressed in advanced lung cancer patients with poor prognosis[19, 20]. Furthermore, knocking down of IKK2 or Timp-1 by shRNAs in tumour cells significantly reduced Erk activation and cell proliferation, thereby attenuated tumour progression in our mouse model.

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 Figure 1a, a typical lenti-vector for this purpose has a combination of tissue specific oncogene (or Cre) and several shRNAs to knockdown tumour suppressor genes. We successfully induced lung

adenoma and adenocarcinoma in the LSL-Kras^{G12D} mouse with carbonic anhydrase II (CA2) driven Cre lentiviral vector (Supplementary Fig. S1b). Notably, the result from CA2-Cre virus infection in LSL-Rosa26^{lacZ} reporter mice indicated that the CA2 promoter is expressed mainly in alveolar epithelial cells[21] 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 (i-iii) shows the evolution of tumour lesion from an atypical adenomatous hyperplasia (AAH), to small adenoma and to advanced adenocarcinoma[22]. Twelve weeks after infection, majority of the tumour lesions reached Grade 3 and Grade 4, showing pleomorphic nuclei and aberrant mitosis (Fig. 1d). More importantly, massive stromal cell infiltration was found in ~20% of the advanced tumours, which recapitulates well the pathology observed in human malignancies (Fig. 1e,f). All tumours we have analyzed in CA2Cre-shp53 lentiviral model were typical adenocarcinomas with SPC (pro-surfactant protein C) positive and CC10 (Clara-cell specific antigen) negative staining patterns (Fig. 1g,h). The Ki-67 positive rate is 5-20% in these tumours, which is similar to what has been reported in human adenocarcinomas[23] (Fig. 1i).

IKK2 knockout in tumour cells impairs tumour proliferation

Given that the NF-κB pathway plays an important role in tumour development and that IKK2 is the seminal kinase responsible for NF-κB pathway activation, we were particularly interested in exploring the therapeutic potential of IKK2 inhibition in non-small cell lung cancers. We therefore crossed IKK2$^{fl/fl}$ and Kras^{G12D} mice so that we could simultaneously activate Kras^{G12D} to initiate tumour formation and inactivate IKK2, upon transduction by Cre lentiviral vectors. Compared to Kras^{G12D}IKK2^{WT} mice (WT), Kras^{G12D}IKK2$^{fl/fl}$ mice (IKK2-/-) developed lung tumours with much longer latency (162 days median survival time vs. 114 days when infected with CA2Cre-shp53 vector, Fig. 2a). At 100 days post lentiviral infection, the average tumour burden in IKK2-/- mice was only about 1/3-1/2 of that in WT mice (Fig. 2b). Although IKK2-/- mice had less tumour burden than WT mice when collected at an early time-point, they developed comparable tumours at the endpoint (Fig. 2b,c). The tumours found in both genotypes of mice were adenocarcinomas, exhibiting SPC + and CC10− staining (Fig. 2d,e). Similar percentages of the tumour areas were scored positive for Erk phosphorylation (Erk-p), indicating their advanced tumour stage[24, 25] (Fig. 2f).

Since in various cancers, inflammatory cell infiltration stimulates tumour growth, we hypothesized that IKK2-deficient tumours might produce less chemokines and attract less inflammatory cells. We therefore analyzed inflammatory cell infiltration in the bronchioalveolar lavage (BAL) at different time-points after tumour initiation. Macrophage counts from WT mice increased significantly during the 3 months of tumour development. IKK2-/- mice showed less macrophage infiltration for the first 3 months, however, the cell numbers were similar to those found in WT mice at the endpoint, suggesting that inflammatory cell infiltration was only correlated with the size of tumour burden instead of IKK2 status of the tumour (Fig. 2g,k). 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 due to reduced macrophage chemotaxis and inflammation.

