BIOCHEMISTRY

Membrane localization of acetylated CNK1 mediates a positive feedback on RAF/ERK signaling

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Spatiotemporal control is a common mechanism that modulates activity and function of signal transducers in the signaling network. We identified acetylation of CNK1 (connector enhancer of kinase suppressor of Ras-1) as a late step in the activation of CNK1 signaling, accompanied with prolonged stimulation of extracellular signal–regulated kinase (ERK). We identified the acetyltransferase CREB (cyclic adenosine 3′,5′-monophosphate response element–binding protein)–binding protein and the deacetylase SIRT2 (sirtuin type 2) as novel binding partners of CNK1, modulating the acetylation state of CNK1. Acetylation of CNK1 at position Lys^{414} located in the pleckstrin homology domain drives membrane localization of CNK1 in growth factor–stimulated cells. Inhibition of ERK signaling abolishes CNK1 acetylation. Cosmic database search identified CNK1 mutants at position Arg^{426} near the acetylation site in several human tumor types. These mutants show constitutive acetylation and membrane localization. CNK1 mutants substituting Arg^{426}, the acetylation mimetic mutant CNK1-K414Q, and membrane-anchored CNK1 mutants all interact with the protein kinase CRAF and stimulate ERK-dependent cell proliferation and cell migration. In RAS-transformed cells, CNK1 is acetylated and membrane-bound and drives cell proliferation. Thus, growth factor–stimulated ERK signaling induces CNK1 acetylation, and acetylated CNK1 promotes ERK signaling, demonstrating a novel function of CNK1 as positive feedback regulator of the RAF/MEK (mitogen-activated protein kinase kinase)/ERK pathway. In addition, acetylation of CNK1 is an important step in oncogenic signaling, promoting cell proliferation and migration.

INTRODUCTION

Cellular signaling processes rely on the correct temporal and spatial regulation of the signal transduction network (*1*). Altering subcellular localization of signaling molecules is an important step in the regulation of signaling events. Furthermore, negative and positive feedback loops control the dynamics of signaling pathways and decide on the biological response (*2*). In growth factor signaling, activated receptor tyrosine kinases (RTKs) use phosphotyrosine residues to recruit SRC homology 2 (SH2) domain–containing proteins, such as the adaptors SHC and GRB2, leading to the activation of plasma membrane–anchored RAS proteins (*3*). Active guanosine 5′-triphosphate (GTP)–bound RAS recruits cytoplasmic RAF to the plasma membrane, followed by stimulation of the RAF/MEK (mitogen-activated protein kinase kinase)/ERK (extracellular signal–regulated kinase) protein kinase cascade. The regulatory p85 subunit of class 1A phosphoinositide 3-kinase (PI3K) recruits through its SH2 domain the holoenzyme to activated RTKs (*4*). Membrane-bound PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to phosphatidylinositol-3,4,5-trisphosphate (PIP$_3$), an important second messenger. Pleckstrin homology (PH) domains target cellular membranes by binding to phosphoinositides and mediate protein-protein interactions, thereby mediating various cellular functions (*5*–*7*). PH domains binding specifically to the PI3K product PIP$_3$ have attracted the most attention because they allow stimulus-dependent recruitment of PH domain–containing protein to the plasma membrane. AKT and its activator PDK1 (phosphoinositide-dependent protein kinase 1) both have PH domains targeting them to PIP$_3$-enriched regions in the plasma membrane (*8*). Reversible acetylation of lysine residues in their PH domains regulates binding of AKT and PDK to the plasma membrane (*9*, *10*).

CNK (connector enhancer of kinase suppressor of Ras) represents a family of scaffold proteins linked to RAF and AKT signaling (*11*–*13*). CNK proteins share a common domain structure (Fig. 1A) (*14*). The three protein-protein interaction domains SAM (sterile α motif), CRIC (conserved region in CNK), and PDZ (postsynaptic density protein/*Drosophila* disc large tumor suppressor/zonula occludens-1 protein) are followed by a PH domain and a C-terminal coiled-coil region. The N-terminal SAM domain of human CNK1 is a target of tyrosine and serine phosphorylation, resulting in its clustering accompanied with stimulation of CNK1 signaling (*15*–*17*). Depending on the composition of the clusters induced by the signaling strength applied, CNK1 activates the RAF/MEK/ERK pathway or the AKT pathway (*16*). Growth factors induce transient membrane localization of CNK1 as an early step in CNK1 signaling (*17*, *18*). Plasma membrane recruitment of CNK1 may depend on one of the three N-terminal protein-protein interaction domains of CNK1 interacting with membrane-bound proteins or the CNK1 PH domain binding to phosphoinositides. Posttranslational modifications may modulate the binding affinity of CNK1 to the membrane or even directly target CNK1 to a respective binding protein localized at the plasma membrane.

Here, we identified acetylation of CNK1 within the PH domain as a late step in the activation of CNK1 signaling, accompanied by prolonged stimulation of ERK. An acetylation mimetic mutant and acetylated CNK1 mutants found in human cancers localize to the plasma membrane, interact with CRAF, and drive cell proliferation and cell migration through ERK signaling. ERK signaling induces CREB (cyclic adenosine 3′,5′-monophosphate response element–binding protein)–binding protein (CBP)–dependent acetylation of CNK1, supporting a novel function of CNK1 as a positive regulator of feedback of ERK signaling.

RESULTS

Plasma membrane–anchored CNK1 activates ERK signaling

Transient localization of CNK1 to the plasma membrane is an early step in growth factor–induced CNK1 signaling (*17*, *18*). To analyze the impact of membrane-localized CNK1 in signal transduction, we fused the C-terminal plasma membrane targeting CaaX motif of KRAS to CNK1

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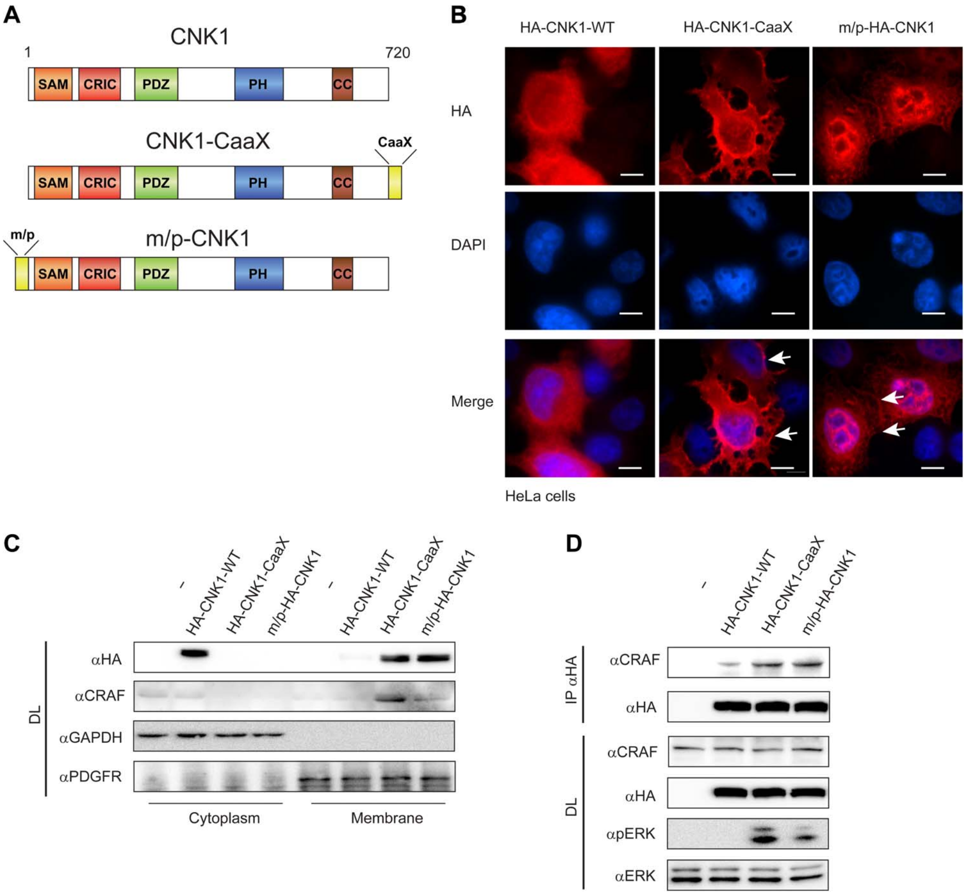

