IKKα promotes lung adenocarcinoma growth through activation of ERK signaling via DARPP-32-mediated inhibition of PP1 activity

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Abstract

Non–small cell lung cancer (NSCLC) accounts for 80-85% cases of lung cancer cases. Diagnosis at advanced stages is common, after which therapy-refractory disease progression frequently occurs. Therefore, a better understanding of the molecular mechanisms that control NSCLC progression is necessary to develop new therapies. Overexpression of IκB kinase α (IKKα) in NSCLC correlates with poor patient survival. IKKα is an NF-κB-activating kinase that is important in cell survival and differentiation, but its regulation of oncogenic signaling is not well understood. We recently demonstrated that IKKα promotes NSCLC cell migration by physically interacting with dopamine- and cyclic AMP-regulated phosphoprotein, Mr 32000 (DARPP-32), and its truncated splice variant, t-DARPP. Here, we show that IKKα phosphorylates DARPP-32 at threonine 34, resulting in DARPP-32-mediated inhibition of protein phosphatase 1 (PP1), subsequent PP1-mediated dephosphorylation of ERK, and activation of ERK signaling to promote lung oncogenesis. Correspondingly, DARPP-32 ablation in human lung adenocarcinoma cells reduced their anchorage-independent growth in soft agar. Mice challenged with IKKα-ablated HCC827 cells exhibited less lung tumor growth than mice orthotopically administered control HCC827 cells. Our findings suggest that IKKα drives NSCLC growth through activation of ERK signaling via DARPP-32-mediated inhibition of PP1 activity.

Introduction

Lung cancer is the second most frequently diagnosed cancer in both men and women and the leading cause of cancer-related deaths worldwide, with an estimated 2.2 million new cases and 1.8 million deaths per year[1,2]. Non–small cell lung cancer (NSCLC) is the most common type of lung cancer and accounts for 85% of total diagnoses[3]. Substantial improvements in the application of predictive biomarkers, smoking cessation, and modification of current treatment paradigms have led to notable progress in managing NSCLC and have transformed outcomes for many patients[4-6]. However, the 5-year relative survival of lung cancer patients is dismal (22.9%) due to the emergence of therapy-resistant disease and metastasis[7,8]. Therefore, improving the general understanding of disease biology, implementing screening programs to diagnose patients early, and identifying alternative treatment strategies to circumvent treatment-refractory disease progression is required to improve the lung cancer survival rate. Here, we introduce a new mechanism for the molecular regulation of oncogenic signaling that builds upon current knowledge of lung cancer biology and may inform the development of novel anticancer therapies.

Inhibitor-κB kinase α (IKKα), a serine/threonine protein kinase, is encoded by the conserved helix-loop-helix ubiquitous kinase (*CHUK*) gene[9]. Phosphorylation of IκBα, a nuclear factor-κB (NF-κB) inhibitor, by IKKα and IKKβ, catalytical subunits of the IKK complex, promotes IκBα protein degradation, which initiates nuclear translocation of NF-κB dimers. In the nucleus, NF-κB functions as a transcription factor to regulate immunity, infection, lymphoid organ/cell development, cell death/growth, and tumorigenesis[9-13]. In noncanonical signaling, NF-κB–inducing kinase activates IKKα protein via phosphorylation upon activation of upstream membrane-bound receptors by their cognate ligands. Active IKKα then phosphorylates and cleaves the p100 protein to generate p52, which complexes with the RelB NF-κB subunit, resulting in nuclear translocation of the p52/RelB dimer to regulate several immune functions,

including lymphoid organ development, the priming function of dendritic cells, B-cell survival, generation and maintenance of effector- and memory- T cells, and antiviral innate immunity[9,14,15].

The tumor-promoting role of IKKα has been documented in breast, prostate, nonmelanoma skin, and lung cancer[16-18]. Aberrant overexpression of IKKα protein is associated with decreased patient survival and promotes the growth of lung adenocarcinoma; it may therefore be used as a biomarker to predict clinical response in lung adenocarcinoma patients[19]. In a separate study, investigators showed that overexpression of cytosolic and nuclear IKKα protein promotes NSCLC cell proliferation, survival, and migration through activating the ERK, p38/MAPK, and mammalian target of rapamycin (mTOR) cell signaling pathways. Additionally, activation of protumorigenic cell signaling pathways depends on the subcellular localization of IKKα[18]. Although the role of IKKα in promoting cancer has been well established, in the context of lung cancer driven by *Kras*-activating mutations it may have tumor-suppressing activity: in a $Kras^{G12D}$-driven spontaneous mouse model of NSCLC, lung-specific *Ikkα* deletion induced by intratracheally injected adenovirus-Cre recombinase promoted NSCLC initiation and growth by elevating the expression of inflammatory cytokines and chemokines, including NF-κB targets[20]. We sought to understand the role of IKKα protein overexpression in tumor growth and progression in *Kras*-wildtype NSCLC.

Dopamine- and cyclic AMP–regulated phosphoprotein, Mr 32000 (DARPP-32), is primarily expressed in the brain, including the caudate nucleus, cerebral cortex, and striatum. It acts as a downstream signaling molecule through dopamine receptor 1 (D_1R) and is negatively regulated by dopamine receptor 2 (D_2R) and glutamate signaling[21-23]. Phosphorylation of DARPP-32 in response to cAMP in dopamine-responsive nerve tissue attenuates protein phosphatase 1 (PP1) activity, affecting the regulation of several cell signaling pathways[24]. Although expression of DARPP-32 proteins is typically restricted to neuronal cell types in the brain, DARPP-32 and

its truncated isoform t-DARPP are aberrantly overexpressed in many types of cancer, including lung cancer[25-31]. t-DARPP, which was originally discovered in gastric cancer tissues, lacks the N-terminal domain responsible for modulating PP1 function[28]. It is phosphorylated by cyclin-dependent kinase (CDK) 1 and 5 and activates protein kinase A (PKA), thereby conferring resistance to trastuzumab, a HER2-targeted anticancer agent, via sustained signaling through the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/AKT pathway[32,33]. Since this discovery, the DARPP-32 and t-DARPP isoforms overexpressed in breast, colon, esophageal, gastric, pancreas, prostate, lung, and ovarian cancer tissues have been shown to activate robust anti-apoptotic signaling through the activation of the AKT and ERK cell signaling pathways; to increase metabolism by forming a complex with the insulin-like growth factor 1 receptor (IGF1R); and to promote cell survival in the presence of receptor tyrosine kinase inhibitors, including gefitinib and trastuzumab[25-27,29,30,34-37]. Our previous work, which serves as the rationale for this current study, revealed that DARPP-32 isoforms increase NSCLC cell migration via increasing the expression of NF-κB2–controlled migratory genes by establishing a direct physical interaction with IKKα[25]. However, the precise role of the DARPP-32/IKKα complex in regulating NSCLC progression has yet to be determined.

In this study, we report that IKKα protein inhibits PP1 function through phosphorylation of the DARPP-32 protein at the Thr-34 position. Pharmacologic inhibition of PP1 activates ERK cell signaling pathways, leading to NSCLC growth promotion in vitro. Furthermore, we show in an orthotopic mouse model that depletion of IKKα protein reduces NSCLC growth. Taken together, our findings suggest that IKKα protein directly phosphorylates DARPP-32 to stimulate oncogenic kinase activity through the inhibition of PP1 function to promote NSCLC growth and oncogenesis.

Results

Phosphorylation of DARPP-32 at Thr-34 is regulated by IKKα

Given our prior observation that the physical association between IKKα and DARPP-32 promotes NSCLC cell migration[25], we postulated that DARPP-32 phosphorylation is controlled by the kinase function of IKKα. To test our hypothesis, we first performed immunoprecipitation experiments in three separate human NSCLC cell lines, which confirmed that IKKα establishes a direct physical interaction with DARPP-32 (Fig. 1a-c). We next performed nonradioactive in vitro kinase assays using commercially available kinase-active IKKα protein. Briefly, DARPP-32 and its short isoform, t-DARPP, were purified from lysates of four different lung adenocarcinoma cell lines using anti-FLAG M2 affinity beads and then incubated with purified IKKα protein in kinase assay buffers containing ATP. Reaction end products were subjected to immunoblotting with anti-phosphorylated DARPP-32 (both T34 and T75) and -total DARPP-32 antibodies. Our western blotting results confirm that purified full-length DARPP-32 protein (but not t-DARPP) serves directly as a substrate for IKKα (Fig. 2a-b). Based on our results, it is evident that IKKα phosphorylates DARPP-32 at the Thr-34 position only (Fig. 2a-b). As expected, IKKα does not phosphorylate t-DARPP because it lacks the first 36 amino acids of full-length DARPP-32 (Fig. 2a,c). However, the presence of strong signals on the immunoblot using anti-phosphorylated DARPP-32 (T75) suggests that t-DARPP is phosphorylated at Thr-75 by unknown endogenous kinase(s) (Fig. 2a,c). In summary, our results indicate that IKKα physically associates with DARPP-32 protein and phosphorylates full-length DARPP-32 protein at the Thr-34 position.