To investigate the real contribution of IKK2 and NF-κB pathway in this lung cancer model, we examined other possible roles of NF-κB, such as pro-proliferation, anti-apoptosis and pro-angiogenesis. We were unable to find any significant differences between IKK2-/- and WT 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 WT tumours (12% Ki-67 positive in WT tumours vs. 6% in IKK2-/- tumours at 11-week, and 8% in WT vs. 2% in IKK2-/- at endpoint, Fig. 2i,j,l). Low Ki-67 staining was found throughout the whole period of tumour progression in IKK2-/- mice (Supplementary Fig. S2a). Similarly, cell lines derived from IKK2-/-tumours had less BrdU incorporation in cell culture (Supplementary Fig. S2b). The microarray data further support this notion because majority of the differences between WT and IKK2-/- 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-κB activation in lung cancer

Constitutive NF-κB activation has been reported in various tumours, including different forms of lung cancer[26]. Therefore, we next examined NF-κB activity in our primary lung tumour samples. Nuclear extracts from WT tumours showed high NF-κB binding activity, while IKK2-/- tumours remained at a basal level. Super-shift using specific antibodies confirmed the bands as canonical NF-κB 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-κB activation in these cells. Using a tetracycline responsible elements (TRE) regulated Kras expressing system, we detected high NF-κB activity within 3-4 days of $Kras^{G12D}$ expression (Fig. S3a). Kras expression up-regulated PI3K-Akt and MEK-Erk pathway, as well as DNA damage response, which all activated the NF-κB pathway[27, 28] (Fig. 3b and Supplementary Fig. S3b-d). In addition, inhibitors of MEK, PI3K and ATM all suppressed the NF-κB reporter activity induced by Kras (Fig. 3c).

As anticipated, IKK2 was required for the NF-κB activation by Kras, since IKK2 shRNA knockdown as well as the small molecule inhibitor TPCA-1 significantly reduced NF-κB activity. Furthermore, p53 shRNA knockdown resulted in higher NF-κB activity (Fig. 3d), consistent with a previous report[4]. All these results supported the idea that oncogenic Kras, together with p53 deficiency, contributed to NF-κB activation in these tumour cells.

Timp-1 is one of the mediators for NF-κB induced tumour proliferation

We next sought to elucidate how NF-κB contributed to tumour proliferation. Multiple NF-κB regulated cytokines stimulate tumour cell proliferation through autocrine and paracrine pathways[29]. We thus investigated the expression of a set of 40 cytokines in WT and $IKK2^{-/-}$

tumour lysates using an antibody array, and further verified by using quantitative RT-PCR (Supplementary Fig. S4a-c).

Timp-1 (Tissue inhibitor of metalloproteinases-1) was identified among the cytokines that were significantly down-regulated in the IKK2$^{-/-}$ 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 they might be from tumour-infiltrating inflammatory cells. Interestingly, Timp-1 has been reported to be expressed in different human tumours, including lung adenocarcinomas[30]. Although the role of Timp-1 in cancer growth and metastasis is controversial[31], high Timp-1 expression levels are always linked to poor prognoses in lung cancer patients[19, 20]. The Timp-1 gene promoter has putative NF-κB binding sites[32], so its expression could be suppressed by the IκBαM super-repressor, or IKK2 shRNA, in WT tumour cells (Fig. 3e). The difference of Timp-1 expression in primary WT and IKK2$^{-/-}$ tumours was then further verified at mRNA and protein levels (Fig. 3f-h and Supplementary Fig. S4e).

These observations led to the question: does high Timp-1 expression in WT tumours contribute 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 WT and IKK2-/- primary tumours, respectively. WT line 4A3 formed a much larger tumour mass than did IKK2$^{-/-}$ line 1D3, within 3 weeks after transplantation. Knocking down either IKK2 or Timp-1 expression in 4A3 reduced tumour size considerably (Fig. 3i). While ectopic expression of IKK2 in line 1D3 completely rescued its proliferation as judged by the size of the tumours (Fig. 3j), surprisingly, ectopic expression of Timp-1 did not significantly influence tumour size. It thus appears that IKK2 and NF-κB play an essential role in tumour cell proliferation, whereas Timp-1 may be a necessary but not sufficient mediator of this effect.

Timp-1 stimulates cell proliferation through Erk pathway

There is increasing evidence which suggests that Timp-1 has paradoxical effects on tumour growth[31, 33]. When Kras^{G12D} was expressed in U2OS cells, significant activation of Erk, Akt and NF-κB pathways was observed, together with up-regulation of Timp-1 expression (Fig. 4a and Supplementary Fig. S5a,b). Given that the Erk pathway is critical for cell proliferation, we asked the question if Timp-1 expression stimulates cell proliferation in a positive feedback loop through Erk activation. Interestingly, Erk but not Akt phosphorylation was severely impaired when IKK2 was knocked down in U2OS cells (Fig. 4a). Moreover, doxycycline-induced expression of Timp-1 completely rescued the defect of Erk phosphorylation in IKK2 knockdown cells, which had low endogenous Timp-1 induction due to lack of NF-κB activation (Fig. 4a). Similarly, an Erk phosphorylation defect was seen in Timp-1 knockdown cells (Fig. S5c). These results point to a positive feedback loop: Kras^{G12D}—Erk-p—NF-κB—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 Timp-1 expression and slow proliferation. When ectopic Timp-1 expression in these cells

was turned on by addition of doxycycline, 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 Erk pathway mediates the pro-proliferation effect of Timp-1 (Fig. 4c).