Fig. 1. Plasma membrane–anchored CNK1 activates ERK signaling. (**A**) Scheme of the multidomain protein CNK1. SAM, sterile alpha motif; CRIC, conserved region in CNK; PDZ, postsynaptic density protein/*Drosophila* disc large tumor suppressor/zonula occludens-1 protein; PH, pleckstrin homology; CC, coiled coil; CaaX, C-terminal membrane targeting motif of K-RAS; m/p, N-terminal membrane targeting site of LCK. (**B**) HeLa cells overexpressing HA (hemagglutinin)–CNK1–WT, HA-CNK1-CaaX, or m/p-HA-CNK1 were immunostained with anti-HA antibody detected by Alexa Fluor 594 rabbit anti-mouse immunoglobulin G (IgG) (red). DAPI (4′,6-diamidino-2-phenylindole) was used to visualize the nuclei (blue). Scale bars, 10 μm. (**C**) Cytoplasmic and membrane fractions of human embryonic kidney (HEK) 293 cells expressing HA-CNK1-WT, HA-CNK1-CaaX, or m/p-HA-CNK1 were immunoblotted with the antibodies indicated. αHA, anti-HA; αCRAF, anti-CRAF; αGAPDH, anti–glyceraldehyde-3-phosphate dehydrogenase; αPDGFR, anti–platelet-derived growth factor receptor; DL, direct lysates. (**D**) Lysates of HEK293 cells expressing HA-CNK1-WT, HA-CNK1-CaaX, or m/p-HA-CNK1 were immunoprecipitated with anti-HA (IP αHA) and immunoblotted with anti-HA for HA-tagged CNK1 proteins and with anti-CRAF for coprecipitating CRAF. Direct lysates were immunoblotted as indicated.

(CNK1-CaaX) or the N-terminal membrane targeting the myristoylation and palmitoylation signal of lymphocyte-specific protein tyrosine kinase (LCK) to CNK1 (m/p-CNK1) (Fig. 1A) (*19*, *20*). Immunofluorescence studies and cell fractionation experiments confirmed membrane localization of CNK1-CaaX and m/p-CNK1, whereas wild-type CNK1 (CNK1-WT) showed diffuse cytoplasmic distribution (Fig. 1, B and C). In addition, we observed increased membrane localization of CRAF in cells expressing CNK1-CaaX and m/p-CNK1 but not in cells expressing CNK1-WT (Fig. 1C). Recruitment to the plasma membrane is a crucial step in the activation of CRAF, resulting in stimulation of MEK and, subsequently, of ERK. In addition, compared to CNK1-WT, membrane-anchored CNK1 showed increased coprecipitation with CRAF, correlating with activation of ERK monitored by phosphorylated ERK (Fig. 1D). Thus, CNK1-CaaX and m/p-CNK1 interact with CRAF and stimulate ERK, indicating that membrane-anchored CNK1 activates the RAF/MEK/ERK signaling cascade.

Acetylation of CNK1 in its PH domain mediates plasma membrane localization

PH domains are phosphoinositide-binding modules involved in the localization of proteins to the plasma membrane (*5*, *6*). The PH domain of CNK1 binds weakly and nonspecifically to phosphoinositides (*21*). However, the affinity of the PH domain to phosphoinositides can be modulated by their oligomeric state or by acetylation (*5*, *9*). In case of AKT, reversible acetylation of Lys^{20} located in the variable loop connecting the β1 strand and the β2 strand of the PH domain regulates membrane binding and activation of AKT (Fig. 2A) (*9*). AKT binds and becomes acetylated by the lysine acetyltransferases p300 (the paralog of CBP) and p300/CBP-associated factor and deacetylated by sirtuin type 1 (SIRT1) (*9*). Because CNK1 also contains a lysine residue, Lys^{414}, in the β1-β2 loop (Fig. 2A), we hypothesized that Lys^{414} is a target for acetylation and that acetylation regulates membrane binding of CNK1. Therefore, we treated HeLa cells expressing CNK1– green fluorescent protein (GFP) with nicotinamide, an inhibitor of lysine deacetylases of the sirtuin family (*22*). Nicotinamide induced recruitment of CNK1-GFP to the plasma membrane (Fig. 2B). In addition, nicotinamide strongly elevated acetylation of CNK1 that was monitored by affinity-purified CNK1 immunoblotted with acetyl-Lys–specific antibodies (Fig. 2C, left). Substitution of Lys^{414} with Arg (CNK1-K414R) abolished nicotinamide-induced acetylation of CNK1, indicating that Lys^{414} is the major acetylation site of CNK1 (Fig. 2C,