Increased expression of p-ERK is regulated by IKKα via DARPP-32/PP1α signaling

A seminal report suggested that the neuronal phosphoprotein DARPP-32 acts as a potent inhibitor of PP1 following phosphorylation by PKA at the Thr-34 position[24]. On the basis of this report, we hypothesized that IKKα-mediated DARPP-32 phosphorylation inhibits PP1α activity in

NSCLC cells and promotes oncogenic growth by activating cell signaling pathways. To test our premise, we transiently overexpressed constitutively active and kinase-dead IKKα plasmids in HCC827 and H1650 cells and performed an immunoblotting experiment with antibodies directed against phosphorylated DARPP-32 (T34). In line with our previous in vitro kinase results, we observed that expression of phosphorylated DARPP-32 increases more in HCC827 and H1650 cell lysates overexpressing active IKKα than in GFP- or kinase-dead IKKα–expressing cell lysates (Fig. 3a-b). Phosphorylation of PP1α by cdc2 kinases inhibits PP1α phosphatase activity in a cell cycle–dependent manner[38], and phosphorylation of DARPP-32 at the T34 position leads to DARPP-32–mediated phosphorylation and inactivation of PP1α in neurons and cancer cells[24,39]. We therefore sought to determine the effect of IKKα expression on the levels of inactive PP1α protein in immunoblotting experiments using anti-phosphorylated PP1α antibodies. Expression of phosphorylated (inactive PP1α proteins increased in cells overexpressing active IKKα (Fig. 3a-b), suggesting that overexpression of IKKα leads to increased DARPP-32 phosphorylation at the T34, which inhibits PP1 phosphatase activity. To test how repression of PP1 function by the IKKα/DARPP-32 complex stimulates downstream oncogenic cell signaling, we focused on the ERK/MAPK signaling pathway because pharmacologic inhibition of PP1 activity has been reported to increase ERK activity[40]. In immunoblotting experiments, we observed an increase in the expression of phosphorylated ERK in HCC827 and H1650 cells exogenously expressing active IKKα (Fig. 3a-b). Expression of phosphorylated ERK proteins remained unchanged in cells expressing GFP or overexpressing kinase-dead IKKα (Fig. 3a-b). To validate our theory that phosphorylation of ERK protein is controlled by PP1α phosphatase, we performed western blotting experiments in HCC827 cells treated with a pharmacological inhibitor of PP1α, calyculin A. The expression of phosphorylated (i.e., phosphatase-inactivated) PP1α as well as phosphorylated (i.e., activated) ERK was higher in calyculin A–treated HCC827 cells than in vehicle-treated cells (Fig. 3c). In summary, our

results indicate that overexpression of kinase-active IKKα protein positively regulates the ERK-MAPK pathway through the DARPP-32/PP1α axis.

IKKα controls the inhibition of PP1α phosphatase activity

To test our hypothesis that IKKα prevents PP1α phosphatase activity in NSCLC cells by phosphorylating DARPP-32 at Thr-34, we performed an in vitro phosphatase assay in lung adenocarcinoma cells stably overexpressing DARPP-32 protein. Briefly, kinase-dead, full-length, and constitutively active IKKα plasmids, as well as GFP-expressing control plasmids, were transiently transfected into HCC827 and H1650 cells stably overexpressing DARPP-32 protein. Endogenous PP1α was immunoprecipitated from the cell lysates and subjected to phosphatase assays. We observed a decrease in PP1α phosphatase activity (i.e., lower concentrations of released phosphates) in the lysates of cells overexpressing full-length or kinase-active IKKα than in lysates of GFP-expressing cells (Fig. 4a-b). As expected, overexpression of kinase-dead IKKα in both cell lines failed to inhibit PP1α phosphatase activity (Fig. 4a-b). To ensure that equal amounts of immunoprecipitated PP1α were used in the in vitro phosphatase assay, we performed immunoblotting experiments to measure the expression level of PP1α in different groups. We observed that equal amounts of PP1α were immunoprecipitated in HCC827 and H1650 cells exogenously expressing kinase-dead, full-length, or constitutively active IKKα or GFP (Fig. 4c-d). To further test whether IKKα blocks PP1α phosphatase activity via DARPP-32 phosphorylation at Thr-34, we stably overexpressed mutant DARPP-32 (T34A) in HCC827 and H1650 cells and repeated the in vitro phosphatase assay. As expected, no PP1α inhibition activity was seen in cells overexpressing full-length or constitutively active IKKα in the presence of mutant DARPP-32 (Supplementary Fig. 1a-d). Taken together, our findings indicate that IKKα-mediated DARPP-32 phosphorylation inhibits PP1α phosphatase activity.

Depletion of IKKα expression in tumor cells inhibits oncogenic growth advantage

To test the premise that IKKα promotes oncogenic tumor growth, we first performed a soft agar anchorage-independent growth assay in human lung adenocarcinoma HCC827 and PC9 cells because anchorage-independent growth is considered one of the most reliable markers of malignant transformation[41]. We observed less anchorage-independent growth (number of colonies formed on the soft-agar plates) of HCC827 and PC9 cells transduced with IKKα shRNAs than of LacZ shRNA–transduced controls (Fig. 5a-c), suggesting that IKKα promotes anchorage-independent oncogenic growth in lung cancer. We then tested whether IKKα ablation reduces lung tumor growth in an orthotopic xenograft mouse model. Briefly, luciferase-labeled human HCC827 NSCLC cells were injected into the left thorax of anesthetized SCID mice. After establishment of the lung tumor, mice were imaged for bioluminescence signals weekly over the course of 7 weeks. Mice challenged with IKKα-ablated HCC827 cells showed less lung tumor growth than those transduced with control LacZ shRNA (Fig. 6a-b). Taken together, our in vitro cell line and in vivo mouse data suggest that IKKα protein drives lung oncogenic tumor growth, and ablation of IKKα expression reduces lung cancer growth.

Discussion

The IKK complex, consisting either of IKKα, -β, and -γ kinases (canonical) or IKKα homodimers (noncanonical), has been studied in the context of inflammation and innate immunity as a regulator of interferon regulatory factors and NF-κB signaling[42-44]. Recently, it has been appreciated that IKKα and related kinases also phosphorylate proteins involved in regulating biological processes, including cell growth, metabolism, apoptosis, cell cycle, cell migration, and invasion, independent of NF-κB–regulated cell signaling pathways[43,45,46]. Here, we show that the kinase function of IKKα promotes lung tumor growth by activating the ERK cell signaling pathway through the DARPP-32/PP1 axis. Overexpression of constitutively active IKKα

189 influences the proliferation of mammary epithelium through regulation of RANK signaling in a
190 genetically engineered mouse model[47]; thus, it is expected that aberrant IKKα expression
191 promotes breast tumorigenesis. Indeed, Bennett et al. reported that overexpression of cytosolic
192 IKKα protein is associated with reduced time to recurrence and worsened disease-free survival
193 in estrogen receptor–positive breast cancer patients[48]. Additionally, the role of IKKα in
194 promoting breast cancer growth in the presence of anti-estrogen therapy via activation of the
195 Notch pathway has been well studied and provides a mechanism for hormone therapy
196 resistance in an NF-κB–independent manner[49]. Recently, Dan and colleagues reported that
197 IKKα protein activates the AKT cell signaling pathway by phosphorylating the mTOR complex 2
198 in cervical, prostate, lung, and pancreatic cell lines, establishing the oncogenic role of IKKα
199 protein in promoting tumor growth[50]. Additionally, transcripts of *CHUK* (IKKα), but not *IKBKB*
200 (IKKβ), are overexpressed in lung adenocarcinoma tissues compared with normal lung
201 tissues[19]. A previous study by our group also demonstrated that NSCLC patients with elevated
202 IKKα expression have significantly shorter overall survival than those with low IKKα expression,
203 and that IKKα regulates NSCLC cell migration by forming a complex with DARPP-32 to
204 influence the noncanonical NF-κB cell signaling pathway[25]. Here, we propose an alternative
205 mechanism in which activated IKKα protein promotes NSCLC growth through a DARPP-32/PP1
206 cell signaling cascade in an NF-κB–independent manner.