Indeed, there was higher Timp-1 expression in WT tumours than in IKK2-/- tumours at all stages of tumour development, due to NF-κB activation (Supplementary Fig. S4e), correlating with the rapid proliferation and high percentage of Erk-p staining observed in WT tumours, compared to IKK2-/- tumours (Fig. 4d). Although majority of the IKK2-/- tumours at endpoint showed elevated Erk-p staining, as observed with WT 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-/- tumours, due to the low Erk activation (Fig. 4e, tumours #3, 4 and 5 and Supplementary Fig. S5e). Overall, Erk phosphorylation was found in ~30% of WT tumour lesions, as compared to less than 10% in IKK2-/- tumours. These results clearly indicate a critical role for IKK2 and Timp-1 in maintaining high proliferation and advanced pathology in lung cancers.

Activation of Erk pathway by Timp-1 requires CD63

Since Timp-1 was originally identified as a tissue inhibitor of metalloproteinases (MMPs), we first asked the question if this inhibitory property is responsible for the its pro-proliferation activity. Addition of recombinant Timp-1 protein (rTimp-1) to IKK2-/- tumour cells precipitously increased both FAK and Erk phosphorylation, while a 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[34]. We used two shRNAs to knockdown CD63 expression in the IKK2-/- tumour cells (Fig. 5c) and checked if CD63 knockdown would eliminate the effect of Timp-1 treatment. As shown in Figure 5d and 5e, Timp-1 induced Erk phosphorylation and cell proliferation were impaired in shCD63-1# and shCD63-2# cells, but not in shCtrl cells. Finally, FAK inhibitor (FAK I-14) successfully repressed Erk phosphorylation induced by ectopic Timp-1 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.

Timp-1 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 which included shRNAs against IKK2 or Timp-1 (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 shIKK2 and shTimp-1 vectors (Fig.

6b). Immunostaining confirmed that shIKK2 and shTimp-1 tumours had less Timp-1 expression, less Erk phosphorylation and lower Ki-67 percentage as compared to controls (Fig. 6b,c). Mice receiving either shIKK2 or shTimp-1 vectors, consistently survived much longer than those receiving control vectors (202 days and 192 days vs. 129 days median survival time, Fig. 6d). All these results strongly support the possibility 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[35, 36] in treating lung cancer in our mouse model. We induced tumours in $Kras^{G12D}Rosa26^{luc}$ 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 compared to vehicle control (Fig. 7b,c). We were not able to continue treatment during the whole tumour development, due to the toxicity of chronic NF-κB 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 haven't been able to test Timp-1 neutralizing antibodies in treating primary mouse lung cancer due 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 to the widely used adeno-Cre vector[18], 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 $Rosa26^{lacZ}$ reporter mouse experiment, we found that less than 100 cells were transduced and gene expression was observed when 5×10^4 lentiviral particles carrying CA2Cre were given intra-tracheally (Supplementary Fig. S1a). The tissue-specific promoter allows tumour initiation from a defined type of cells, 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[1, 7]. 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)[3], IKK2 (this study), or by over-expressing IκBαM[4]. Contrary to expectation, none of these groups identified changes in classic anti-apoptotic genes expression when NF-κB was inhibited (Supplementary Fig. S4d). In contrast, we observed significant decrease of cell proliferation markers (both Ki-67 and PCNA) in IKK2-/- tumours. Consistent with this result, we identified predominantly changes of cell cycle profiling in microarray analysis of WT and IKK2-/- tumours samples (Supplementary Fig. S2d and Table S1), and impressively, the changes of cell cycle-related genes could be rescued in the IKK2-/- tumour cells by IKK2 reconstitution (Supplementary Fig. S2e). In the search for NF-κB target genes that are related to stimulating cell proliferation, we have identified Timp-1. 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[24, 25] (Supplementary Fig. S5d). Furthermore, complete Erk1 and Erk2 ablation eliminated $Kras^{G12V}$ induced lung cancers[37]. In experiments reported here, Timp-1 or IKK2 knockdown in tumour cells dramatically reduced Erk phosphorylation, and hence impaired tumour proliferation (Fig. 6). These results identify an important role of NF-κB as well as Timp-1 in maintaining Erk activation and accelerating lung cancer progression.