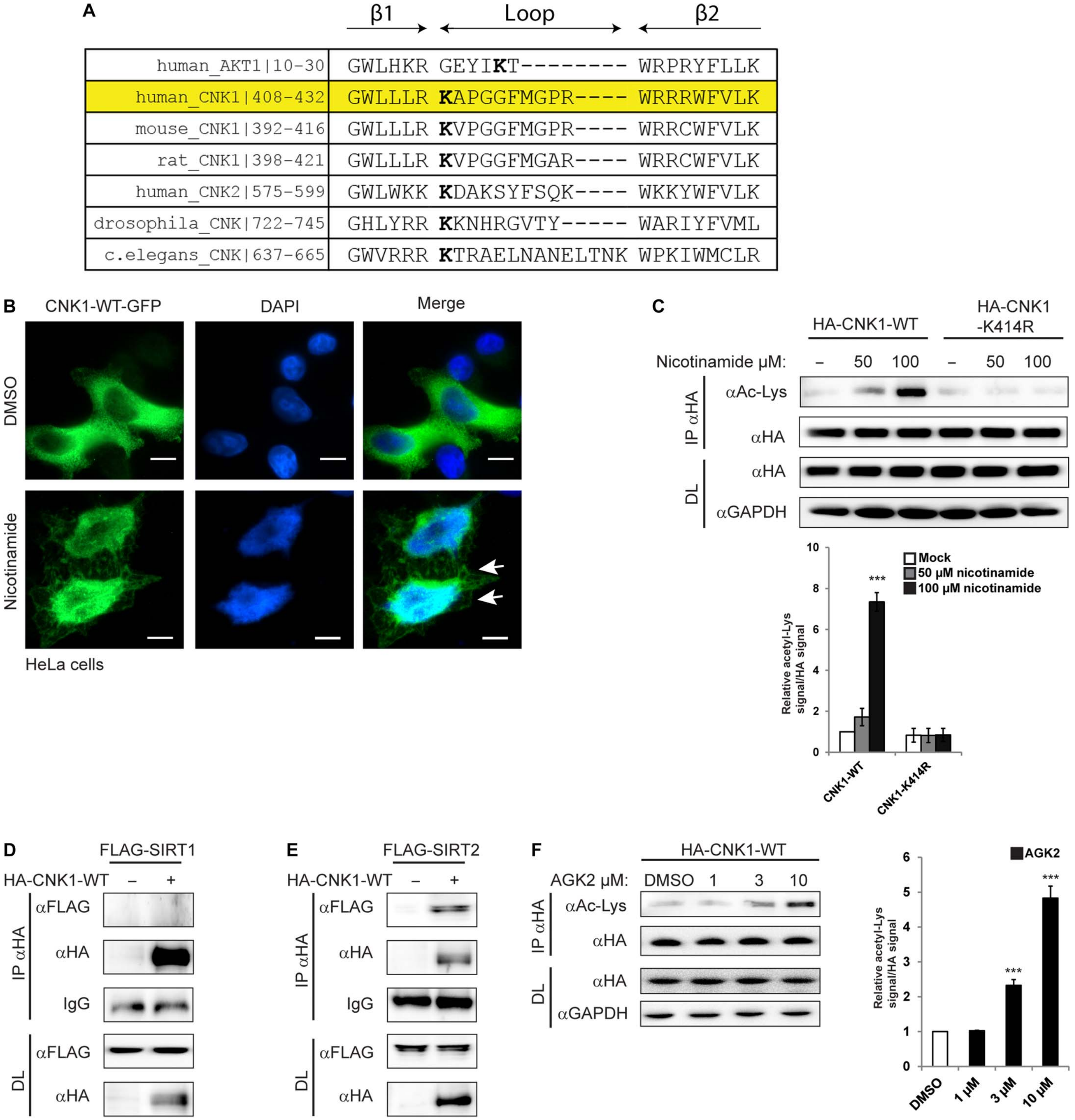

Fig. 2. Acetylation of CNK1 at Lys^{414} in its PH domain regulates membrane localization. (**A**) Amino acid sequence alignment of PH domains of selected CNK proteins and AKT1 performed by clustal analysis (*45*). Sequences according to their UniProt entry are as follows: human AKT1 (P31749), human CNK1 (Q969H4), mouse CNK1 (Q14CE3), rat CNK1 (Q499SO), human CNK2 (Q8WXI2), *Drosophila melanogaster* CNK (Q7KNQ9), and *Caenorhabditis elegans* CNK (G5EEW9). (**B**) Fluorescence images of GFP-CNK1 expressed in HeLa cells treated for 2 hours with nicotinamide (100 μM) or dimethyl sulfoxide (DMSO) for control. DAPI staining was used for visualizing nuclei (blue). Scale bars, 10 μm. (**C**) Acetylation state of HA-CNK1-WT or HA-CNK1-K414R expressed in HEK293 cells was monitored by anti-HA immunoprecipitation (IP αHA), followed by immunoblotting with anti–acetyl-Lys (αAc-Lys). Bar chart represents quantification of immunoblot signals of three independent experiments. ±SD, two-tailed Student's *t* test, $^{***}P < 0.001$. (**D** and **E**) Lysates of HEK293 cells coexpressing HA-CNK1 and FLAG-SIRT1 (D) and FLAG-SIRT2 (E) were immunoprecipitated with anti-HA and subsequently immunoblotted with anti-HA or anti-FLAG. (**F**) HEK293 cells expressing HA-CNK1-WT were treated with the Sirt2 inhibitor AGK2. Anti-HA immune complexes were immunoblotted with anti–Ac-Lys to detect acetylated CNK1. Bar chart represents quantification of immunoblot signal of three independent experiments. ±SD, two-tailed Student's *t* test, $^{***}P < 0.001$.

right). Among the seven known sirtuins, SIRT1 is mainly expressed in the nucleus, but also in the cytoplasm, and deacetylates AKT, whereas SIRT2 is the isoform predominantly expressed in the cytoplasm (*23*–*25*). SIRT3 to SIRT7 localize to the mitochondria or nucleus. Coexpression of CNK1 with SIRT1 and SIRT2 revealed that SIRT2 coimmunoprecipitated with CNK1 whereas SIRT1 did not (Fig. 2, D and E). Accordingly, treatment of cells with the SIRT2 inhibitor AGK2 strongly enhanced CNK1 acetylation (Fig. 2F). These findings indicate that acetylation of CNK1 at Lys^{414} triggers the recruitment of CNK1 to the plasma membrane. Binding of SIRT2 to CNK1 reverts acetylation of CNK1.

Growth factor–stimulated ERK signaling induces acetylation of CNK1 through CBP

Growth factor stimulation mediates membrane recruitment of CNK1 (*17*, *18*). To study the impact of growth factor stimulation on the acetylation state of CNK1, we treated HEK293 with epidermal growth factor (EGF) or insulin-like growth factor (IGF). EGF and IGF significantly enhanced acetylation of CNK1, as monitored by anti–acetyl-Lys immunoblotting (Fig. 3A). The mutant CNK1-K414R showed strongly reduced acetylation in growth factor–treated cells compared to CNK1-WT (Fig. 3B). Next, we determined which acetyltransferase can modify CNK1. It has been shown that CNK1 promotes cell proliferation via the AKT/forkhead box O (FOXO) pathway (*26*). Transcriptional activity of FOXO is regulated by CBP-induced acetylation (*27*). CBP represents a class of lysine acetyltransferases that functions as transcriptional coactivator but also participates in cell growth, transformation, and development (*28*, *29*). Coimmunoprecipitation experiments revealed that CBP interacted with CNK1 (Fig. 3C). In addition, the CBP inhibitor C646 blocked EGF-induced acetylation of CNK1 (Fig. 3D). It should be noticed that pretreatment with C646 did not affect stimulation of ERK by EGF treatment for 30 min, indicating that CNK1 acetylation takes place downstream or independent of ERK stimulation. However, pretreatment of cells with the MEK inhibitor U0126 abolished not only EGF-induced ERK phosphorylation but also CNK1 acetylation (Fig. 3E). This indicates that CNK1 can be acetylated and that acetylation depends on MEK/ERK signaling.

The acetylation mimetic mutant CNK1-K414Q stimulates the RAF/MEK/ERK pathway

Acetylation of CNK1 correlates with its plasma membrane localization (see Fig. 2, B and C). To support the notion that acetylation controls the recruitment of CNK1 to the plasma membrane, we analyzed the acetylation mimetic mutant CNK1-K414Q that resembles the acetylated Lys in terms of charge and the acetylation blocking mutant CNK1-K414R that conserves the net positive charge of the amino acid. As expected from the results obtained in nicotinamide-treated cells (Fig. 2B), the mutant CNK1-K414Q localized to the plasma membrane, whereas the mutant CNK1-K414R showed diffuse cytoplasmic localization (Fig. 4A). According to the membrane-anchored mutants CNK1-CaaX and m/p-CNK1 (Fig. 1D), the acetylation mimetic mutant CNK1-K414Q localized at the plasma membrane showed enhanced binding to CRAF and enhanced level of phosphorylated ERK in serum-starved cells compared to CNK1-WT (Fig. 4B). This was not the case for the acetylation blocking mutant CNK1-K414R (Fig. 4B). Thus, the acetylation mimetic mutant as well as membrane-anchored CNK1 stimulates ERK signaling.