207 DARPP-32 protein, encoded by the *PPP1R1B* gene, has been well studied in the nervous
208 system to understand the complexity of signal transduction in neurons, especially striatal
209 projection neurons[51]. The function of DARPP-32 in amplifying responses to many external
210 stimuli is tightly regulated by its phosphorylation on multiple sites by different protein kinases.
211 Notably, DARPP-32 phosphorylation at Thr-34 by PKA in response to extracellular signals has
212 been shown to inhibit PP1 function in neurons[23,51]. In agreement with the previous finding
213 showing DARPP-32 is phosphorylated by PKA, Hansen and colleagues showed that Wnt-5A-

mediated phosphorylation of DARPP-32 Thr-34 reduces breast cancer cell migration, and DARPP-32 phosphorylation is tightly regulated by intracellular cAMP levels and subsequent PKA activation[39]. Recently, it was shown that breast cancer patients with elevated DARPP-32 expression but low PP1 expression have worse overall survival than those with low expression of DARPP-32[52], suggesting a strong inverse correlation between PP1 and DARPP-32 proteins in patient outcome. This supports the notion that DARPP-32–mediated inactivation of PP1 functions via phosphorylation leads to increased activation of kinases involved in oncogenic signaling pathways. Moreover, PKA protein expression in breast tumor tissues shows a strong correlation with DARPP-32 and PP1 protein expression, warranting further investigation to understand their molecular role in regulating breast tumorigenesis[52]. In a separate study, Hansen et al. reported that PKA protein activated by Wnt-5a ligands regulates breast cancer cell migration by phosphorylating DARPP-32 at Thr-34 in a PP1/CREB-dependent manner[39]. In line with this observation, our current study provides strong evidence that NSCLC cell growth is regulated by the IKKα/DARPP-32/PP1/ERK cell signaling pathway. Overexpression of t-DARPP has been shown to confer resistance to trastuzumab, a HER2-targeted monoclonal antibody, via activation of PKA and PI3K/AKT cell signaling in $HER2^{+}$ breast cancer cells[53,54]. The molecular mechanism has been recently uncovered in which t-DARPP phosphorylated by CDK -1 and -5 activates PKA kinase function by forming a direct complex with PKA regulatory subunits in breast cancer cells overexpressing t-DARPP[32,33]. Here, we demonstrate that phosphorylation of t-DARPP is not regulated by IKKα, as expected, due to the absence of first 36 amino acids. The strong presence of t-DARPP protein phosphorylated at Thr-39 (equivalent to DARPP-32 Thr-75) in our assays requires further investigation to identify the molecular mechanism of t-DARPP regulation by upstream kinases in NSCLC cells.

The catalytic subunit of PP1, a major protein phosphatase in human cells composed of α, β, and γ subunits, regulates critical cellular processes including cell cycle progression, apoptosis, and

metabolism by catalyzing dephosphorylation of a wide range of proteins[55]. The role of PP1 as a tumor suppressor or oncogene depends on the type of cancer, the cancer staging, and the regulatory proteins that interact with it. The pathways are further complicated because both oncogenes and tumor suppressor proteins are known substrates of PP1, and dephosphorylation events can activate or downregulate downstream cell signaling pathways[56]. Therefore, detailed mechanistic insight is needed to understand the role of PP1 in lung cancer. The complex of PP1 with the leucine-rich repeat protein SHOC2 promotes tumor growth in a subset of *KRAS*-mutant NSCLC cell lines by dephosphorylating a critical inhibitory site on RAF kinases, resulting in RAF-ERK pathway activation. Moreover, genetic inhibition of SHOC2 suppresses tumor development in autochthonous murine *Kras*-driven lung cancer models[57]. In contrast, activated PP1, upon forming a complex with protein 4.1N, a neuronal homolog of the erythrocyte membrane cytoskeletal protein 4.1, inhibits lung tumor progression by depressing the JNK cell signaling pathway[58]. Our results indicate that PP1-mediated dephosphorylation of ERK is inhibited by the DARPP-32/PP1 complex, which in turn promotes lung tumor growth by increasing ERK activity, which is associated with increased oncogenic potential due to the central position of ERK downstream of several oncogenic growth signaling pathways.

Manipulation of PP1 activity has long been considered a potential approach to treating cancer because of the involvement of PP1 in several cancer-related cellular processes. The small molecule inhibitors calyculin A and okadaic acid have been used to mitigate PP1 and PP2A activity, thereby impairing progression of hormone therapy-resistant prostate cancer by stimulating cell death[59]. However, PP1 small molecule inhibitors have unwanted cellular toxicity because PP1 is involved in a broad range of cellular processes. Moreover, homology of the active sites among different phosphatases contributes to the limited efficacy of these inhibitors in treating cancer. Therefore, targeting PP1 complexes, instead of focusing on the catalytic sites of PP1, is a promising solution to suppress sustained growth and survival in cancer.

Recent findings of novel phosphorylation substrates of IKK family kinases, including DARPP-32 in this study, expands current knowledge of critical biological and disease-related mechanisms. To comprehensively understand the function of these pleiotropic kinases, further experiments are needed to assess the roles of IKK family members in regulating phosphorylation-dependent substrates in different settings and diseases. In this regard, it will be interesting to see whether DARPP-32 phosphorylation is regulated by IKKα protein in the presence of anticancer agents routinely used in the clinic to treat lung cancers. Another critical question—which upstream kinases regulate IKKα activation—warrants further investigation because EGFR and KRAS are highly mutated in lung cancer patients[60]. Targeting IKK and IKK-related kinases with the small molecule IKK inhibitors SAR-113945 and MLN-0415 has shown encouraging results in preclinical studies, although they failed to meet the primary endpoints of a phase 2 clinical trial and the safety profile of a phase 1 clinical trial, respectively[61]. Because NF-κB functions in many different systems, targeting IKKα and IKK-related kinases to treat disease, including cancers, can result in unpredictable adverse events. Therefore, development of more selective, isoform-specific, non-ATP-competitive inhibitors against IKK family kinases to use in combination therapies and/or as part of a targeted delivery approach is required, particularly in cancers that aberrantly express IKKα protein.

Methods

Cell lines and inhibitors

Human NSCLC cell lines A549 and H1650, as well as a transformed human embryonic kidney epithelial cell line, HEK-293T, were purchased from the American Type Culture Collection. The epidermal growth factor receptor (EGFR)-mutated human NSCLC cell lines HCC827, PC9, and H1975 were kindly provided by Dr. Pasi A. Jänne at the Dana-Farber Cancer Institute[62], Dr. Aaron N. Hata at Massachusetts General Hospital[63], and Dr. Anthony C. Faber at Virginia

Commonwealth University[64], respectively. Dulbecco's modified Eagle's medium (DMEM; Corning) supplemented with 10% fetal bovine serum (FBS; Millipore) was used to grow HEK-293T cells. Human NSCLC cell lines A549, H1650, HCC827, PC9, and H1975 were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Corning) supplemented with 10% FBS (Millipore), 1% penicillin/streptomycin antibiotics (Corning), and 25 µg/mL plasmocin prophylactic (Invivogen). All cell lines were routinely authenticated via morphologic inspection and tested negative for mycoplasma contamination. A serine/threonine protein phosphatase inhibitor, calyculin A, purchased from Cell Signaling Technology (CST), was used to mitigate PP1α function.

Generation of stable cell lines

Human full-length DARPP-32 and mutant DARPP-32 (T34A) cDNAs cloned into the pcDNA3.1 vector were kindly provided by Dr. Wael El-Rifai at University of Miami Health System[65]. Retrovirus containing FLAG-tagged full-length and mutant DARPP-32 cDNAs were prepared by following a previously described procedure[66]. NSCLC cells seeded at a density of 3×10^5 cells per 10-cm cell culture dish were transduced with 1 mL retrovirus diluted in 5 mL fresh medium supplemented with 10 µg/mL polybrene solution (Millipore). Cells were used for subsequent experiments 48 h after transduction.