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-κB has been found aberrantly up-regulated in large number of human cancers[1, 8], and targeting NF-κB with small molecules for the treatment of cancer as well as other diseases has attracted much attention in the last decades. However, safety and efficacy are still of concern due to the involvement of NF-κB in so many biological activities[8, 38]. Our work of identifying NF-κB target genes that mediate its role in tumour development may offer an alternate approach. For example, Timp-1 inhibition reduced tumour size and prolonged animal survival similarly to what 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 likely has fewer side effects. Our study indicates 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 C-terminus region that is required for binding to CD63 (ref. 34, 39)[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 currently available.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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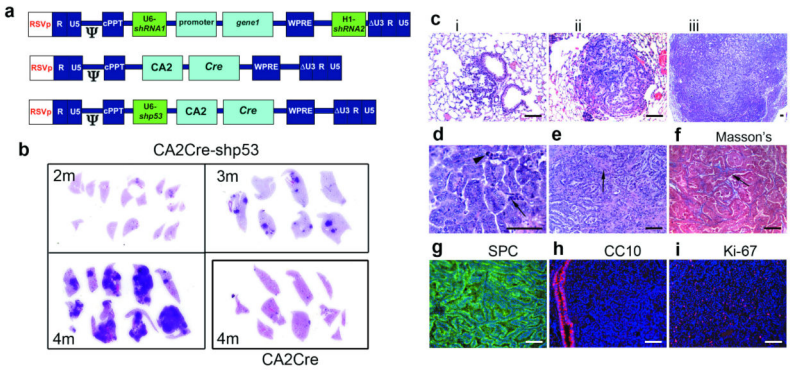

Figure 1.

Lentiviral vector mediated mouse lung cancer model. (**a**) Diagram of lentiviral vector designing. Oncogene or Cre is controlled by tissue-specific promoter and shRNAs are driven by U6 or H1 promoter. CA2, carbonic anhydrase 2 promoter. (**b**) $Kras^{G12D}$ mice were infected intra-tracheally with CA2Cre or CA2Cre-shp53 lentivirus. Mice were harvested at 2, 3 or 4 months after the infection. Lung sections showed different tumour burdens. (**c**) Histology of atypical adenomatous hyperplasia (AAH, i), adenoma (ii) and adenocarcinoma (iii). (**d**) Pleomorphic nuclei (arrow head) and aberrant mitosis (arrow) were found in advanced adenocarcinoma. (**e,f**) Stromal cell infiltration (arrows) identified by H&E staining and Masson's trichrome staining. (**g-i**) Tumour sections stained with SPC, CC10 and Ki-67 antibodies respectively. Scale bars, 100 μm.