CNK1 mutants in Arg^{426} promote cell proliferation and cell migration

The data presented so far indicate that acetylation in the PH domain as well as an acetylation mimetic mutant promotes recruitment of CNK1 to the plasma membrane, correlating with enhanced ERK activation. Recently, we identified phosphorylation of Ser^{22} located in the SAM domain of CNK1 as an activation mechanism for CNK1 signaling and the phosphomimetic mutant CNK1-S22D and the mutant CNK1-S22F found in human tumors as constitutive activators of AKT signaling (*15*). By searching the COSMIC (Catalogue of Somatic Mutations in Cancer) database that lists mutants found in human tumors, we identified mutants of CNK1 located in the PH domain (http://cancer.sanger.ac.uk/cosmic) (*30*). In several tumors, Arg at position 426 is replaced by cysteine, histidine, or serine. Arg^{426} is part of a conserved basic motif in the beginning of the β2 strand (Fig. 2A). To investigate the role of CNK1 mutants targeting Arg^{426} in CNK1, we generated the respective mutant CNK1 constructs. All three mutants, CNK1-R426C, CNK1-R426H, and CNK1-R426S, were acetylated in serum-starved cells comparable to membrane-bound CNK1-CaaX (Fig. 5A). Acetylation of the CNK1 mutants correlated with their predominant localization at the plasma membrane, similar to what the acetylation mimetic mutant CNK1-K414Q did (Fig. 5B). Accompanied by increased membrane localization of these CNK1 mutants, endogenous CRAF was preferentially detected in the membrane fraction (Fig. 5B). In addition, enhanced levels of CNK1 mutants and CRAF at the plasma membrane correlated with increased coimmunoprecipitation of CRAF with CNK1 and elevated the levels of phosphorylated ERK similar to membrane-anchored CNK1-CaaX (Fig. 5C).

Aberrant activation of the RAF/MEK/ERK pathway has been linked to oncogenesis (*31*, *32*). To test the stimulatory effect of the CNK1 mutants targeting the PH domain, we performed cell proliferation and cell migration assays. Overexpression of CNK1-WT was sufficient to significantly increase cell proliferation, and this effect depends on AKT signaling, as proven by treatment of the cells with the AKT inhibitor MK2206 (Fig. 5D) (*14*, *16*). The three CNK1 mutants targeting Arg^{426}, the acetylation mimetic mutant CNK1-K414Q, and the membrane-anchored mutant CNK1-CaaX all stimulated cell proliferation in a range similar to what CNK1-WT did, although the mutant CNK1-R426S showed even a significantly higher cell proliferation rate (Fig. 5D). Cell proliferation induced by these CNK1 PH mutants was insensitive to the AKT inhibitor. In contrast, the MEK inhibitor U0126 blocked cell proliferation stimulated by these mutants. This fits with the observation that these PH mutants predominantly signal via the MEK/ERK pathway. The acetylation-defective mutant CNK1-K414R behaved similarly as CNK-WT did. This mutant promoted proliferation in an AKT-dependent and ERK-independent manner (Fig. 5D). Thus, acetylation and the activating mutants inside the PH domain switched signaling from AKT, as is the case for CNK1-WT, to ERK. To further analyze the biological effects of the CNK1 PH mutants, we performed cell migration assays. The mutants CNK1-R426S, CNK1-K414Q, and CNK1-CaaX expressed in HEK293 cells strongly increased cell migration (Fig. 5E). The MEK inhibitor U0126, but not the AKT inhibitor MK2206, abolished cell migration of these CNK1 mutants, indicating that cell migration depends on the activation of ERK signaling. Consistently, CNK1-WT and the mutant CNK1 K414R were unable to stimulate ERK and did not induce cell migration. Thus, substitutions of Arg^{426} in the PH domain of CNK1 found in a subset of human tumors resulted in acetylated and membrane-localized CNK1 mutants that promote cell proliferation and cell migration by constitutively stimulating the RAF/MEK/ERK pathway.

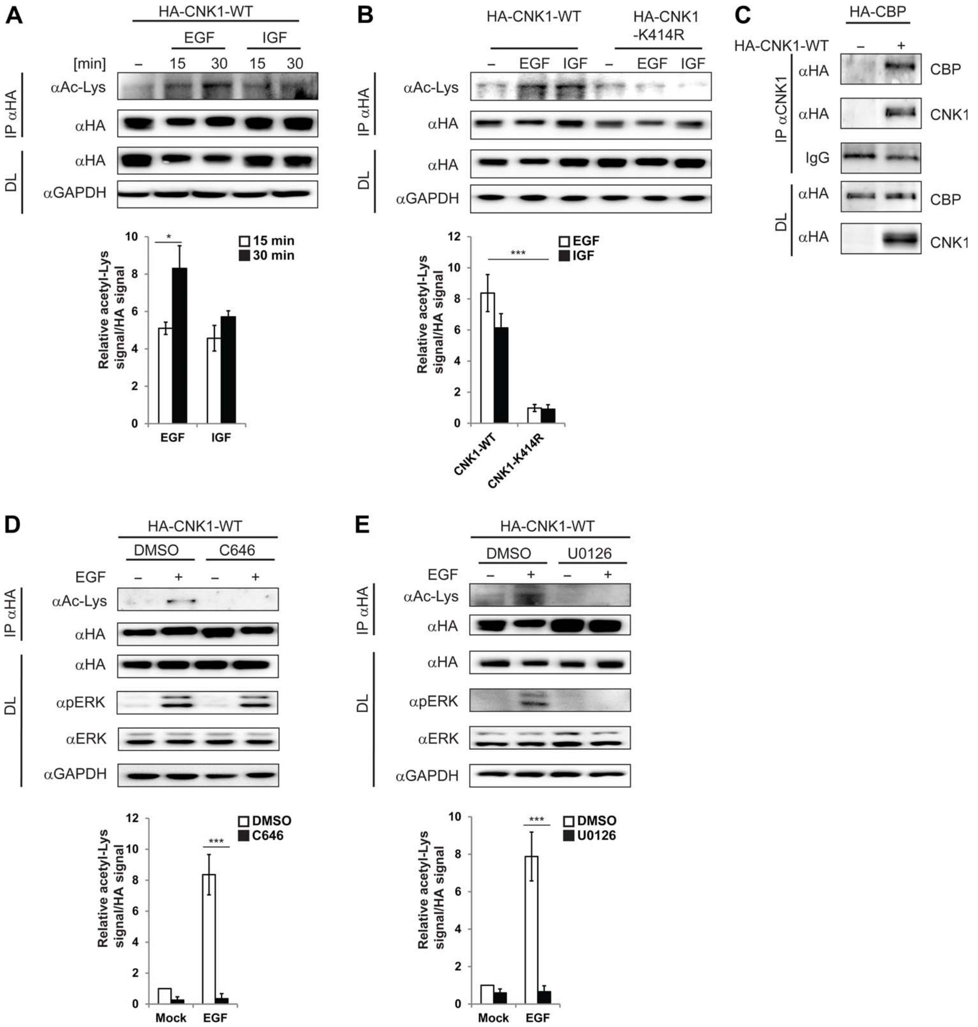

Fig. 3. Growth factor–induced acetylation of CNK1 depends on ERK signaling. (**A**) HEK293 cells expressing HA-CNK1-WT were stimulated with EGF (20 ng/ml) or IGF (20 ng/ml). Anti-HA immunocomplexes (IP αHA) were immunoblotted with anti–Ac-Lys to detect acetylated HA-CNK1. Bar chart represents quantification of Western blot signal of three independent experiments. ±SD, quantification shows fold increased signal to untreated control. (**B**) HEK293 cells expressing HA-CNK1-WT or HA-CNK1-K414R were stimulated for 30 min with EGF (20 ng/ml) or IGF (20 ng/ml), and anti-HA immunocomplexes were immunoblotted with anti–Ac-Lys. Bar chart represents quantification of Western blot signal of three independent experiments. ±SD, quantification shows fold increased signal to untreated control. (**C**) Lysates of HEK293 cells coexpressing HA-CNK1-WT and HA-CBP were immunoprecipitated with anti-CNK1 antibody (IP αCNK1) and immunoblotted with anti-HA to monitor HA-CBP. Direct lysates (DL) were shown for control. (**D**) HEK293 cells expressing HA-CNK1-WT were pretreated with the CBP inhibitor C646 for 2 hours (5 μM) before being incubated for 30 min with EGF (20 ng/ml). Anti-HA immunocomplexes were immunoblotted with anti–Ac-Lys. Bar chart represents quantification of Western blot signal of three independent experiments. ± SD, two-tailed Student's *t* test, $^{***}P < 0.001$. Direct lysates were immunoblotted with the antibodies indicated. (**E**) HEK293 cells expressing HA-CNK1-WT were pretreated with U0126 (10 μM) for 2 hours before being incubated for 30 min with EGF (20 ng/ml). Anti-HA immunocomplexes were immunoblotted with anti–Ac-Lys to detect acetylated HA-CNK1. Bar chart represents quantification of Western blot signal of three independent experiments. Direct lysates were immunoblotted with the antibodies indicated. ±SD, two-tailed Student's *t* test, $^{***}P < 0.001$.