Human HEK-293T cells transfected with either LacZ shRNA (control) or IKKα shRNAs (Sigma) along with their corresponding packaging plasmids were used to prepare lentiviruses from cell culture medium 48 h after transfection. Lentiviruses concentrated in Lenti-X concentrator (Takara) were used immediately to transduce HCC827 and PC9 lung cancer cell lines, as reported previously[67]. Transduced cells were incubated in puromycin (Sigma)-containing medium for 72 h to select stable IKKα knockdown cells.

Lentiviruses containing the luciferase gene were prepared in HEK-293T cells as described previously[26]. Luciferase-labeled stable human NSCLC cells were used to determine tumor growth in orthotopic murine models.

Antibodies

Primary antibodies (1 µg/µl) identifying two different phosphorylated sites on DARPP-32 (T34: cat no. 12438; dilution 1:1000; and T75: cat no. 2301; dilution 1:1000), phosphorylated PP1α (T320; cat no. 2581; dilution 1:1000), IKKα (cat no. 2682; dilution 1:1000), phosphorylated p44/42 MAPK (T202/Y204; cat no. 4370; dilution 1:1000), and total p44/42 MAPK (cat no. 4695; dilution 1:1000) were purchased from CST. Antibodies (200 µg/ml) against DARPP-32 (cat no. sc-398360; dilution 1:200), PP1 (cat no. sc-7482; dilution 1:100), and α-tubulin (cat no. sc-5286; dilution 1:500) were obtained from Santa Cruz Biotechnology. Horseradish peroxidase (HRP)–conjugated secondary antibodies against the heavy chains of anti-rabbit (cat no.: 7074; dilution 1:5000) and anti-mouse (cat no.: 7076; dilution 1:5000) IgG were purchased from CST.

Plasmids

Expression vectors of full-length (#15467), kinase-dead (#15468), and constitutively kinase-active (#64608) IKKα were purchased from Addgene. Briefly, the investigators constructed full-length IKKα in-frame with DNA encoding an N-terminal FLAG epitope in pCR-3 vectors[68]. Kinase-dead IKKα (K44A) was generated from full-length IKKα expression plasmids by using a site-directed mutagenesis kit[68]. Expression vectors for V5 epitope–tagged kinase-active IKKα (S176E, S180E) were constructed in destination/expression vector pcw107 via the Gateway cloning system[69]. Expression plasmids (pCMV) encoding GFP used as transfection controls were kindly shared by Dr. Georgiy Aslanidi at The Hormel Institute, University of Minnesota.

Immunoblotting

Radioimmunoprecipitation assay (RIPA) buffer (Millipore) supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitors (Millipore) were used to lyse human NSCLC cells. Equal amounts of cell lysates were separated via 4-20% gradient SDS-PAGE (Bio-Rad) and transferred to polyvinyl difluoride membranes (Millipore). Prior to primary antibody incubation, membranes were incubated in Tris-buffered saline (Growcells) containing 5% bovine serum albumin (Sigma) at room temperature for 1 h. Incubation of diluted primary and secondary antibodies was carried out overnight and for 2 h, respectively. Chemiluminescence substrate (Thermo Fisher Scientific) was used to detect antibody-reactive protein bands in the membranes, and signals were captured electronically using an ImageQuant™ LAS 4000 instrument (GE Healthcare).

Purification of DARPP-32 isoforms

Human lung adenocarcinoma A549, HCC827, PC9, and H1975 cells stably overexpressing FLAG-tagged DARPP-32 or t-DARPP proteins were lysed on ice using 1× lysis buffer (50 mM Tris HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) supplemented with protease inhibitor cocktail (Roche). Immunoprecipitation of FLAG fusion proteins was performed using an anti-FLAG M2 affinity gel (Sigma) by following the manufacturer's instructions. Protein elution was carried out under native conditions by competition with commercially available 3× FLAG peptide (Sigma). Eluted proteins separated in 4-20% polyacrylamide gels in denatured conditions were visualized for purity after Coomassie blue (Bio-Rad) staining.

In vitro kinase assay

Human DARPP-32 isoforms purified from NSCLC cells were incubated with kinase-activated human IKKα protein (SignalChem) for in vitro kinase assays by following previously described

methods[70]. Briefly, 3 µg purified DARPP-32 isoforms as well as 5 µl ATP (New England Biolabs) in kinase dilution buffer III (SignalChem) were incubated with 1 µg commercially available human IKKα protein (SignalChem) for 30 minutes at 30°C, then at 95°C for 5 minutes, in which 1× Laemmli sample buffer (Bio-Rad) supplemented with 10% β-mercaptoethanol (Bio-Rad) was added to stop the kinase reaction. Phosphorylation of DARPP-32 by kinase-activated human IKKα protein was validated via immunoblotting using monoclonal primary antibodies against phosphorylated DARPP-32 (T34 and T75, CST).

Transient transfection

Human NSCLC cell lines, HCC827 or H1650, were plated in 6-well cell culture plates at a concentration of 2×10^5 cells per well. Cells were washed with PBS (Corning) on the following day prior to transfection, and complete RPMI-1640 medium (Corning) was added to each well. Based on the protocols from the manufacturer, 2.5 µg of plasmid DNA and 5 µl P3000 reagent (Invitrogen) diluted in OPTI-MEM medium (Gibco) were incubated with 10 µl Lipofectamine-3000 transfection reagent (Invitrogen) for 15 min at room temperature. The DNA:Lipofectamine mixture was then added to each well in a dropwise manner and incubated for 48 h.

Immunoprecipitation

Human NSCLC cell lines transiently transfected with either control GFP or one of three different IKKα plasmids were lysed in RIPA buffer (Millipore) supplemented with protease inhibitors (Roche). The concentration of harvested cell lysates was measured by using the Bradford reagent (Bio-Rad). Anti-PP1α antibody (2 µg) was added to the supplied spin column (Catch and Release Immunoprecipitation Kit; cat no. 17-500; Millipore) along with the cell lysates (500 mg) to immunoprecipitate the proteins following the manufacturer's protocol. The eluted proteins in native form were subsequently used to perform the in vitro phosphatase assay.

In vitro phosphatase assay

The in vitro phosphatase assay was performed in accordance with the manufacturer's protocol . Briefly, 5 µl PP1α substrates (GRPRTS[p]SFAEG; cat no. P50-58; Signal Chem) or 5 µl control histone H1 peptides (GGGPATP-KKAKKL-COOH; cat no. H10-58; Signal Chem) diluted in phosphatase dilution buffer II (cat no. P22-09; Signal Chem) was incubated for 15 min at 37 °C with human PP1α protein in its native form immunoprecipitated from human NSCLC cells. The amount of free phosphate molecule generated by the reaction was colorimetrically quantified with a Phosphate Assay Kit (Abcam). The amount of released phosphate was determined from a standard curve generated after plotting the absorbance value against increasing known concentrations of free phosphate molecule.

Soft agar colony formation assay

Five milliliters of complete RPMI-1640 medium (Corning) containing 0.75% melted agar (Sigma) was added to 60-mm cell culture dishes to create a bottom layer. Cells of the human NSCLC lines HCC827 and PC9 transduced with lentivirus encoding LacZ shRNA (control) or IKKα shRNAs were suspended in complete RPMI-1640 medium containing 0.36% melted agar and were plated on top of the bottom layer at a concentration of 2.5×10^4 cells per dish. After 2 weeks of incubation, images of colonies that had grown on the soft-agar cell culture plates were captured using a 4× Plan S-Apo 0.16 NA objective on an EVOS FL cell imaging system (Thermo Fisher Scientific). The colonies were counted by using ImageJ software and plotted by using GraphPad Prism 9 software.

In vivo orthotopic lung cancer model

Six- to eight-week-old pathogen-free SCID/NCr mice were purchased from Charles River Laboratories. Mice were allowed one week to acclimate to their surroundings, then bred,

maintained under specific-pathogen-free conditions in a temperature-controlled room with alternating 12 h light/dark cycles, and fed a standard diet in accordance with protocols approved by the University of Minnesota Institutional Animal Care and Use Committee. For each mouse, luciferase-labeled human HCC827 lung cancer cells (1×10^6) transduced with either LacZ shRNA (control) or IKKα shRNAs were suspended in 80 µl PBS and Matrigel. The cells were then orthotopically injected in the right thoracic cavity of 8- to 12-week-old male and female mice and allowed to establish tumors over 1 week. Luminescence images of mice were taken weekly over 7 weeks using an In-Vivo Xtreme xenogen imaging system (Bruker). The luciferase intensity (total photon count) of each mouse was calculated using Bruker molecular imaging software and plotted over time in GraphPad Prism 9 software.