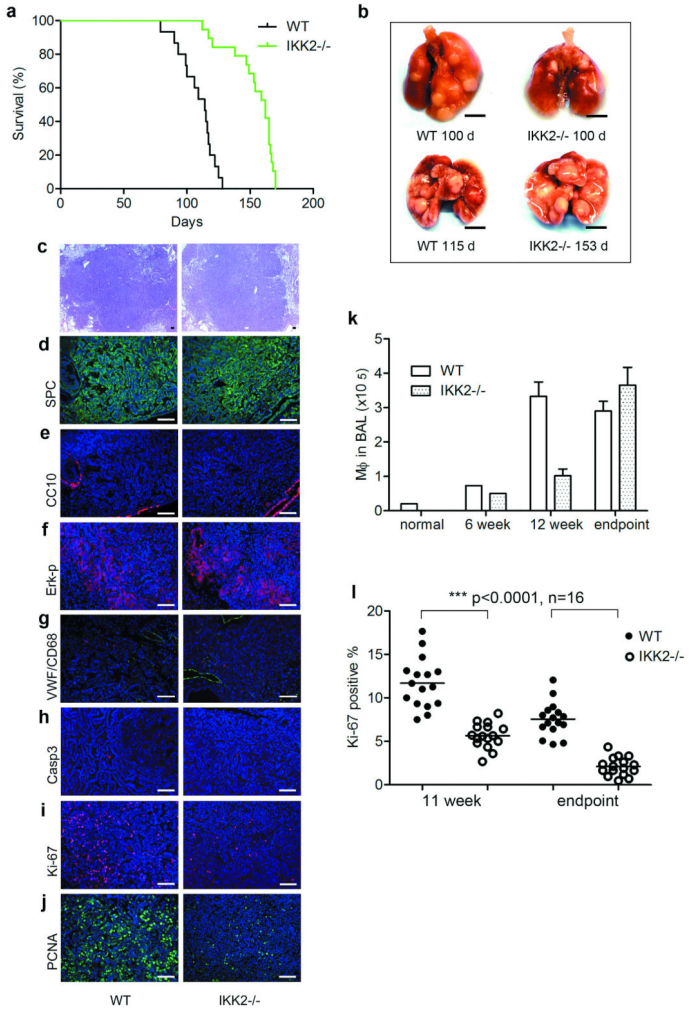

Figure 2.

IKK2 depletion in $Kras^{G12D}$ tumour cells prolonged mice survival. (**a**) Kaplan-Meier curve showing $Kras^{G12D}$ (WT) and $Kras^{G12D}IKK2^{fl/fl}$ (IKK2-/-) mice survival after CA2Cre-shp53 lentivirus infection. Median survival time of the two groups was 114 days (WT, n=15) and 162 days (IKK2-/-, n=19) respectively (p<0.0001). (**b**) Representative whole lungs collected from WT and IKK2-/- mice at indicated time after infection. Scale bars, 5 mm. (**c-j**) Tumours from WT and IKK2-/- mice collected at endpoint were stained with H&E, anti-SPC, CC10, Erk-p, von Willebrand Factor (green), CD68 (red), cleaved caspase 3, Ki-67 and PCNA antibodies respectively. Scale bars, 100 μm. Quantification of Ki-67 positive cells at multiple fields is shown in (**l**). (**k**) Bronchioalveolar lavage (BAL) macrophage counts were analyzed from WT and IKK2-/- mice at indicated time after infection. Error bars, s.d. (Normal mice, *n*=2; 6 week, *n*=1; 12 week, *n*=4; endpoint, *n*=3.)

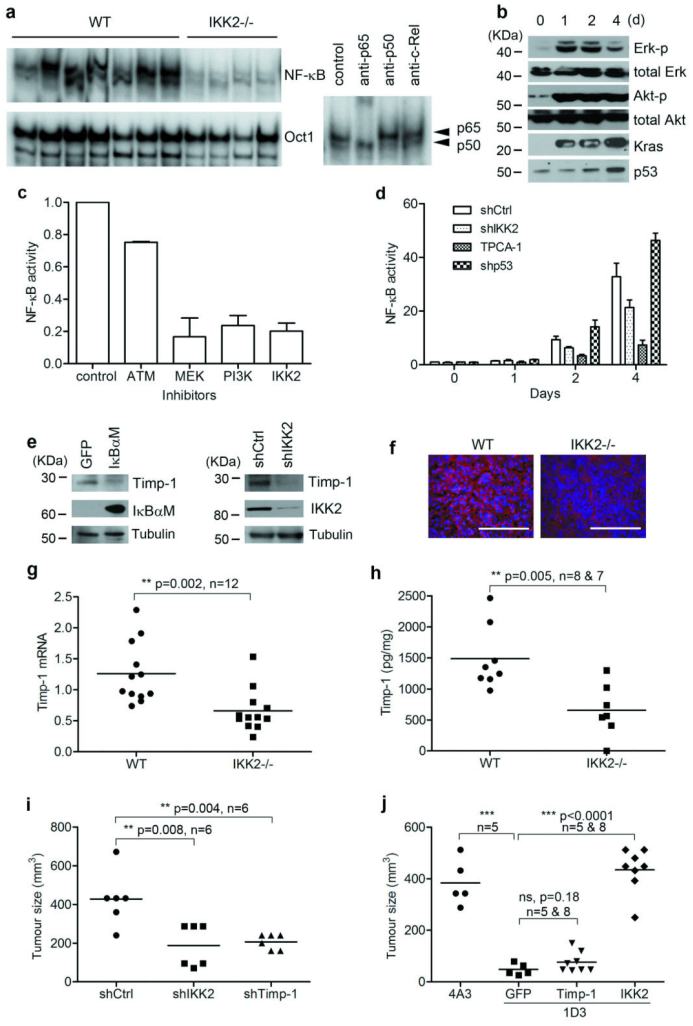

Figure 3.