CNK1 mediates RAS-driven oncogenic signaling

So far, we have demonstrated that ERK signaling facilitates acetylation of CNK1 and that acetylated CNK1 localizes to the plasma membrane and induces constitutive activation of ERK signaling. By identifying acetylation as a promoter of CNK1 signaling, we next studied the function of CNK1 in RAS-transformed cells. Sbcl-2 melanoma cells express oncogenic NRAS-Q61K, leading to constitutive activation of the RAF/MEK/ERK pathway (*33*). In Sbcl-2 cells, CNK1 was acetylated and coimmunoprecipitated with CRAF (Fig. 6A). The interaction between CNK1 and CRAF was sensitive to the CBP inhibitor C646 (Fig. 6A). In line with our result that acetylation of CNK1 localizes CNK1 to the membrane, treatment of Sbcl-2 cells with C646 shifted CNK1 from the membrane fraction to the cytoplasmic fraction (Fig. 6B, compare DMSO and C646). This correlated with a change in subcellular localization of CRAF from the membrane fraction in DMSO-treated cells to the cytoplasmic fraction in C646-treated cells. Moreover, knockdown of CNK1 in Sbcl-2 cells abolished membrane-localized CRAF in favor of cytoplasmic CRAF (Fig. 6B, compare siControl and siCNK1-a). This indicates that in RAS-transformed Sbcl-2 cells, CNK1 is acetylated and complexed with CRAF at the plasma membrane. Increased proliferation is a hallmark of transformed cells. Similar to the farnesyltransferase inhibitor Salisarib targeting RAS, C646 reduced proliferation of Sbcl-2 cells (Fig. 6C). Moreover, knockdown of CNK1 inhibits cells proliferation comparable to Salisarib and C646, demonstrating that CNK1 acts downstream of

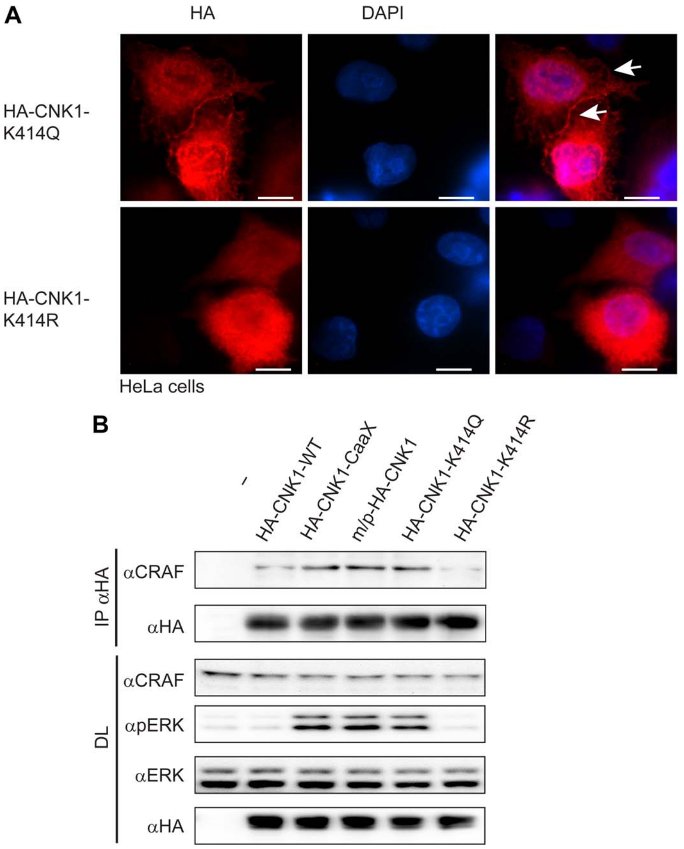

Fig. 4. Constitutive membrane-bound CNK1 and the acetylation mimetic mutant CNK1-K414Q activate ERK. (**A**) Immunostaining of HA-CNK1-K414Q and HA-CNK1-K414R expressed in HeLa cells was performed with anti-HA antibody detected by Alexa Fluor 594 rabbit anti-mouse IgG (red). DAPI was used for visualizing nuclei (blue). Scale bars, 10 μm. (**B**) HEK293 cells were transfected with the HA-CNK1 constructs indicated. Anti-HA immunocomplexes were immunoblotted with anti-CRAF. Direct lysates (DL) were analyzed for active ERK by anti–phosphorylated ERK (pERK) immunoblotting.

RAS. Together, these data hint at CNK1 as a crucial mediator of oncogenic RAS signaling.

DISCUSSION

Stimulation of signaling pathways and signal transmission depends on spatiotemporal regulation of the components of the respective pathways. Here, we demonstrate that a late step in the activation of CNK1 signaling involves acetylation of CNK1 within its PH domain and subsequent translocation from the cytoplasm to the plasma membrane as part of a positive feedback mediated by CNK1 on ERK signaling. Constitutive acetylation of CNK1-WT in RAS-transformed cells and of CNK1 mutants found in human tumors deregulates CNK1 signaling and promotes cell proliferation and cell migration.

Lysine acetylation is a reversible posttranslational modification controlled by lysine acetylases and lysine deacetylases and contributes to the regulation of many cellular processes (*34*–*36*). CBP interacts with CNK1, and growth factor–induced acetylation of CNK1 facilitates plasma membrane translocation correlating with stimulation of CNK1 signaling (Fig. 3). We identified Lys^{414} inside the β1-β2 loop of CNK1's PH domain as the major target for acetylation (Fig. 2). The acetylation mimetic mutant CNK1-K414Q constitutively binds to the plasma membrane. The acetylation blocking mutant CNK1-K414R conserving the positive charge of lysine no longer binds to the membrane (Fig. 5). This differs from the effect of acetylation on AKT regulation. In case of AKT, Lys^{14} located at the end of the β1 strand and Lys^{20} located inside the β1-β2 loop are acetylated under basal conditions, blocking membrane localization and subsequent activation of AKT (*9*, *10*). Growth factors induce deacetylation of Lys^{14} and Lys^{20}. Lys^{14} seems to be the target for ubiquitination facilitating the recruitment to the plasma membrane. Lys^{20} located in the PIP$_3$ binding loop elevates affinity to PIP$_3$ by its positive charge. The mutant AKT1-K20R that conserves the net charge of the amino acid but prevents neutralization by acetylation binds to PIP$_3$ and is constitutively active, indicating that the charge, but not Lys as such, is essential at this position. In contrast, Lys^{414} of CNK1 located in the putative PIP$_3$ binding loop seems to be essential for membrane targeting, however not by binding, to PIP$_3$. This is in line with a previous study showing that the PH domain of CNK1 binds only weakly and nonspecifically to phosphoinositides (*21*). The PH domain of CNK1 was postulated to be a protein-protein interaction domain that binds to GTP-bound RHO GTPases (guanosine triphosphatases) and mediates RHO-induced signaling (*21*). Binding of other PH domains to active monomeric GTPases and Gα subunits has also been described (*7*). Here, we demonstrate that acetylation of the PH domain drives localization of CNK1 to the plasma membrane. The underlying mechanism of how acetylation recruits CNK1 to the plasma membrane is under further investigation.