Statistics

Statistically significant differences between multiple groups (greater than 2) were determined by performing one-way analysis of variance (ANOVA) followed by Dunnett's test. Statistically significant differences in tumor growth over time between two groups in the mouse experiments were determined with two-way ANOVA followed by Sidak's test. Values of $P \leq 0.05$ were considered significant. Data are expressed as mean ± SEM of at least three independent experiments.

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Author contributions: S.K.A. conducted in vitro cell line-based experiments, including immunoprecipitation experiments, soft agar colony formation assays, and in vitro kinase and phosphatase assays. S.K.A. and L.H.H. managed the immunocompromised SCID/NCr mouse colony and performed tumor studies in mice. S.K.A. and L.W. conducted murine in vivo bioluminescence imaging and necropsy. L.W. purified full-length and truncated DARPP-32 protein from mammalian cells. S.K.A. and Z.Z. performed western blotting experiments. Z.Z. assisted with imaging and analysis of soft agar colony formation assays. S.K.A, L.W., and L.H.H. provided technical and scientific support. S.K.A. and L.H.H. performed experimental troubleshooting, reviewed relevant scientific literature, critically analyzed data, prepared figures, and wrote the manuscript. L.H.H. conceived the aims, led the project, and acquired funding to complete the reported research.

Data Availability: The authors declare that the data supporting the findings of this study are available within the article and its supplementary information. Any other associated data supporting the findings of this study are available from the corresponding author upon request.

Competing interests: The authors declare no competing interests.

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Figure 1

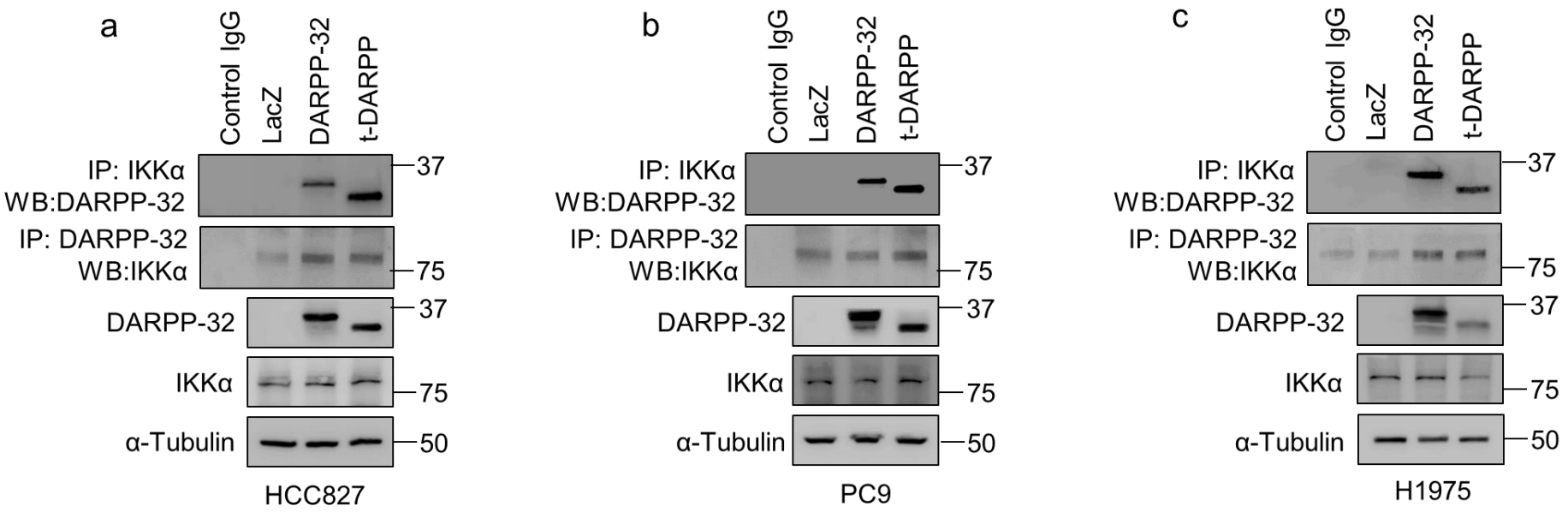
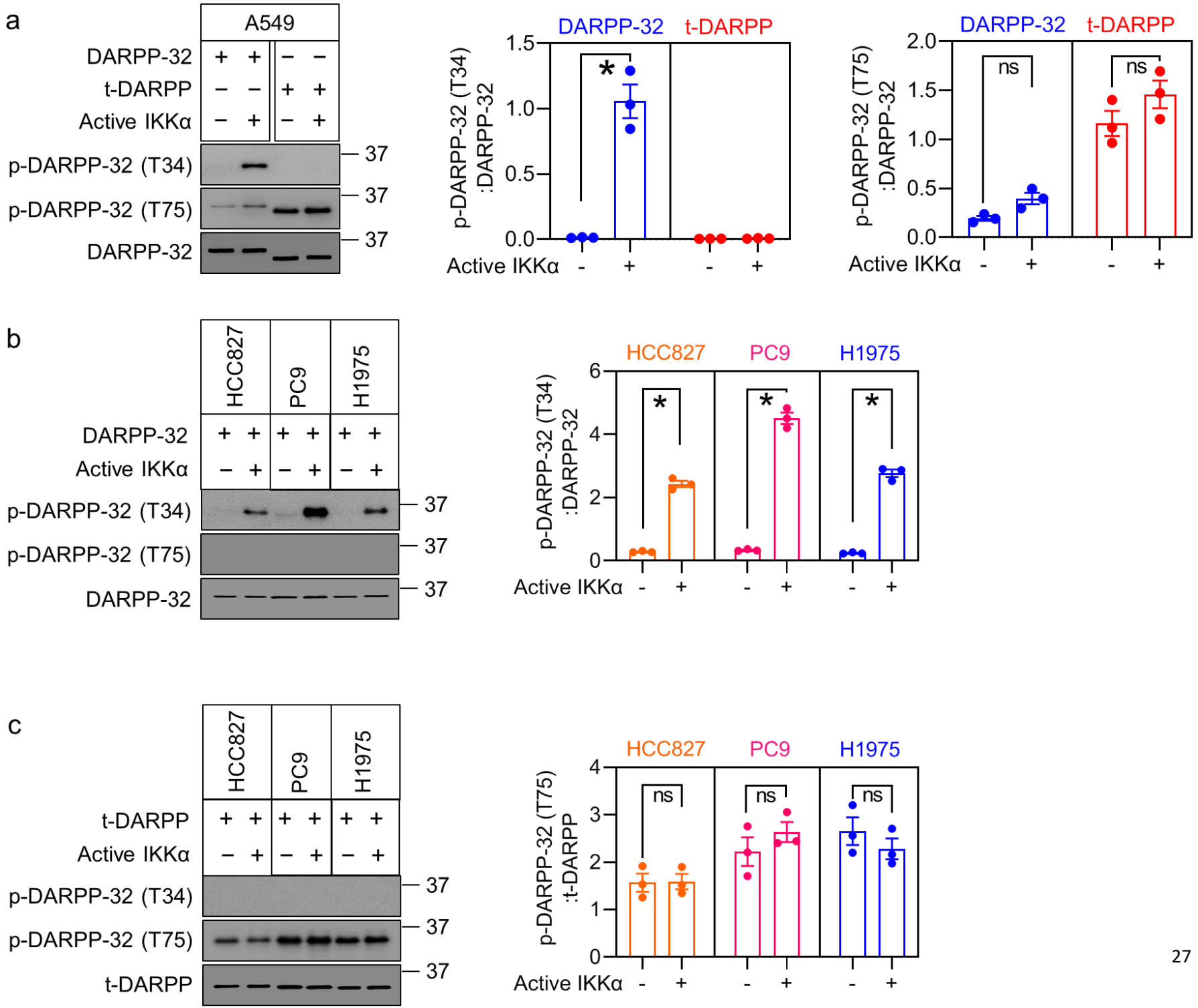


Figure 1: IKKα physically associates with DARPP-32 isoforms. a-c. Human lung adenocarcinoma cell lines HCC827 (a), PC9 (b), and H1975 (c) stably overexpressing FLAG-tagged human DARPP-32 isoforms were lysed and subjected to immunoprecipitation using either anti-IKKα or anti-FLAG antibody (IP:DARPP-32). Immunoprecipitated lysates were separated in SDS-PAGE and immunoblotted with antibodies against IKKα, DARPP-32, and α-tubulin (loading control).