Timp-1 expression induced by NF-κB activation in Kras^{G12D} tumours. (**a**) Nuclear extracts from primary tumour lesions were analyzed by EMSA for NF-κB binding. Antibodies were used in super-shift to specify the composition of NF-κB dimers. (**b**) Kras^{G12D} was expressed under the regulation of tetracycline responsible elements (TRE) in U2OS cells. Cell lysates were collected at different time-points after adding doxycycline and subjected to immunoblotting analysis. (**c**) Exogenous Kras^{G12D} 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^{G12D} was down- or up-regulated by different shRNAs or IKK2 inhibitor TPCA-1. Error bars, s.d. (**c**,**d**, *n*=4 biological replicates). (**e**) Timp-1 expression was reduced in WT tumour cells stably infected with IκBαM super repressor (GFP fusion) or IKK2 shRNA virus. (**f**) Immunostaining of Timp-1 in WT and IKK2-/- tumours. Scale bars, 100 μm. (**g**,**h**) Timp-1 mRNA and protein levels were examined by quantitative RT-PCR and ELISA in individual primary tumours collected from WT and IKK2-/- mice. (**i**) shRNA knockdown of IKK2 or Timp-1 reduced 4A3 (WT) cell proliferation in nude mice xenograft model. (**j**) 1D3

(IKK2-/-) cell proliferation in xenograft model was rescued by exogenous IKK2 expression, but not Timp-1.

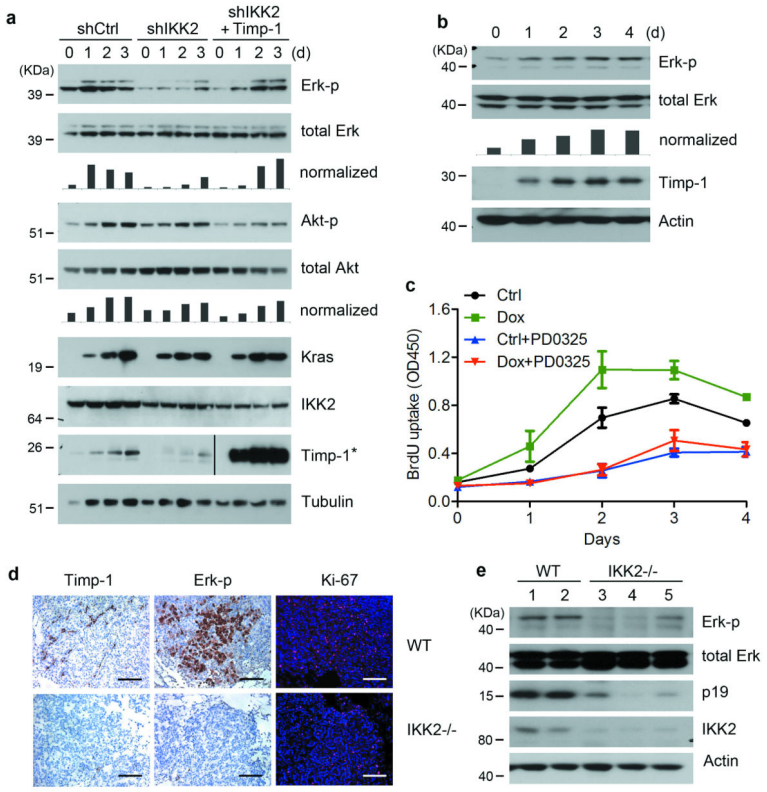

Figure 4.