Growth factors stimulate the RAF/MEK/ERK and PI3K/AKT pathways, although with different intensities (*37*). CNK1 is an effector of growth-stimulated RTKs and promotes ERK and AKT signaling in a mutually exclusive manner (*16*). Early in signaling and at low signal intensity, CNK1 forms complexes with CRAF, leading to ERK activation. Later and/or at higher signal intensities, CNK1 associates with CRAF and AKT and acts as platform for the AKT/RAF cross-talk, that is, AKT-dependent phosphorylation and inactivation of CRAF. CBP is a downstream target of ERK signaling (*38*). Both, CNK1 and AKT interact with and are acetylated by p300/CBP, however, with contrary effects (Fig. 3) (*9*). Stimulated CBP results in acetylated, membrane-bound CNK1 that recruits CRAF and prolongs ERK signaling, whereas acetylation of AKT silences AKT activity. Deacetylation of CNK1 is exerted by the cytoplasmic SIRT2 (Fig. 2), and AKT1 seems to be preferentially deacetylated by SIRT1. This indicates that acetylation of CNK1 and AKT favors CNK1-mediated ERK signaling compared with AKT signaling at late steps in growth factor stimulation. Feedback loops are an important mechanism to control signaling pathways. In growth factor–induced signaling, ERK phosphorylation negatively regulates upstream elements, such as the EGF receptor, the RAS activator SOS, and RAF (*2*). A positive feedback loop results from ERK-dependent phosphorylation and inactivation of the RAF inhibitory protein RKIP (*39*). ERK phosphorylates and activates CBP (*38*) that, in turn, acetylates CNK1. Acetylated CNK1 stimulates the RAF/MEK/ERK cascade. Thus, CNK1 presents a novel positive feedback mediator that controls ERK signaling and ERK-dependent effects.

Mutants in acetylation sites occur frequently and are suggested as a driver mechanism of cancer (*40*). Here, we identified constitutive acetylation of Lys^{414} induced by substitution of Arg^{426} by Cys, His, and Ser as activating CNK1 mutants constitutively stimulating ERK signaling (Fig. 5). For AKT1, it has been reported that the substitution of Glu^{17} to Lys (E17K) localizes the PH domain to the plasma membrane in the

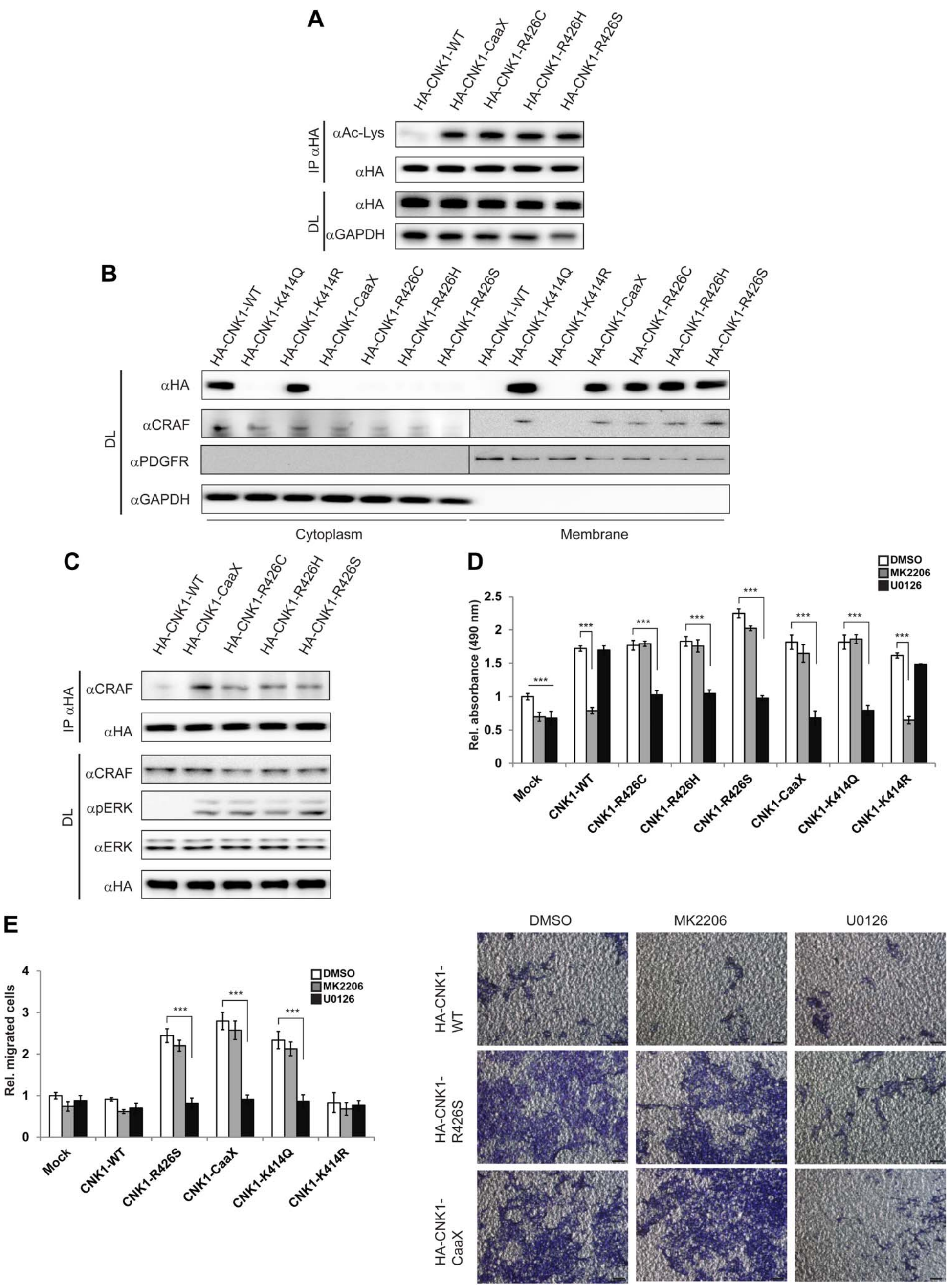

Fig. 5. Oncogenic potential of CNK1 mutated in Arg^{426} that is located in the PH domain. (**A**) Acetylation state of HA-CNK1-WT and the indicated CNK1 mutants expressed in HEK293 cells was monitored by anti-HA immunoprecipitation (IP αHA) followed by immunoblotting with anti–Ac-Lys. (**B**) Cytoplasmic and membrane fractions of HEK293 cells expressing CNK1-WT or the CNK1 mutants indicated were immunoblotted HEK293 with anti-HA and anti-CRAF. PDGFR and GAPDH were monitored as markers for the plasma membrane and the cytoplasmic fraction, respectively. (**C**) CNK1-WT and the CNK1 mutants indicated were expressed in HEK293 cells. Anti-HA immunocomplexes were immunoblotted with anti-CRAF to detect CRAF coprecipitating with HA-CNK1 proteins. (**D**) HEK293 cells overexpressing the indicated HA-CNK1 constructs were treated for 48 hours with the AKT inhibitor MK2206 (10 μM), the MEK inhibitor U0126 (10 μM), or DMSO for control. Cell proliferation was analyzed by an MTT assay. ±SD, two-tailed Student's *t* test, ***$P < 0.001$. (**E**) HEK293 cells expressing HA-CNK1-WT, HA-CNK1-R426S, HA-CNK1-CaaX, HA-CNK1-K414Q, and HA-CNK1-K414R were transferred into wells of a Boyden chamber and incubated for 48 hours with the AKT inhibitor MK2206 (10 μM), the MEK inhibitor U0126 (10 μM), or DMSO for control. Cells migrated through the porous membrane were monitored by staining with Giemsa solution. Right: Images for the selected samples. Bar chart shows quantification of three independent experiments. Scale bars, 100 μm. ±SD, two-tailed Student's *t* test, ***$P < 0.001$.