Figure 2


a
A549
DARPP-32
t-DARPP
Active IKKα
p-DARPP-32 (T34)
p-DARPP-32 (T75)
DARPP-32
37
37
37
DARPP-32
t-DARPP
p-DARPP-32 (T34) :DARPP-32
Active IKKα
DARPP-32
t-DARPP
p-DARPP-32 (T75) :DARPP-32
ns
ns
Active IKKα
b
HCC827
PC9
H1975
DARPP-32
Active IKKα
p-DARPP-32 (T34)
p-DARPP-32 (T75)
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HCC827
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p-DARPP-32 (T34) :DARPP-32
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p-DARPP-32 (T75)
t-DARPP
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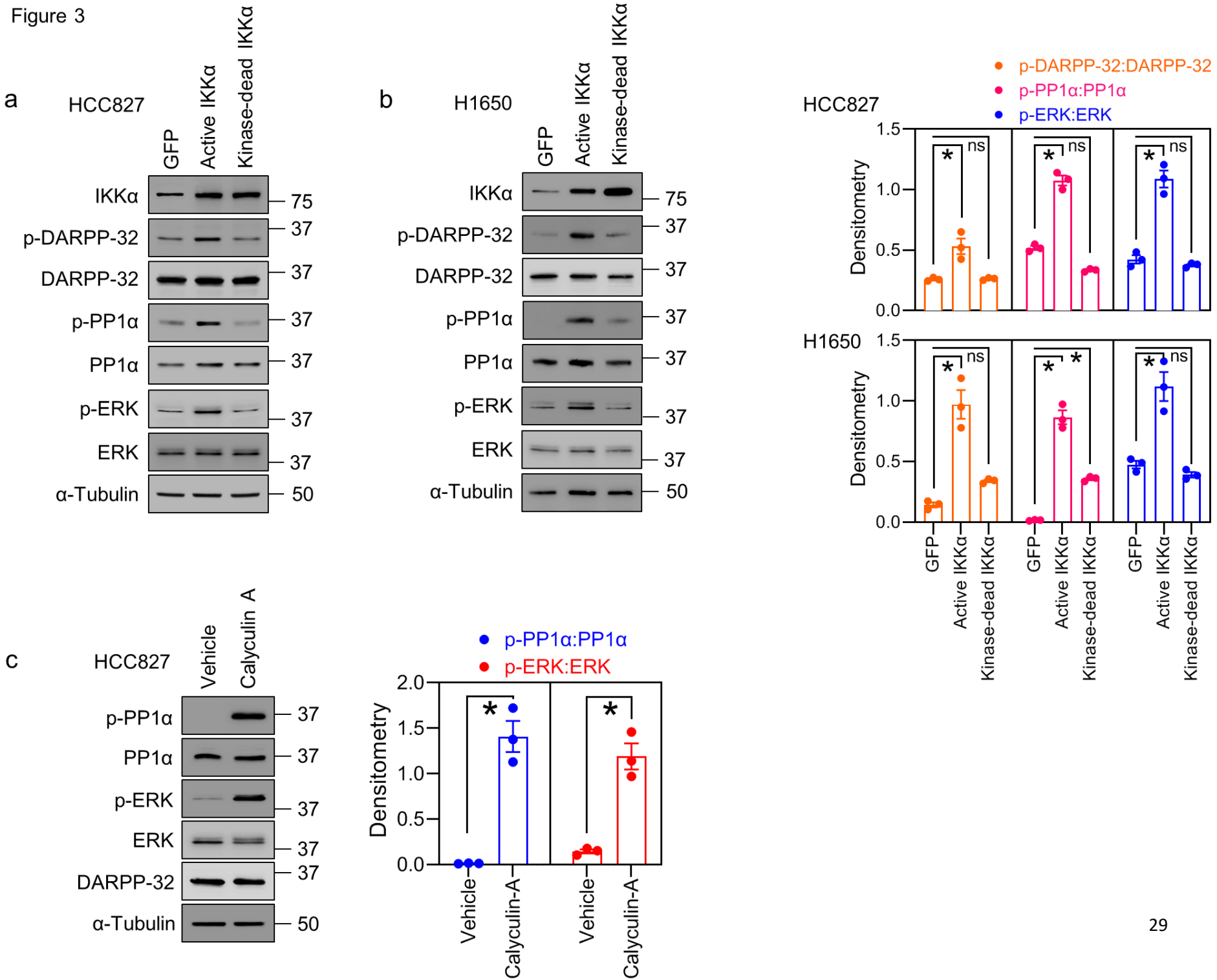
H1975
p-DARPP-32 (T75) :t-DARPP
ns
Active IKKα

Figure 2: IKKα phosphorylates DARPP-32 at Thr-34. a. Human A549 cell lines stably overexpressing FLAG-tagged human DARPP-32 isoforms (DARPP-32 and t-DARPP) were lysed and subjected to immunoprecipitation using anti-FLAG antibody–conjugated agarose beads. Immunoprecipitated lysates were used to perform nonradioactive in vitro kinase assays following incubation with commercially available active IKKα protein. At the end, the reaction mixtures were subjected to immunoblotting using antibodies against DARPP-32 phosphorylated on Thr-34 or Thr-75 and total DARPP-32 protein. **b-c**. Human HCC827, PC9, and H1975 lung adenocarcinoma cell lines retrovirally transduced with either FLAG-tagged human DARPP-32 (b) or t-DARPP (c) cDNA plasmids were lysed, immunoprecipitated, incubated with active IKKα protein, and subjected to western blotting using anti-phosphorylated (Thr-34 or Thr-75) DARPP-32 and anti-DARPP-32 antibodies. Data from one experimental replicate are shown. The experiments were repeated three times independently; each circle in a bar represents one experiment. *$P < 0.05$; ns, not significant.

Figure 3


a
HCC827
GFP
Active IKKα
Kinase-dead IKKα
IKKα
p-DARPP-32
DARPP-32
p-PP1α
PP1α
p-ERK
ERK
α-Tubulin
75
37
37
37
37
37
37
50
b
H1650
GFP
Active IKKα
Kinase-dead IKKα
IKKα
p-DARPP-32
DARPP-32
p-PP1α
PP1α
p-ERK
ERK
α-Tubulin
75
37
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37
50
p-DARPP-32:DARPP-32
p-PP1α:PP1α
p-ERK:ERK
HCC827
H1650
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0.0
0.5
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GFP
Active IKKα
Kinase-dead IKKα
c
HCC827
Vehicle
Calyculin A
p-PP1α
PP1α
p-ERK
ERK
DARPP-32
α-Tubulin
37
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p-PP1α:PP1α
p-ERK:ERK
Densitometry
0.0
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1.5
2.0
*
Vehicle
Calyculin-A

Figure 3: Overexpression of constitutively active IKKα activates ERK signaling. a-b. Human lung cancer cells, HCC827 (a) and H1650 (b), transfected with GFP (control), constitutively active IKKα, or kinase-dead IKKα were lysed using 1× RIPA buffer supplemented with protease and phosphatase inhibitors. Equal amounts of proteins were separated with 4-20% SDS-PAGE and transferred to polyvinyl difluoride membranes. Antigen-coated membranes were incubated overnight with primary antibodies against IKKα, phosphorylated DARPP-32 (Thr34), total DARPP-32, phosphorylated PP1α, total PP1α, phosphorylated ERK, total ERK, and α-tubulin (loading control). **c.** Vehicle (DMSO)- or calyculin-A-treated human HCC827 cells were lysed with 1× RIPA buffer and subjected to immunoblotting using anti-phosphorylated PP1α, -total PP1α, -phosphorylated ERK, -total ERK, -DARPP-32, and -α-tubulin (loading control) antibodies. Chemiluminescence signals were detected after incubating membranes with HRP-tagged secondary antibodies. Representative images from one experiment are shown, but results were validated by performing three independent biological repeats. Bar graphs at the right show quantification of the results from the three western blotting experiments. *$P < 0.05$; ns, not significant.