IKK2 and Timp-1 are required to maintain high Erk activation. (**a**) TRE-Kras^{G12D} was expressed in U2OS cells stably infected with shCtrl, shIKK2 or shIKK2 plus TRE-Timp-1. Erk and Akt phosphorylation was examined by immunoblotting analysis and quantified with Image J. *Dox-induced Timp-1 expression was shown at a shorter exposure time. (**b,c**) Dox-induced Timp-1 expression in 1D3 (IKK2-/-) cells up-regulated Erk phosphorylation and BrdU incorporation. The increase of BrdU incorporation was abolished by adding MEK inhibitor PD0325901. Error bars, s.d. (*n*=3 biological replicates.) (**d**) Tumour samples from WT and IKK2-/- mice collected at 11 weeks were analyzed for Timp-1, Erk-p and Ki-67 staining. Scale bars, 100 μm. (**e**) Individual tumour lesions collected at 11 weeks (1 and 2, WT; 3, 4 and 5, IKK2-/-) were lysed and protein expression was analyzed by immunoblotting.

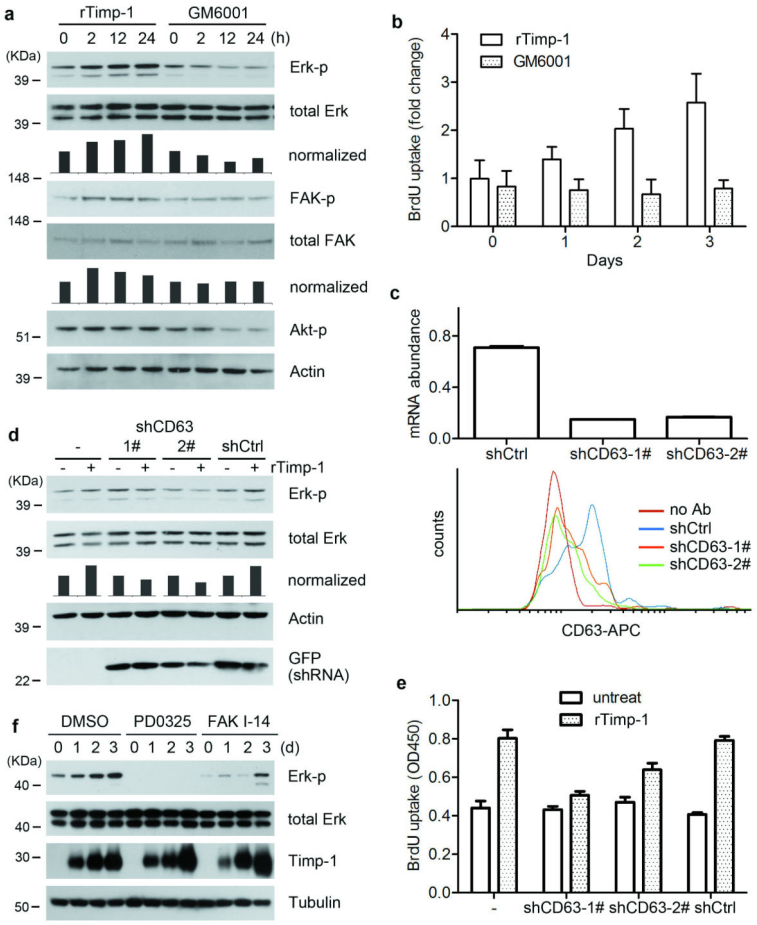

Figure 5.

Activation of FAK-Erk pathway and cell proliferation requires Timp-1 and CD63. (**a**) IKK2-/- tumour cells were treated with 0.5 μg/ml recombinant Timp-1 (rTimp-1) or 10 μM MMP inhibitor GM6001 for different time. Erk, Akt and FAK phosphorylation was examined by immunoblotting and quantified with Image J. (**b**) Cell proliferation was analyzed by BrdU incorporation assay. Error bars, s.d. (*n*=3 biological replicates.) (**c**) CD63 mRNA levels were examined in cells infected with control shRNA (shCtrl) or CD63 shRNAs (shCD63-1# and -2#) by quantitative RT-PCR (top). Error bars, s.d. (*n*=4 biological replicates.) CD63 protein levels were examined by flow cytometry (bottom). (**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 performed 2 days after rTimp-1 treatment. Error bars, s.d. (*n*=3 biological replicates.) (**f**) Timp-1 expression was induced by Dox in the cells for 1, 2 or 3 days, and the cells were treated PD0325901 or FAK I-14 for 4 h before the collection. Phosphorylation of Erk was examined by immunoblotting.