serum-starved cells. AKT1-E17K increases AKT activation, promotes transformation of cells, and is found in human solid tumors (*41*, *42*). In RAS-transformed Sbcl-2 melanoma cells, we demonstrate that CNK1-WT is constitutively acetylated, membrane-bound, and associated with CRAF (Fig. 6). Accordingly, not only farnesyltransferase inhibitors targeting RAS but also acetyltransferase inhibitors prevented oncogenic signaling. RAS GTPases are also targeted by lysine acetylation, reducing the active GTP-bound state. The RAS mutant NRAS-Q61K

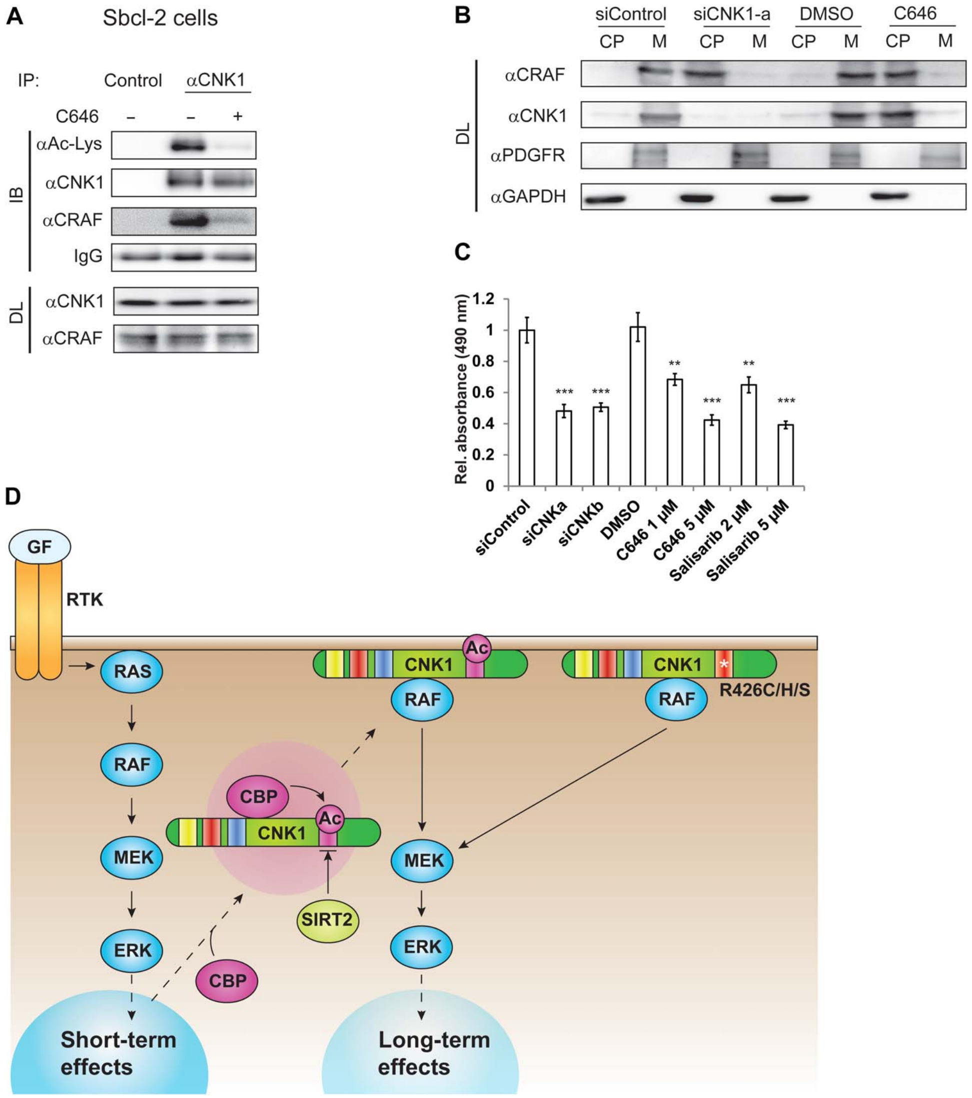

Fig. 6. CNK1 mediates RAS-driven oncogenic signaling. (**A**) The acetylation state of CNK1 was studied in Sbcl-2 melanoma cells expressing oncogenic NRAS ($NRAS_{Q61K}$). Cells were pretreated for 2 hours with C646 (5 μm) and analyzed by anti-CNK1 immunoprecipitation (IP αCNK1) followed by immunoblotting (IB) with anti–Ac-Lys, anti-CRAF, or anti-CNK1. (**B**) Sbcl-2 cells transfected with CNK1-specific small interfering RNA (siRNA) (siCNKa) or control siRNA (siControl) for 48 hours or treated with C646 (5 μM) or DMSO for 2 hours were subjected to subcellular fractionation. Cytoplasmic (CP) and membrane (M) fractions were immunoblotted with anti-CNK1 anti-CRAF. Detection of PDGFR and GAPDH was used as membrane and cytoplasmic markers, respectively. (**C**) Sbcl-2 cells were treated for 48 hours either with CNK1-specific siRNA (siCNKa or siCNKb) or control siRNA (siControl) or with the acetylase inhibitor C646, the farnesyltransferase inhibitor salisarib, or DMSO. Cell proliferation was analyzed by an MTT assay. ±SD, two-tailed Student's *t* test, ***$P < 0.001$. (**D**) Scheme showing the effect of acetylation on CNK1 signaling. Mutants in CNK1-R426 found in human tumors are bound to the plasma membrane and constitutively stimulate RAF/MEK/ERK signaling. GF, growth factor.

expressed in Sbcl-2 evades negative regulation by acetylation (*43*). This hints for acetylated CNK1 as an important downstream effector of oncogenic RAS signaling.

In summary, we identified reversible acetylation as a novel regulation mechanism of CNK1 and revealed CNK1 as a mediator of a positive feedback mechanism for ERK signaling. Our data also support the notion of CNK1 as an oncoprotein (*12*, *26*). Activating mutations in the SAM domain drive clustering of CNK1 and stimulate CNK1-mediated AKT signaling (*15*). Activating mutations in the PH domain induces constitutive acetylation and membrane localization of CNK1, stimulating ERK signaling. Both constitutive ERK signaling and AKT signaling are connected to tumorigenesis and tumor progression, indicating that CNK1 is an inducer of oncogenic signaling.