Figure 4

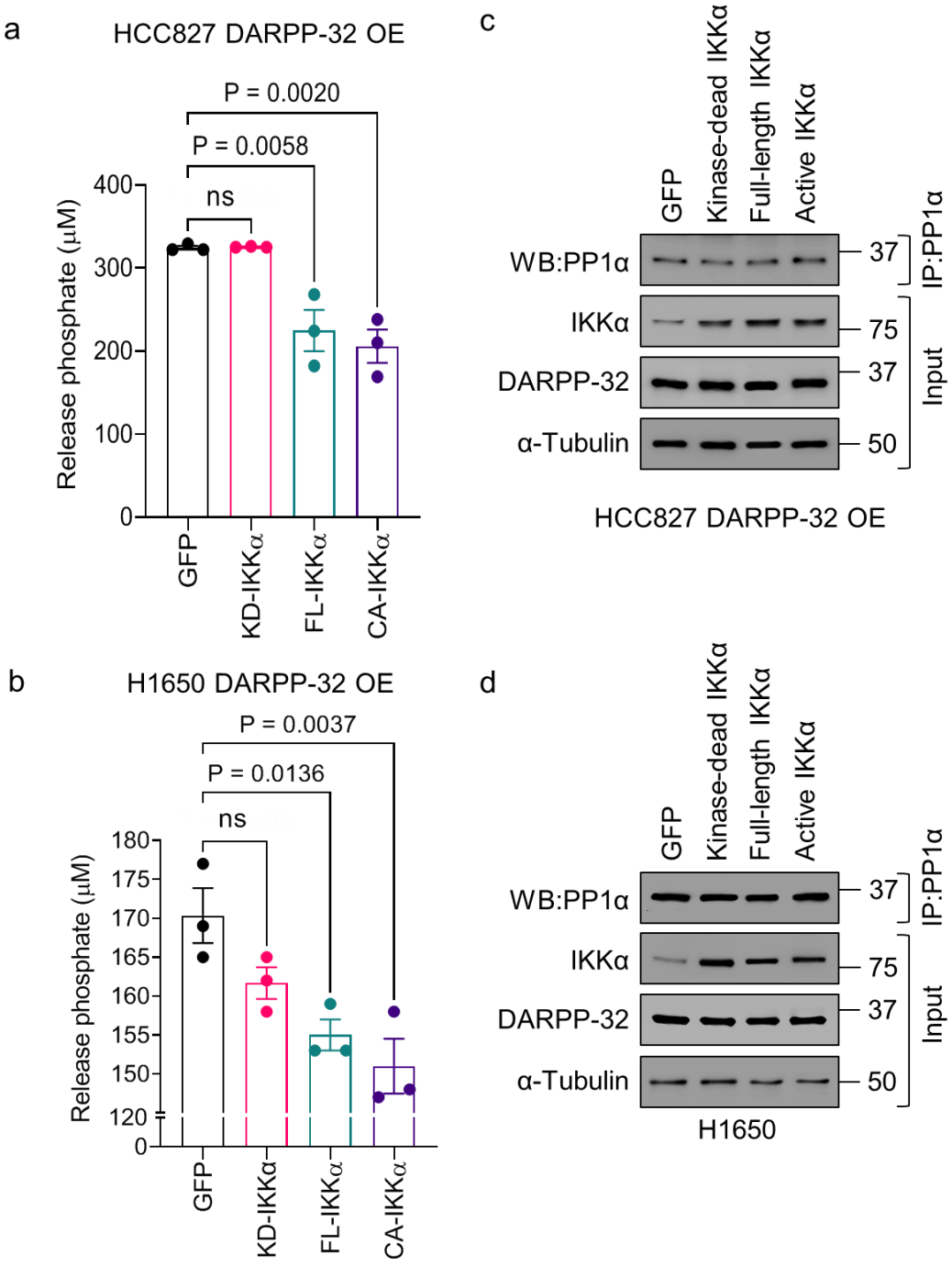


Figure 4: Overexpression of IKKα inhibits phosphatase activity of PP1α. a-b. Human NSCLC HCC827 (a) and H1650 (b) cells transfected with GFP (control), kinase-dead (KD), full-length (FL), and constitutively active (CA) IKKα cDNAs were lysed using 1× RIPA buffer supplemented with

protease inhibitors only. Equal amounts of proteins (500 ng) were immunoprecipitated using anti-PP1α antibodies. Immunoprecipitated cell lysates were subjected to in vitro phosphatase assays following incubation with either PP1α substrate or histone H1 peptide (control). Released phosphates in each reaction tube were determined by using a phosphate detection reagent. In vitro phosphatase experiments were repeated three times independently. Bar graphs represent mean ± SEM of the three repeats, with each circle in a bar representing an independent experiment. A value of $P \leq 0.05$ was considered significant, ns, not significant, one-way ANOVA followed by Dunnett's test. **c-d** Immunoprecipitated HCC827 (c) and H1650 (d) cell lysates separated with 4-20% SDS-PAGE were subjected to western blotting using anti-PP1α antibodies. Input cell lysates were blotted with antibodies against IKKα, DARPP-32, and α-tubulin (loading control).

Figure 5

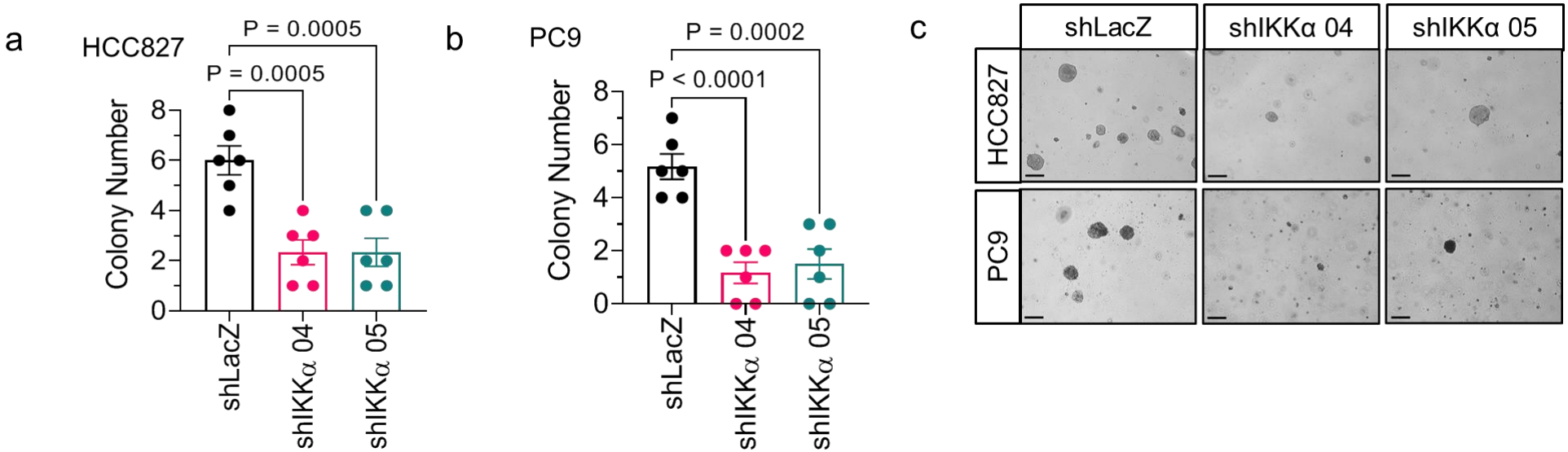


Figure 5: Knockdown of IKKα expression blocks anchorage-independent NSCLC cell growth. a-b. Human NSCLC HCC827 (a) and PC9 (b) cells transduced with lentivirus designed to silence LacZ (control) or IKKα expression were subjected to soft agar colony formation assays to determine anchorage-independent cell growth. ImageJ was used to count colonies on the cell culture dishes after 2 weeks of incubation, and the number of counted colonies was plotted. Each circle on a graph represents an independent experiment. Soft agar colony formation experiments were repeated at least six times. Error bars indicate SEM (n=6). A value of $P \leq 0.05$ was considered significant, one-way ANOVA followed by Dunnett's test. **c.** Representative images of HCC827 and PC9 cells transduced with lentivirus encoding either LacZ shRNA or IKKα shRNAs forming colonies on soft-agar cell culture dishes 2 weeks after plating.