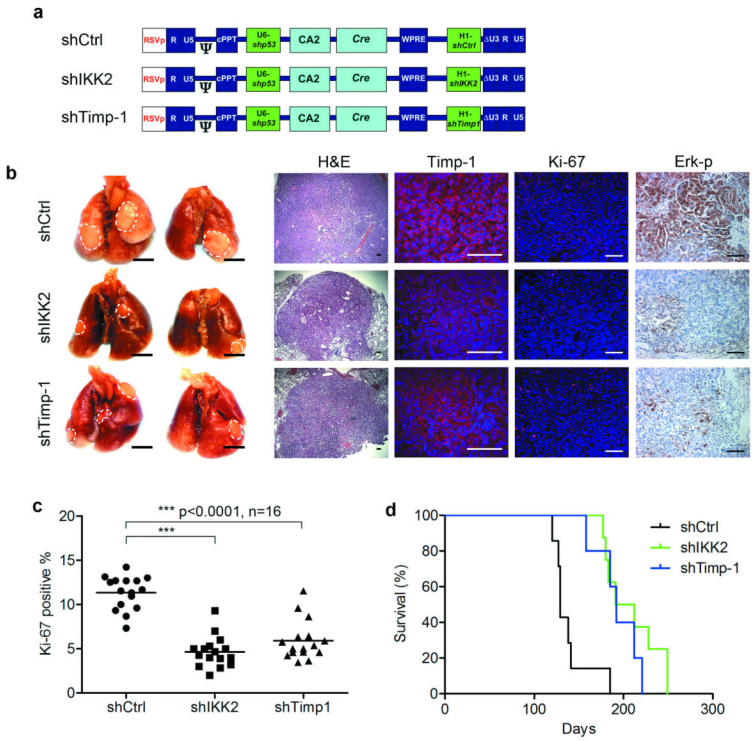

Figure 6.

Knockdown of IKK2 or Timp-1 attenuated tumour growth. (**a**) Diagram of the lentiviral vectors used in the experiments. (**b**) $Kras^{G12D}$ mice infected with 2×10^4 shCtrl, shIKK2 or shTimp-1 lentiviral particles were collected at 4 months. Scale bars, 5 mm. Tumour histology, Timp-1, Ki-67 and Erk-p immunostaining were examined. Scale bars, 100 μm. Quantification of Ki-67 positive cells at multiple fields is shown in (**c**). (**d**) Kaplan-Meier curve showing survival of mice infected with shRNA vectors. The median survival time of shCtrl, shIKK2 and shTimp-1 mice was 129 days (n=7), 202 days (n=8) and 192 days (n=5) respectively.

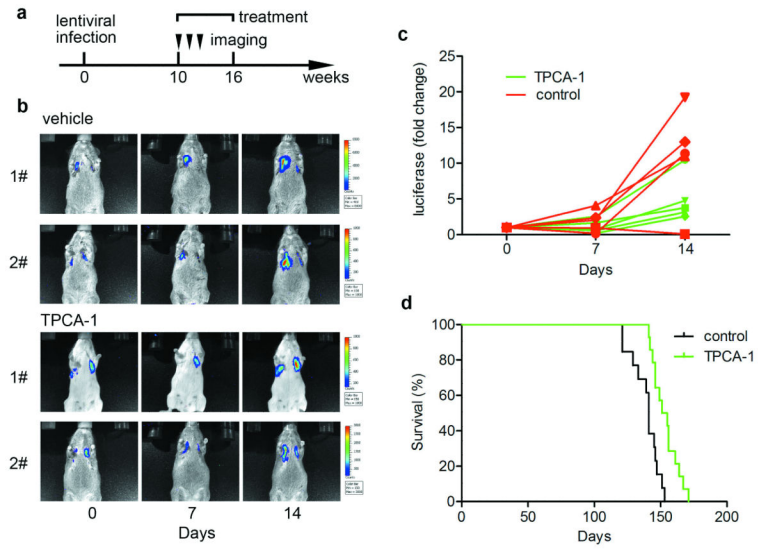

Figure 7.

Lung cancer treatment using IKK2 inhibitor TPCA-1. (**a**) Scheme of TPCA-1 treatment. $Kras^{G12D}Rosa26^{luc}$ mice were induced for lung cancer with 2×10^4 CA2Cre-shp53 lentiviral particles. Mice were given TPCA-1 treatment (15 mg/kg/day) 10 weeks after the infection for 6 weeks. (**b,c**) Tumour burden was monitored by IVIS imaging on day 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 untreated group and TPCA-1 group was 141 days (n=13) and 153 days (n=14) respectively (p=0.0007).