MATERIALS AND METHODS

Plasmids and reagents

FLAG-SIRT1 (#1791), SIRT2-FLAG (#13813), and FLAG-CBP-HA (#32908) were purchased from Addgene. Plasmids coding for HA-CNK1 were described elsewhere (*11*). HA-CNK1-GFP was generated by inserting the HA-CNK1 coding the Hind III–Xba I fragment from pcDNA3-HA-CNK1 into pEGFP-N2 (Invitrogen). HA-CNK1-CaaX was cloned by PCR, inserting the 17–amino acid C-terminal coding sequence of KRAS 3′ to HA-CNK1. m/p-HA-CNK was generated by introducing PCR fragments encoding the 12–amino acid N-terminal sequence of LCK, which carries the myristoylation/palmitoylation signal, and HA-tagged CNK1 into the pcDNA3.1 mammalian expression vector (Invitrogen) using an restriction enzyme–free isothermal assembly method described elsewhere (see Table 1 for primer sequences) (*44*). CNK1 point mutants were generated by site-directed mutagenesis (Invitrogen) using primers listed in Table 1. Mouse anti-HA IgG, rabbit anti-HA IgG, anti-mouse IgG–horseradish peroxidase (HRP), and anti-rabbit IgG-HRP were from Sigma Aldrich. Anti-CNK1 (*46*) IgG was from Santa Cruz. Anti-CRAF (9442), anti–acetyl-lysine (9441), anti-PDGFRβ (28E1) monoclonal antibody, anti–phosphorylated ERK (4370), anti-ERK (4695), and anti-GAPDH (D16H11) monoclonal antibody were from Cell Signaling Technology; Alexa Fluor 594 rabbit anti-mouse IgG (H+L) was from Invitrogen. EGF, IGF, and AGK2 were purchased from Sigma-Aldrich; MK2206 and U0126 were from Selleckchem. C646 was purchased from Abcam. CNK1 siRNA was purchased from QIAGEN (siCNK1-b; catalog no. 1027415) and from Santa Cruz Biotechnology (siCNK1-a; sc-142433). Scrambled siRNA used as control was from Santa Cruz Biotechnology (sc-37007). AGK2 (A8231), nicotinamide (N0636), and salisarib (SML1166) were purchased from Sigma-Aldrich.

Cell culture

HEK293 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 100 U of penicillin, and streptomycin (100 μg/ml). Sbcl-2 cells were provided by M. Herlyn (Philadelphia) via T. Brummer (Freiburg) and cultivated as described previously (*33*).

Table 1. Primers designed and used in this work. PCR, polymerase chain reaction.

Name	Sequence
Primers for site-directed mutagenisis	
O PM K414Q 1 fw	CGTGTTGCGACAGGCACCGG
O PM K414Q 1 rv	CCGGTGCCTGTCGCAACAGG
O PM K414R 1 fw	CCTGTTGCGAAGGGCACCGG
O PM K414R 1 fw	CCGGTGCCCTTCGCAACAGG
O PM R426C 1 fw	CTGGCGCTGCCGCTGG
O PM R426C 1 rv	CCAGCGGCAGCGCCAG
O PM R426C 1 fw	CTGGCGCTGCCGCTGG
O PM R426C 1 rv	CCAGCGGCAGCGCCAG
O PM R426S 1 fw	CTGGCGCAGCCGCTGG
O PM R426S 1 rv	CCAGCGGCTGCGCCAG
Primers for PCR-based cloning	
Name	**Sequence**
bbl fw	CTGCCGCTTACCGGATAC
bbl rv	CCACTGAGCGTCAGACC
O AF 7	GGCTGTGGCTGCAGCTCACACC
	CGGAATACCCCTACGACGTTGCC
O AF 8	TTCCGGGTGTGAGCTGCAGCCA
	CAGCCGGCCATGGCAGCTTGG

Transient transfection and cell lysis

Cells were seeded at a density of 70% confluency. Plasmids were diluted in Opti-MEM (Gibco) and polyethylenimine solution [1 μg/μl (pH 7); Polysciences] was added. After incubation for 15 min, the transfection mix was added to the cells. Thirty hours after transfection, cells were starved overnight by using serum-free DMEM and subsequently incubated with lysis buffer [20 mM tris-HCl (pH 7.5), 1% Triton X-100, 100 mM NaCl, 1 mM sodium orthovanadate, 9.5 mM sodium fluoride, 10 mM sodium pyruvate, 10 mM β-glycerophosphate, 10 mM nicotinamide, 10 mM butyric acid, and one tablet of Roche protease inhibitor] for 10 min on ice. After suspending, the lysates were boiled in 4× Laemmli sample buffer and separated by 10% SDS–polyacrylamide gel electrophoresis. Immunoprecipitation was performed overnight by incubation with 1 μg of antibody per 400 μl of cell lysate on a rotating wheel. Sepharose G (15 μl; Roche) was added and was continuously incubated further for 3 hours. Immunocomplexes were washed three times with lysis buffer and resuspended in 4× Laemmli buffer. Immunoblotting was performed in a wet tank system (Bio-Rad).

Subcellular fraction

Cells were washed two times with ice-cold phosphate-buffered saline (PBS) and incubated with cytoplasm extraction buffer [20 mM Hepes (pH 7.4), 150 mM NaCl, 2 mM $MgCl_2$, 2 mM dithiothreitol, 2 mM EDTA, digitonin (42 μg/ml), 1 mM sodium orthovanadate, 9.5 mM sodium fluoride, 10 mM sodium pyruvate, 10 mM β-glycerophosphate, 10 mM nicotinamide, 10 mM butyric acid, and one tablet of Roche protease inhibitor] for 10 min on ice. Lysates were transferred into reaction tubes and centrifuged at 12,000*g* for 5 min at 4°C. The first supernatant was subjected to ultracentrifugation (100,000*g* for 1 hour), resulting in the cytoplasmic fraction. Cell pellet was incubated with membrane extraction buffer [20 mM tris-HCl (pH 7.5), 1% Triton X-100, 0.5% SDS, 100 mM NaCl, 1 mM sodium orthovanadate, 9.5 mM sodium fluoride, 10 mM sodium pyruvate, 10 mM β-glycerophosphate, 10 mM nicotinamide, 10 mM butyric acid, and one tablet of Roche protease inhibitor] for 30 min on a shaker at 4°C and subsequently centrifuged at 12,000*g* for 5 min at 4°C. The supernatant represented the membrane fraction.

Proliferation assay

HEK293 cells were seeded in a 96-well plate and transfected with 50 ng of plasmid DNA. Twenty-four hours after transfection, cells were starved and further incubated for 36 hours. Cell proliferation MTT assay was performed following the manufacturer's instructions (Roche).

Cell migration assay

HEK293 cells (5×10^4) were seeded in an insert of a Boyden chamber used for cell migration assays (catalog no. 3428, Corning). Two hours after seeding, cells were transfected with 200 ng of plasmid DNA per well. Six hours after transfection, the cell medium was exchanged. After further 36 hours, cells were fixed with 4% formaldehyde, washed, and stained with crystal violet. Samples were analyzed using a Nikon Eclipse TS100 microscope.

Immunofluorescence analysis

Cells were cultivated on collagen-coated coverslips and fixed with 50:50 methanol/acetone. Subsequently, coverslips were incubated at 4°C for 2 hours in PBS. Nonspecific binding was blocked by incubation in blocking buffer [10% (v/v) goat serum, 10% (w/v) bovine serum albumin in PBS] for 1 hour. Antibody incubation was performed for 2 hours, followed by extensive washing steps in 0.02% (v/v) Tween-PBS. DAPI staining was performed for 2 min, followed by washing with deionized H_2O. Coverslips were mounted on microscope slides with ProLong Gold Antifade Reagent (Life Technologies) and dried overnight at room temperature. Samples were analyzed using a Nikon Eclipse TS100 microscope. Data analysis was performed with NIS-Element 4.0 (Nikon).

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Acknowledgments

Funding: This work was supported by the Excellence Initiative of the German Federal and State Governments (grant EXC-294; BIOSS Centre for Biological Signalling Studies). **Author contributions:** G.R. planned and coordinated this study; G.R. and A.F. designed the experiments; A.F. carried out the experiments; B.W. provided the equipment and resources; G.R., A.F., and W.W.D.M. analyzed the data and wrote the manuscript. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper. Additional data related to this paper may be requested from the authors.

Submitted 13 February 2017
Accepted 12 July 2017
Published 11 August 2017
10.1126/sciadv.1700475

Citation: A. Fischer, W. W. D. Mühlhäuser, B. Warscheid, G. Radziwill, Membrane localization of acetylated CNK1 mediates a positive feedback on RAF/ERK signaling. *Sci. Adv.* **3**, e1700475 (2017).