Figure 6

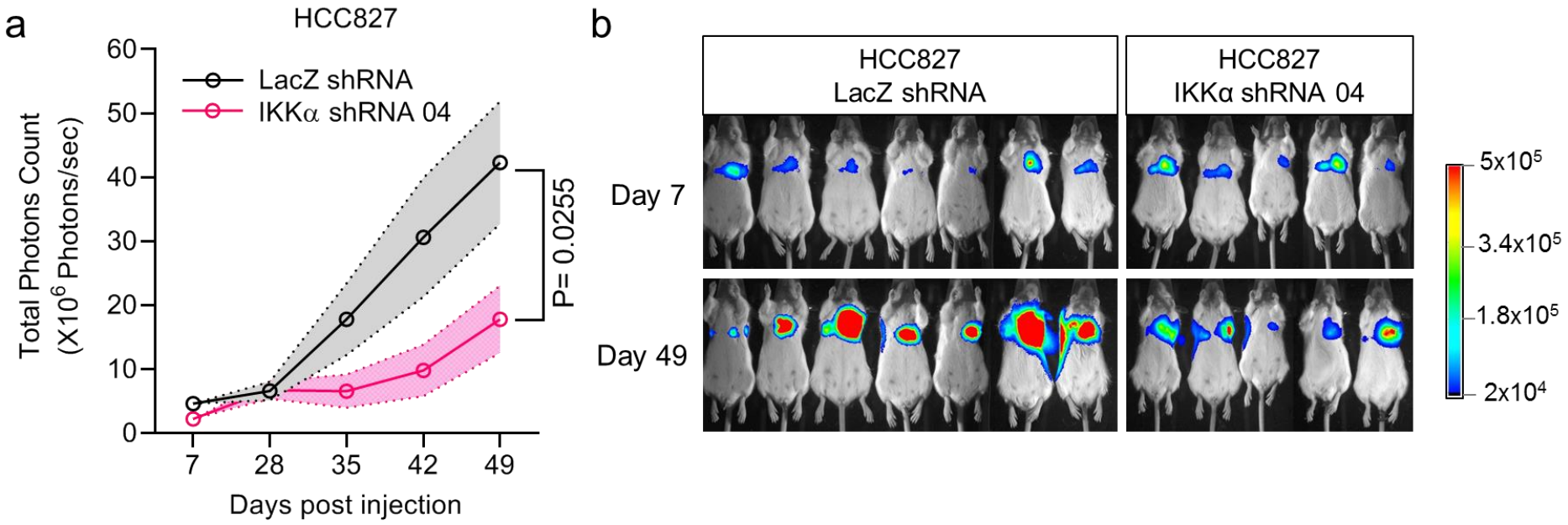
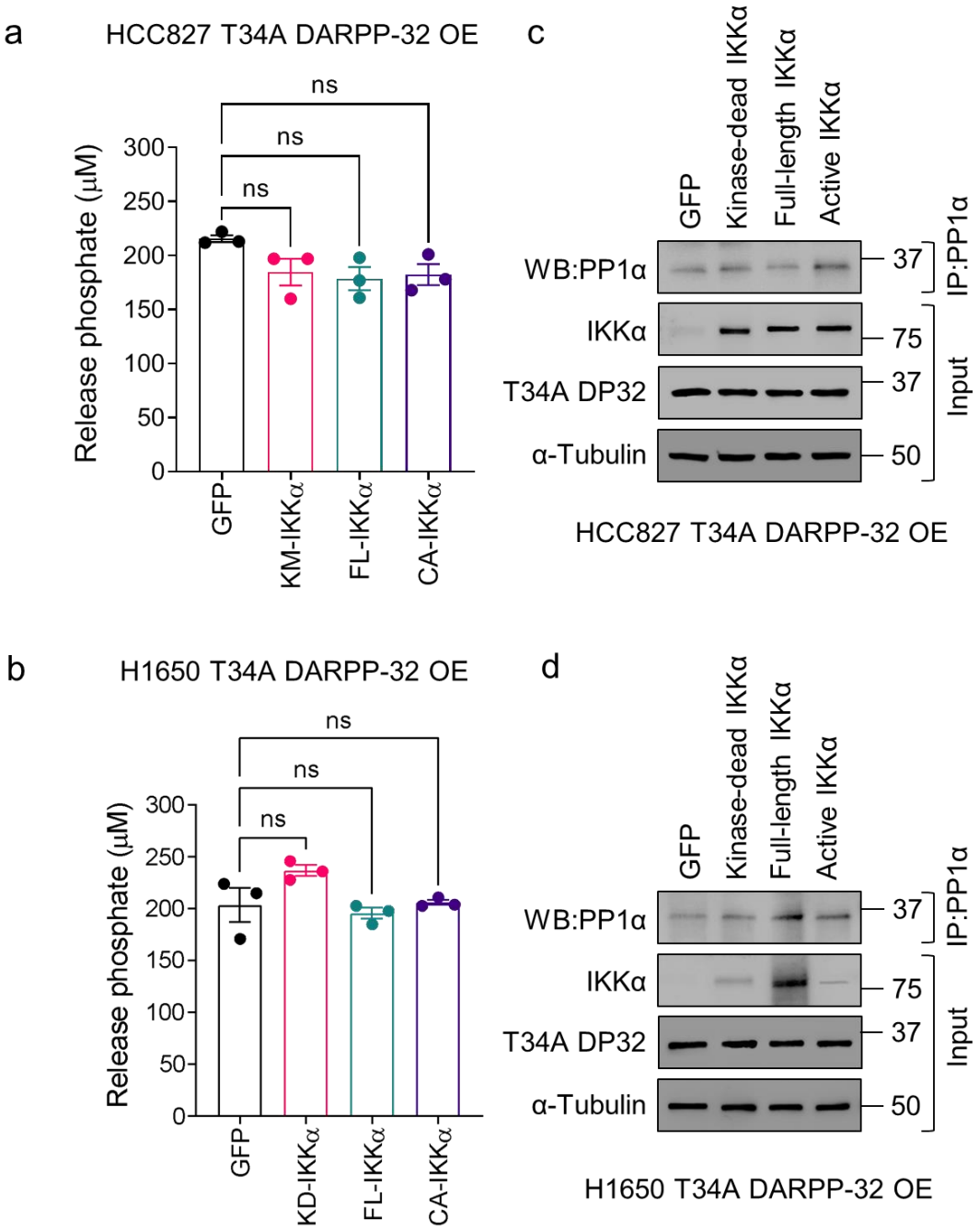


Figure 6: Depletion of IKKα inhibits lung tumor cell growth and proliferation in vivo. a. Luciferase-labeled IKKα-depleted human HCC827 cells were orthotopically injected into the left thorax of SCID mice and imaged for luminescence on the indicated days. Total luminescence intensity (photon count) was calculated using molecular imaging software and plotted as a line graph. Error bars are shown as dotted lines indicating SEM. A value of $P \leq 0.05$ was considered significant, two-way ANOVA followed by Sidak's test. **b.** Images of anesthetized mice were captured to detect luminescence signals on the indicated days.

Supplementary Figure 1



Supplementary Figure 1: PP1α activity is controlled by DARPP-32 phosphorylation.

a-b. Human NSCLC HCC827 (a) and H1650 (b) cells transduced with retrovirus designed to overexpress mutant (T34A) DARPP-32 proteins were transiently transfected with GFP, kinase-dead (KD), full-length (FL), or constitutively active (CA) IKKα expression plasmids. Following

immunoprecipitation with anti-PP1α antibodies, cell lysates were subjected to in vitro phosphatase assays with either PP1α substrate or histone H1 peptide (control). A colorimetric-based phosphate detection reagent was used to determine the amount of phosphate ions released from each reaction. Bar graphs represent mean ± SEM of three repeats, with each circle in a bar representing one experiment. ns, not significant; one-way ANOVA followed by Dunnett's test. **c-d** Immunoprecipitated lysates of HCC827 (c) and H1650 (d) cells were separated with 4-20% SDS-PAGE and blotted using anti-PP1α primary antibodies. Total cell lysates (Input) were subjected to western blotting using primary antibodies against IKKα, DARPP-32, and α-tubulin (loading control).